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Linderane protects pancreatic β cells from streptozotocin (STZ)-induced oxidative damage

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ABSTRACT

Aims: Linderane, an important bioactive compound in Linderae, improved glucose and lipid metabolism in *ob/ob* mice. However, the effect of linderane on streptozotocin (STZ)-induced oxidative damage in INS-1 cells remains unclear.

Main methods: INS-1 cells were pre-treated with different doses of linderane for 2 h and then treated with 3 mM STZ for 12 h. Cell viability was determined by MTT assay. Cell apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit. The level of intracellular ROS was determined using dichlorofluorescein-diacetate (DCFH-DA). The activities of insulin secretion, SOD, catalase (CAT) and GPx were measured using ELISA kits. The expression levels of bax, bcl-2, p38, p-p38, nuclear Nrf2 and HO-1 were measured using western blot.

Key findings: The results showed that STZ-caused inhibitory effects on cell viability and insulin secretion were mitigated by linderane. Furthermore, linderane inhibited apoptosis and oxidative stress in STZ-induced INS-1 cells. Finally, linderane suppressed the activation of p38 MAPK pathway, as well as enhanced the activation of Nrf2 pathway in STZ-induced INS-1 cells. Activation of p38 MAPK pathway or inhibition of Nrf2 significantly reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis.

Significance: The protective effects of linderane on STZ-induced INS-1 cells might be attributed to the inhibition of p38 MAPK and activation of Nrf2 pathway.

1. Introduction

Diabetes mellitus (DM), is a kind of metabolic disorder, which is characterized by prolonged period of high blood sugar [1]. It has been surveyed that around 400 million people suffer from DM worldwide [2]. There are three main types of DM including Type 1 DM, Type 2 DM, and gestational diabetes [1]. Mechanism investigations have proven that major pathological event for DM is loss or destruction of pancreatic islet β cells, which are responsible for insulin secretion and controlling plasma glucose levels [3]. For the past 100 years, the conventional treatment for DM patients is exogenous insulin injection [2]. However, managing insulin dosing is a challenge, thereby insulin injection often cannot achieve overall glycemic control. Furthermore, over time poor glucose control may lead to severe complications and mortality [2]. Therefore, it is of importance to develop new strategies to obtain functional human β cells for DM therapy.

Linderane is an important bioactive compound in Linderae, which is a widely used Chinese herb [4]. Linderane was found to act as an indirect activator of phosphodiesterase 3 (PDE3), a cyclic AMP (cAMP)-degrading enzyme. Linderane inhibits hepatic gluconeogenesis by activating hepatic PDE3 in rat primary hepatocytes [5]. In addition, linderane significantly decreased the mRNA expression levels of phosphoenolpyruvate carboxykinase (*Pck1*) and glucose-6-phosphatase (*G6pc*), two key gluconeogenic genes [5]. Chronic administration of linderane improved glucose and lipid metabolism in *ob/ob* mice [5]. These findings suggest that linderane may be as a potential agent for Type 2 DM therapeutics. However, the role of linderane in pancreatic β cells has not been examined. Thus, in this study, we investigated the effect of linderane on streptozotocin (STZ)-induced β cells oxidative damage.

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2. Materials and methods

2.1. Cell culture and treatment

INS-1 cells (CRL-1672; American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium plus 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA), 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA). Cells were grown in a humidified incubator containing 5% CO_2 at 37 °C.

The INS-1 cells were divided into five groups: a) control group, cells were incubated without any treatment; b) STZ stimulated group, cells were incubated with 3 mM STZ (Sigma-Aldrich, St. Louis, MO, USA) for 12 h; c–e) linderane treatment groups, cells were pre-treated with different doses of linderane (5, 10, 20 μM; Sigma) for 2 h and then treated with 3 mM STZ for 12 h.

2.2. Cell viability assay

Cell viability of INS-1 cells was determined by MTT assay. INS-1 cells were seeded onto 96-well plates at a density of 1×10^4 cells/well. Then the cells were subjected to different treatments. After incubation, MTT (0.5 mg/ml, Sigma) was added to the cells, followed by an incubation for 4 h. Then the DMSO was added to dissolve the formazan precipitate, and the absorbance was determined at 570 nm with a microplate reader (Perlong, Beijing, China).

2.3. Insulin secretion

INS-1 cells (1×10^5 cells/well) were plated into 24-well plates and subjected to indicated treatments. After that, INS-1 cells were incubated with Hanks Balanced Salt Solution containing 5.6 mM and 16.7 mM glucose for 30 min at 37 °C. Culture medium was collected for the determination of insulin using commercial enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA, USA).

2.4. Measurement of reactive oxygen species (ROS) production

Levels of intracellular ROS were determined using dichlorofluorescein-diacetate (DCFH-DA, Sigma), which is a ROS sensitive probe. INS-1 cells with different treatments were collected and incubated with DCFH-DA (10 μM) for 30 min at 37 °C. Then the cells were rinsed twice by PBS, and then the fluorescence was analyzed using flow cytometry with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

2.5. Detection of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity

After different treatments as described above, INS-1 cells were harvested and rinsed. The activities of SOD, CAT and GSH-PX were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

2.6. Cell apoptosis assay

Cell apoptosis was detected using flow cytometry method with an Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA). INS-1 cells were collected and rinsed twice with PBS, followed by staining with Annexin V-FITC and propidium iodide (PI) for 20 min at room temperature. Finally, cells were analyzed by fluorescence-activated cell sorting (FACS) on a flow cytometer (Becton Dickinson, San Jose, CA, USA).

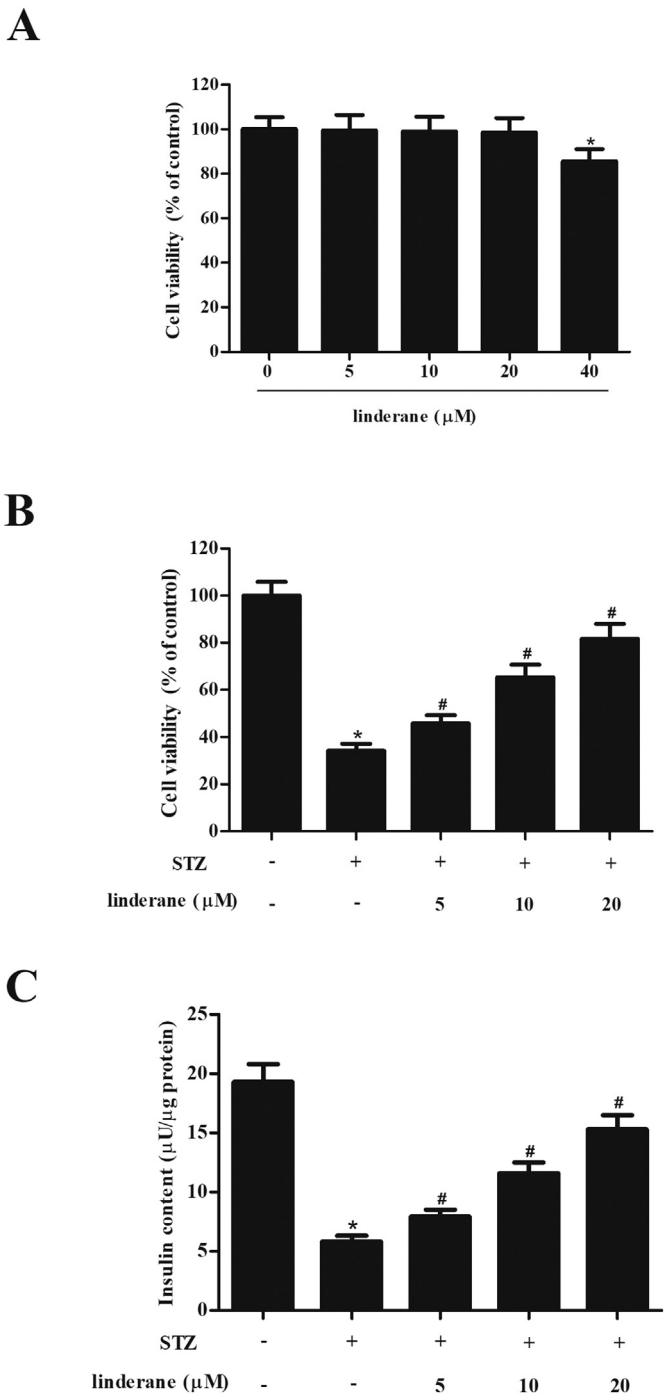

Fig. 1. Linderane improved cell viability and insulin secretion ability in STZ-induced INS-1 cells. (A) INS-1 cells were treated with different doses of linderane (5, 10, 20 and 40 μM) for 24 h, cell viability was determined by MTT assay. (B) Effect of linderane on cell viability in STZ-induced INS-1 cells. (C) Effect of linderane on insulin secretion ability in STZ-induced INS-1 cells. $^*p < 0.05$ versus the control INS-1 cells and $^{\#}p < 0.05$ versus the STZ-induced INS-1 cells.

2.7. Western blot

INS-1 cells were lyzed using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing 1 mM PMSF (Sigma). For detecting the expression of nuclear factor E2-related factor 2 (Nrf2), nuclear protein was extracted using a nucleo protein extraction kit (Sangon Biotech, Shanghai, China). Protein concentrations were

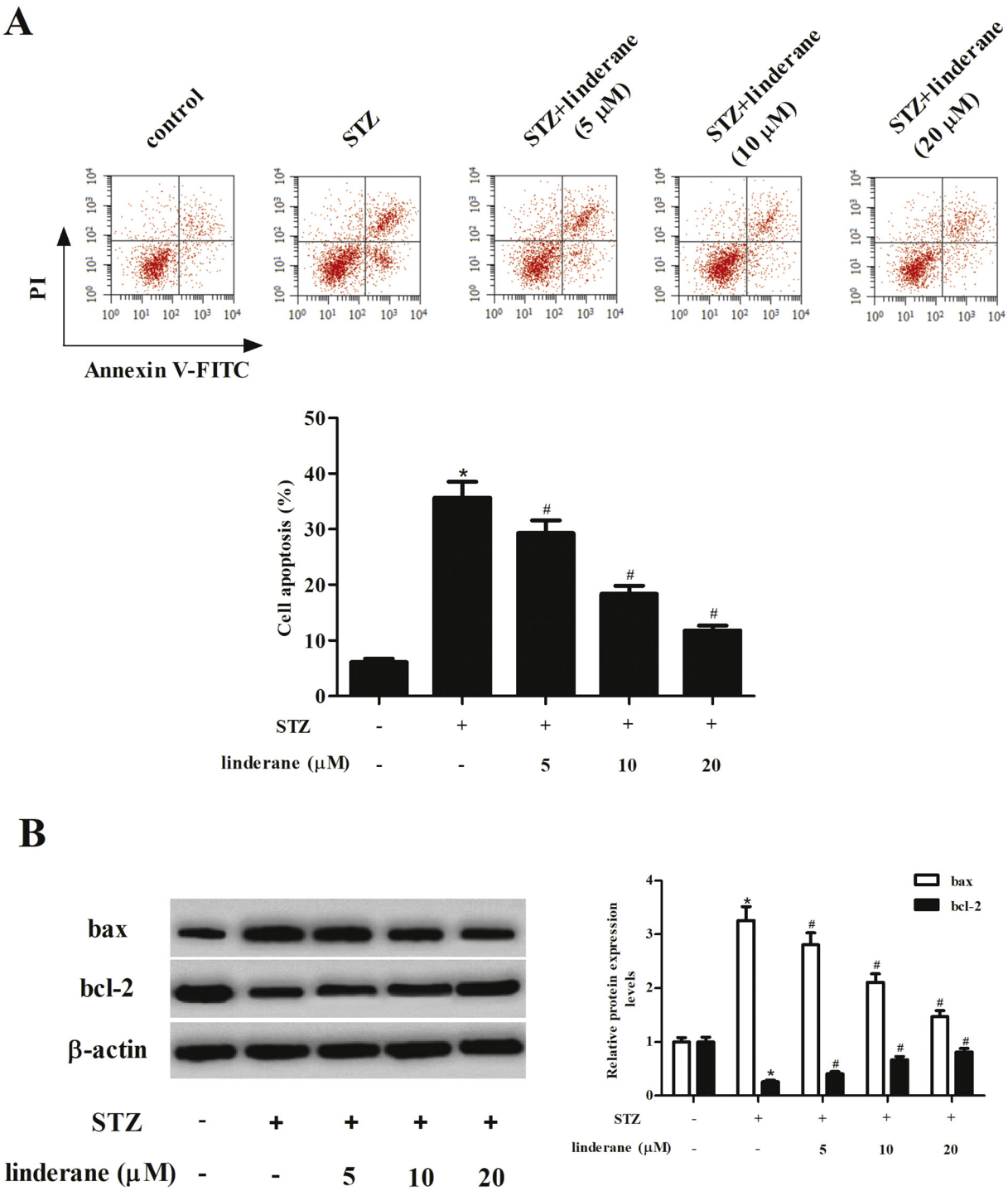

Fig. 2. Linderane reduced apoptosis in STZ-induced INS-1 cells. (A) Effect of linderane on apoptotic rates in STZ-induced INS-1 cells. (B) Effect of linderane on expressions of bax and bcl-2 in STZ-induced INS-1 cells. $^{*}p < 0.05$ versus the control INS-1 cells and $^{\#}p < 0.05$ versus the STZ-induced INS-1 cells.

determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime). Protein samples were separated by sodium dodecyl polyacrylamide (SDS)-polyacrylamide gelelectrophoresis (PAGE) and then transferred to nitrocellulose membrane (Millipore). After blocking with 5% skimmed milk in TBST buffer for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies against bax, bcl-2, p38, p-p38, Nrf2, HO-1 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following incubation with horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz) at room temperature for 1 h, the bands on the membranes were visualized with enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA). Protein bands were quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Statistical analysis

All data were presented as means ± S.D. Statistical analysis was conducted using SPSS statistical software (version 21.0; SPSS Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA) followed by Dunnett's test. A p value < 0.05 was considered statistically significant.

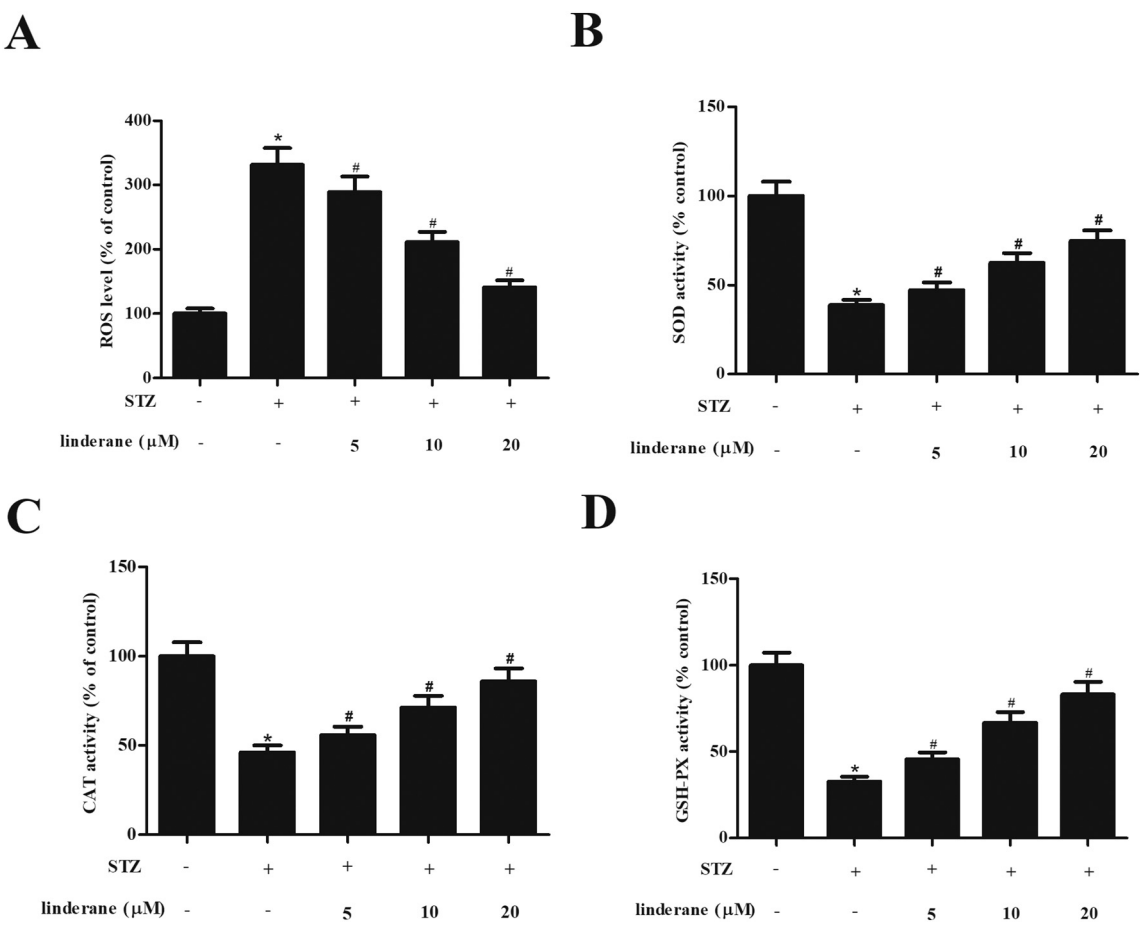

Fig. 3. Linderane alleviated oxditive stress in STZ-induced INS-1 cells. (A) Effect of linderane on ROS generation in STZ-induced INS-1 cells. (B–D) Effect of linderane on activities of SOD, CAT and GSH-PX in STZ-induced INS-1 cells. $^{*}p < 0.05$ versus the control INS-1 cells and $^{\#}p < 0.05$ versus the STZ-induced INS-1 cells.

3. Results

3.1. Linderane improved STZ-caused inhibitory effect on cell viability and insulin secretion ability in INS-1 cells

In order to evaluate the effect of linderane on cell viability, MTT assay was performed. As shown in Fig. 1A, the concentration of 40 μM linderane produced a significant effect while the concentration of 20 μM linderane showed no significant change. In addition, exposure to STZ markedly reduced cell viability of INS-1 cells, while pretreatment with linderane resulted in a significant improvement of cell viability (Fig. 1B). Besides, STZ exposure caused marked decrease in insulin secretion ability in INS-1 cells, whereas linderane induced significant increase in insulin secretion ability (Fig. 1C).

3.2. Linderane attenuated STZ-induced apoptosis in INS-1 cells

To investigate the effect of linderane on cell apoptosis, flow cytometry was carried out to detect the apoptotic rates. As indicated in Fig. 2A, the apoptotic rate in the STZ-induced INS-1 cells was markedly higher than that in control INS-1 cells. After linderane treatment, apoptotic rate was obviously decreased when compared with STZ-induced INS-1 cells. Besides, the expressions of bax and bcl-2 were detected using western blot analysis. STZ stimulation caused significant increase in bax expression and decrease in bcl-2 expression in INS-1 cells. However, STZ-caused changes in expressions of bax and bcl-2 were reversed by linderane (Fig. 2B).

3.3. Linderane suppressed STZ-induced oxidative stress in INS-1 cells

We next examined whether linderane could affect oxidative stress in STZ-induced INS-1 cells. The results in Fig. 3A showed that STZ induced ROS generation, while pretreatment with linderane inhibited the ROS generation. Fig. 3B–D indicated that STZ inhibited the activities of SOD, CAT and GSH-PX, however, the decreased SOD, CAT and GSH-PX activities were elevated by linderane pretreatment.

3.4. Linderane inhibited the phosphorylation of p38 in STZ-treated INS-1 cells

To assess the effect of linderane on the p38 MAPK pathway, the expressions of p38 and p-p38 were measured. Western blot revealed that the p-p38 levels were markedly increased after STZ treatment, while the changes of p38 levels were not obvious. Pretreatment with linderane suppressed the STZ-induced p-p38 expressions (Fig. 4).

3.5. p38 MAPK specific agonist P79350 reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis

To confirm whether p38 MAPK pathway was involved in regulating linderane-mediated cytoprotective effect, we examined the effect of p38 MAPK specific agonist *P79350* on linderane-regulated ROS production and cell apoptosis. The results indicated that *P79350* significantly reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis (Fig. 5A and B).

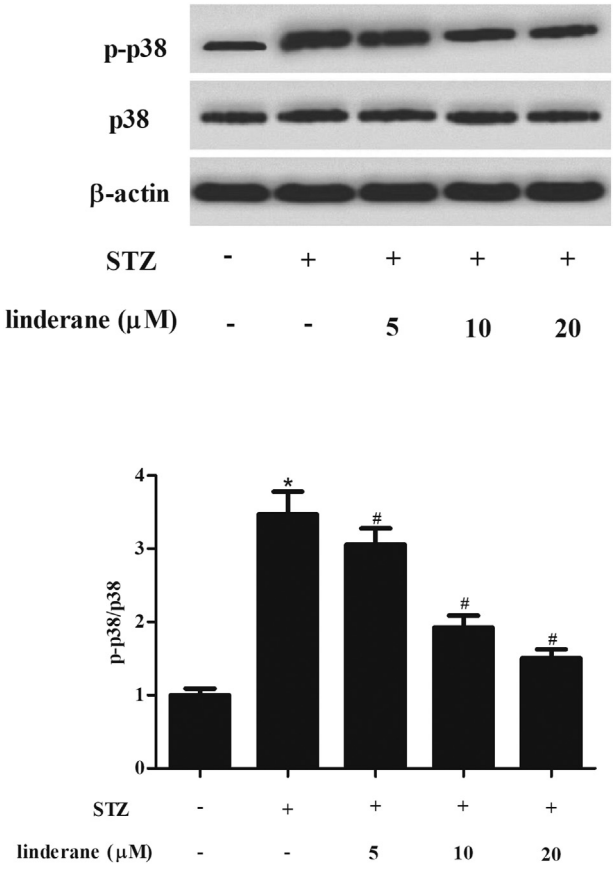

Fig. 4. Linderane inhibited STZ-induction activation of p38 signaling pathway in INS-1 cells. The expressions of p38 and p-p38 in INS-1 cells were measured using western blot to assess the effect of linderane on the p38 MAPK pathway. $^{*}p < 0.05$ versus the control INS-1 cells and $^{\#}p < 0.05$ versus the STZ-induced INS-1 cells.

3.6. Linderane induced Nrf2 activation in STZ-treated INS-1 cells

To evaluate the alternation of Nrf2 signaling pathway, the nuclear Nrf2 levels were measured using western blot. As illustrated in Fig. 6, STZ-treated INS-1 cells exhibited slight increases in nuclear Nrf2 and HO-1 expression as compared to the control cells. Furthermore, significant increases in nuclear Nrf2 and HO-1 expression were observed in the linderane pretreated INS-1 cells when compared with STZ-treated INS-1 cells.

3.7. Inhibition of Nrf2 reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis

To confirm whether Nrf2 pathway was involved in regulating linderane-mediated cytoprotective effect, we investigated the effect of Nrf2 knockdown on linderane-regulated ROS production and cell apoptosis. The results indicated that the protective effects of linderane on STZ-induced ROS production and cell apoptosis in INS-1 cells were partially reversed by Nrf2 inhibition (Fig. 7A and B).

4. Discussion

The main task assigned to the pancreatic β cells is glucose-stimulated insulin secretion [6]. Pancreatic β cells dysfunction and loss lead to the failure to produce insulin in amounts that are sufficient to control blood glucose levels, which is considered to be the major driver for the pathogenesis of DM [6]. Better understanding of the mechanisms underlying β cells dysfunction and loss may facilitate the development of

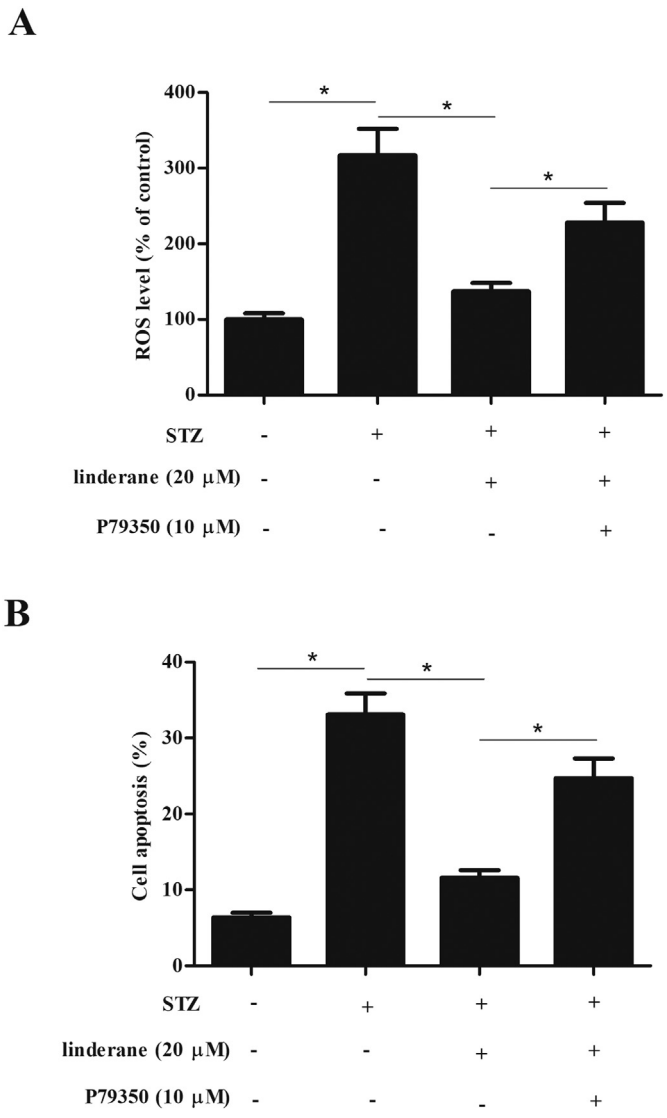

Fig. 5. p38 MAPK specific agonist *P79350* reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis. INS-1 cells were treated with 20 μM linderane and *P79350* (10 μM) before STZ treatment. (A) Intracellular ROS production was determined using DCFH-DA method. (B) Cell apoptosis was evaluated using flow cytometry method with an Annexin V-FITC Apoptosis Detection Kit. $^{*}p < 0.05$.

new therapeutic strategies to DM. Accumulating evidence points to a role for oxidative stress in the loss of normal β cells function [7,8]. Pancreatic β cells are highly dependent on oxidative metabolism for adenosine triphosphate (ATP) synthesis since insulin secretion depends on the production of ATP, which is tightly regulated by extracellular glucose concentrations [9]. Elevated oxygen consumption at high glucose levels is central to the stimulation of insulin secretion [9]. During stimulation with glucose, ROS are an unavoidable by-product of β cells of mitochondrial respiration [10]. Besides, ROS are also generated by NADPH oxidase through the reduction of molecular oxygen [10]. Furthermore, enzymes involved in antioxidant defense are present at unusually low levels or encoded by disallowed genes in β cells. Therefore, β cells are highly susceptible for damage induced by either oxidative stress.

STZ is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent [11]. It exhibits particular toxic to the pancreatic β cells and has been approved by FDA for treating metastatic cancer of pancreatic islet cells. Besides, STZ is wildly used for experimental induction of DM both in vivo and in vitro [11]. There is

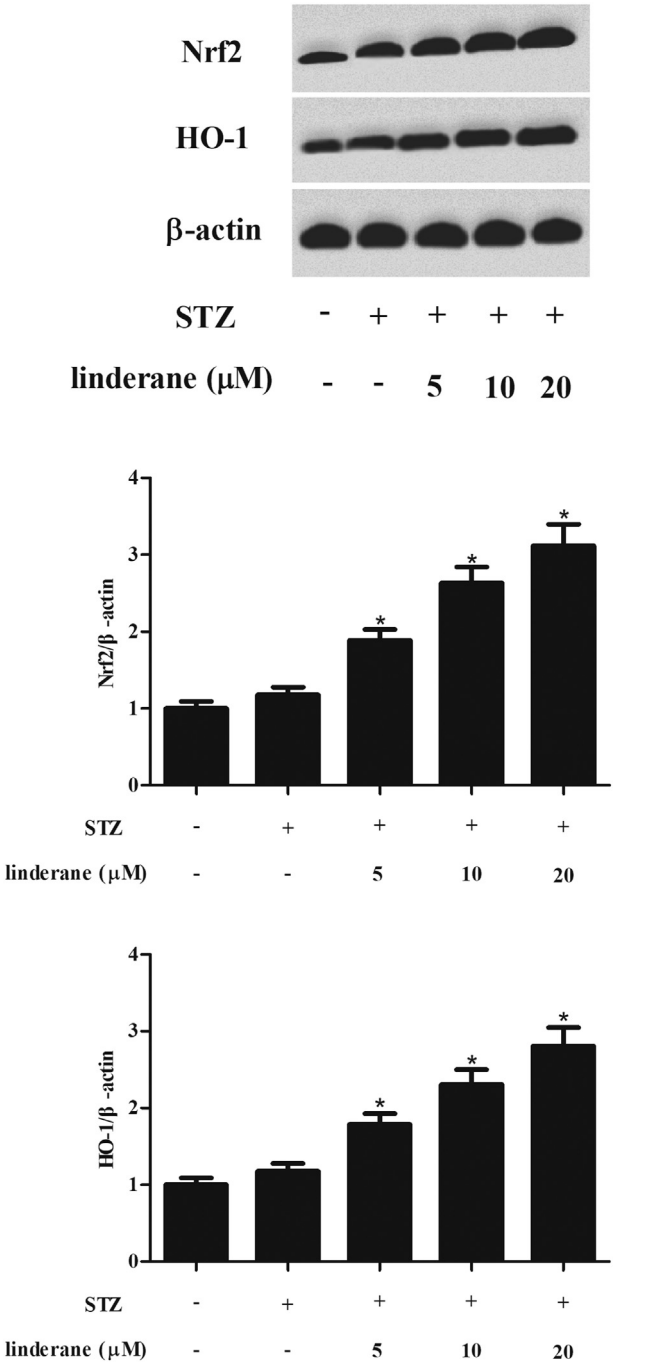

Fig. 6. Linderane enhanced the activation of Nrf2 signaling pathway in STZ-treated INS-1 cells. The expression levels of nuclear Nrf2 and HO-1 were measured using western blot to examine the effect of linderane on the Nrf2 pathway. $^{*}p < 0.05$ versus the control INS-1 cells and $^{\#}p < 0.05$ versus the STZ-induced INS-1 cells.

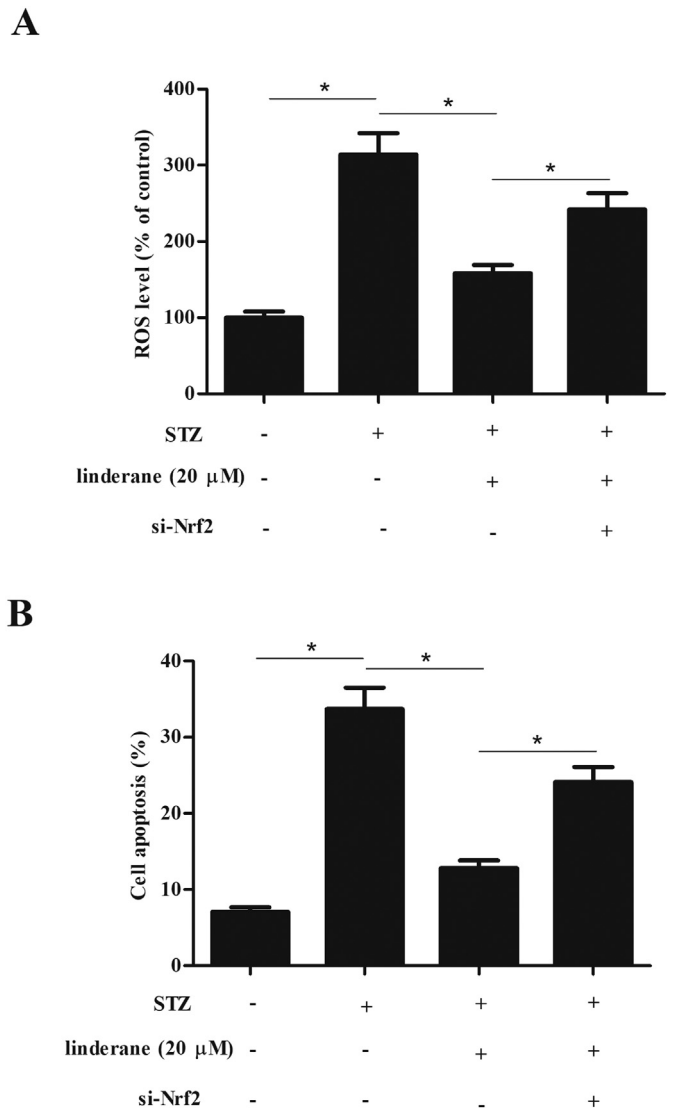

Fig. 7. Inhibition of Nrf2 reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis. INS-1 cells were transfected with si-Nrf2 in the presence of 20 μM linderane and then subjected to STZ treatment. (A) Intracellular ROS production was determined using DCFH-DA method. (B) Cell apoptosis was evaluated using flow cytometry method with an Annexin V-FITC Apoptosis Detection Kit. $^{*}p < 0.05$.

substantial evidence that induction of INS-1 cell apoptosis was performed by using 3 mM STZ [12–14]. In line with the previous studies, in the present study, we also used 3 mM STZ to stimulate INS-1 cells. The results showed that STZ caused inhibitory effects on cell viability and insulin secretion, and resulted in increase in cell apoptosis. However, several studies showed that some concentrations of STZ, such as 0.5 mM, 1 mM and 5 mM were used to induce INS-1 cell apoptosis [15–17], implying that low concentrations of STZ had no significant effect on INS-1 cell phenotype.

STZ stimulation also induced significant oxidative burden in INS-1 cells. Linderane is a natural bioactive compound that has been found to be as a potential agent for Type 2 DM therapeutics [5]. Linderan also inhibited the inactivation of cytochrome P450 2C9 [18]. However, STZ easily penetrates into the cells and then transforms into other sub-compounds, resulting in damage to DNA and organelles. Thus, linderan may inhibit the conversion of the STZ molecule to different compounds by inhibiting cytochrome enzymes and therefore reduced cellular toxicity. Linderane may block glucose transporter such as GLUT and reduce the penetration of STZ into the cells. Whether linderan regulates the conversion of STZ and blocks glucose transporters will require further experiments. Moreover, linderane possesses hepatoprotective activity against H_2O_2-induced oxidative damages on HepG2 cells [19]. In the current study, linderane was found to protect INS-1 cells from STZ-induced oxidative injury.

The redox-sensitive transcription factor Nrf2 serves as an important regulator of cell survival in response to oxidative stress [20]. Nrf2 regulates the expressions of antioxidant-related enzymes through binding to a cis-acting element designated ARE [20]. Hence, Nrf2 is considered as a therapeutic target for countering oxidative stress-

associated diseases [21]. It has been demonstrated that the induction of Nrf2 signaling pathway can be a major mechanism of cellular protection against pancreatic β cells dysfunction. Li et al. [22] reported that honokiol protects pancreatic β cells against high glucose and intermittent hypoxia-induced oxidative injury via activating Nrf2/ARE pathway in both Type 2 DM rats and INS-1 cells. Our results revealed that linderane elevated the activation of Nrf2 signaling pathway in STZ-treated INS-1 cells, and inhibition of Nrf2 significantly reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis.

The mitogen-activated protein kinase (MAPK) pathways are involved in a number of pathogenic responses such as oxidative stress, inflammation and extracellular fibrosis [23]. They can be activated by various stress stimuli including intracellular ROS, via distinct signaling cascades. Among the MAPKs, p38 MAPK is involved in a multitude of different biological functions [24,25]. Growing evidence has indicated that p38 MAPK is aberrantly regulated in diabetic conditions both in vivo and in vitro [26]. Inhibition of p38 MAPK activation significantly prevents the development of DM, implicating p38 MAPK as a novel therapeutic target for DM [26]. Advanced glycation end products (AGEs) are involved in diverse complications of diabetes mellitus since they can lead to decreased insulin secretion and cell apoptosis if pancreatic β cells [27]. The novel role of AGEs is partially due to the activation of p38 MAPK pathway [27]. Zhang et al. [28] demonstrated that ghrelin protects INS-1 cells against dexamethasone-induced apoptosis via ERK and p38 MAPK signaling. In the current study, we found that linderane suppressed STZ-induced activation of p38 MAPK signaling pathway in INS-1 cells, which might contribute to the protective effects of linderane. And, activation of p38 MAPK pathway significantly reversed the protective effects of linderane against STZ-induced ROS production and cell apoptosis.

There existed several limitations in this study. Firstly, we adopted the concentration of STZ (3 mM) in INS-1 cells, however, the concentration of STZ used in animal model remains to be determined. Secondly, there is only one cell line (INS-1) being studied in vitro experiments, it would be very helpful to confirm the role of linderane in pancreatic β cells from other cell lines, such as MIN6 and RIN-5F cell lines. Thirdly, we only evaluated the role of linderane in pancreatic β cells in vitro. An in vivo animal study will be considered in the following studies.

In summary, we found that linderane exhibited protective effects on STZ-induced oxidative damage and impaired insulin secretion in INS-1 cells. These protective effects might be due to the inhibition of p38 MAPK signaling pathway and activation of Nrf2 signaling pathway. These findings suggested that linderane could be a novel pharmacological agent for the treatment of DM.

Declaration of competing interest

The authors declare no conflicts of interest.

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