**Prunella vulgaris** aqueous extract attenuates IL-1β-induced apoptosis and NF-κB activation in INS-1 cells

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**Abstract.** We previously reported that *Prunella vulgaris* aqueous extract (PVAE) promotes hepatic glycogen synthesis and decreases postprandial hyperglycemia in ICR mice. Inflammatory cytokines play a critical role in the pathogenesis of diabetes. This study was designed to examine whether PVAE has a protective effect on IL-1β-induced apoptosis in INS-1 cells. INS-1 pancreatic β cells were plated at 2x10⁴/ml and treated with PVAE (100 μg/ml) 30 min before the cells were challenged with IL-1β (10 ng/ml). Untreated INS-1 cells served as control. INS-1 cell cytotoxicity was examined by MTT and lactate dehydrogenase (LDH) activity assays. Caspase-3 activity and activation of the apoptotic signaling pathway were analyzed by western blotting. NF-κB binding activity was examined by EMSA. The levels of inflammatory cytokines in the supernatant were measured by ELISA. IL-1β treatment significantly induced INS-1 cell death by 49.2%, increased LDH activity by 1.5-fold and caspase-3 activity by 7.6-fold, respectively, compared with control cells. However, PVAE administration significantly prevented IL-1β-increased INS-1 cell death and LDH activity and attenuated IL-1β-increased caspase-3 activity. Western blot data showed that PVAE also significantly attenuated IL-1β-increased Fas, FasL and phospho-JNK levels in the INS-1 cells. In addition, PVAE treatment significantly attenuated IL-1β-increased NF-κB binding activity and prevented IL-1β-increased TNF-α and IL-6 expression in INS-1 cells. Our data suggest that PVAE has a protective effect on IL-1β-induced INS-1 cell apoptosis. PVAE also attenuates IL-1β-increased NF-κB binding activity and inflammatory cytokine expression in INS-1 cells. PVAE may have a benefit for type I diabetic patients.

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**Introduction**

Proinflammatory cytokines, such as IL-1β, IFN-γ and TNF-α play an important role in the damage of insulin-producing pancreatic β cells (1). It has been well documented that these inflammatory cytokines induce the apoptotic or necrotic destruction of β cells. Recent study has shown that attenuation/prevention of pancreatic β-cell damage induced by inflammatory cytokines is an important step for treatment of diabetes (2).

*Prunella vulgaris* (*P. vulgaris*) is a perennial herb with worldwide distribution and has been used as a traditional medicine to reduce sore throat, alleviate fever and accelerate wound healing in China for many years (3,4). *P. vulgaris* contains different bioactive components, including complex carbohydrates, hydrophobic metabolites (triterpenes), polysaccharides and Rosmarinic acid (5,6). Numerous studies have reported that extracts of *P. vulgaris* have properties which are involved in anti-oxidative stress, anti-microbial invasion (7,8), anti-inflammatory responses (9) and anti-DNA damage and caspase-3 activity (10). We previously reported that PVAE significantly reduces postprandial hyperglycemia in ICR mice (11). However, it is unclear whether PVAE has a protective effect on inflammatory cytokine-induced pancreatic β cell damage.

IL-1β induces pancreatic β cell apoptosis, stimulates JNK phosphorylation and activates nuclear factor-κB (NF-κB) (12), which results in increases in inflammatory cytokine expression. In contrast, inhibition of JNK activation protects β cells from IL-1β-induced apoptosis (13). In the present study, we investigated whether PVAE prevents IL-1β-induced pancreatic β cell apoptosis and attenuates IL-1β-increased NF-κB activation. We observed that PVAE significantly prevented IL-1β-induced pancreatic β cell damage and attenuated IL-1β-stimulated NF-κB activation and inflammatory cytokine expression. Our data indicate that PVAE may have a significant benefit for diabetes patients.

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**Materials and methods**

*Preparation of PVAE.* *Prunella vulgaris* plants were collected in Nanjing, China and identified by Dr Junsong Li (College of Pharmacy, Nanjing University of Chinese Medicine). A voucher specimen was deposited at the College of Pharmacy,
Nanjing University of Chinese Medicine. The procedure of PVAE preparation was previously described by Kim et al (14) with modification. Briefly, *P. vulgaris* was extracted with distilled water at 70°C for 5 h. The extracts were filtered through Whatman No. 1 filter paper and the filtrates were lyophilized. The final dried extracts were dissolved in saline and filtered using a 0.45-µm syringe prior to use in the *in vitro* experiments.

**Cell culture and treatment.** INS-1 pancreatic β cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). INS-1 cells were maintained in RPMI-1640 (Cellgro, USA) medium containing 10% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37°C in an incubator with a humidified atmosphere of 5% CO₂ (15). To examine the cytotoxicity of the PVAE preparation, INS-1 cells were plated at 1x10⁴/ml and treated with increasing concentrations of PVAE at 0.01, 0.1, 1, 10 and 100 µg/ml for 48 h. To investigate the protective effect of PVAE on IL-1β-induced cell damage, INS-1 cells (2x10⁶/ml) were treated with PVAE at 100 µg/ml 30 min before the cells were stimulated with IL-1β (10 ng/ml) for 48 h. There were three replicates in each group. The supernatants were harvested for measurement of LDH activity. The cells were harvested for preparing the nuclear and cytoplasmic proteins as described previously (16).

**Cell cytotoxicity assay.** The cell viability was measured using the MTT assay (16). The LDH activity was measured according to the manufacturer’s instructions (Sigma-Aldrich).

**Western blot analysis.** Western blots were performed as described previously (17). Briefly, the cellular proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ). The ECL membranes were incubated with the appropriate primary antibody [anti-P-IκB, anti-IκB, anti-P-P38, anti-p38, anti-P-JNK, anti-JNK, anti-P-ERK, anti-ERK, anti-cleaved caspase-3, anti-Fas, anti-FasL, anti-Bax, anticaspase-3, anti-Bcl-2, anti-GAPDH (Cell Signaling Technology, Inc.)], respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell Signaling Technology, Inc.). The membranes were analyzed by the ECL system (Amersham Pharmacia). The signals were quantified by G:Box (Syngene).

**Electrophoretic mobility shift assay (EMSA).** NF-κB binding activity was examined in the nuclear protein preparation using a LightShift Chemiluminescent EMSA kit (Thermo Scientific) according to the instructions of the manufacturer.

**ELISA.** The levels of cytokines (TNF-α and IL-6) in the supernatants were measured using commercially available ELISA kits (Peprotech, USA) according to the instructions provided by the manufacturer.

**Statistics.** Results are expressed as means ± SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. A significant value was defined as p<0.05.

**Results**

**PVAE attenuates IL-1β-induced cytotoxicity in INS-1 cells.** We examined the effect of PVAE on IL-1β-induced cytotoxicity in INS-1 pancreatic β cells. INS-1 cells were treated with IL-1β (10 ng/ml) in the presence and absence of PVAE. (A) Cell cytotoxicity was measured by MTT assay. (B) LDH activity was measured by a kit. (C) PVAE did not have a cytotoxic effect on INS-1 cells. INS-1 cells were plated in a 96-well plate and various concentrations of PVAE (0.01, 0.1, 1, 10 and 100 µg/ml) were added for 48 h. Cell cytotoxicity was measured by MTT assay. There were three replicates in each group. **p<0.01 compared with indicated groups.**

**Figure 1.** PVAE attenuates IL-1β-induced cytotoxicity of INS-1 cells. INS-1 cells were treated with IL-1β (10 ng/ml) for 48 h in the presence or absence of PVAE. (A) Cell cytotoxicity was measured by MTT assay. (B) LDH activity was measured by a kit. (C) PVAE did not have a cytotoxic effect on INS-1 cells. INS-1 cells were plated in a 96-well plate and various concentrations of PVAE (0.01, 0.1, 1, 10 and 100 µg/ml) were added for 48 h. Cell cytotoxicity was measured by MTT assay. There were three replicates in each group. **p<0.01 compared with indicated groups.**

**PVAE attenuates IL-1β-induced cytotoxicity in INS-1 cells.** We examined the effect of PVAE on IL-1β-induced cytotoxicity in INS-1 pancreatic β cells. INS-1 cells were treated with IL-1β (10 ng/ml) in the presence and absence of PVAE (100 µg/ml) for 48 h. Cell cytotoxicity was examined by MTT and LDH activity assay (16), respectively. IL-1β treatment significantly induced cell death (Fig. 1A) and increased LDH activity by 1.51-fold (Fig. 1B) compared with the untreated cells. In contrast, PVAE administration significantly attenuated IL-1β-induced cell death (Fig. 1A) and reduced IL-1β-increased LDH activity (Fig. 1B). The data suggest that PVAE has a protective effect on IL-1β-induced cytotoxicity in INS cells.
We also examined whether PVAE itself was cytotoxic to INS-1 cells. INS-1 cells were treated with PVAE at increasing concentrations for 48 h. The cell viability was measured by MTT. As shown in Fig. 1C, PVAE treatment did not significantly induce cell death compared with untreated control, suggesting that PVAE does not have cytotoxic effects on INS cells.

**PVAE attenuates IL-1β-activated FasL/Fas-mediated apoptotic signaling in INS-1 cells.** Next, we examined how PVAE attenuates IL-1β-induced cell death. It is well known that IL-1β can induce apoptosis in β cells (18). Therefore, we examined the protective effect of PVAE on IL-1β-induced apoptosis in INS-1 cells. Caspase-3 activity is a specific marker for apoptosis (19). Fig. 2A shows that IL-1β treatment significantly increased caspase-3 activity by 7.6-fold compared with control cells. In the PVAE-treated cells, IL-1β-increased caspase-3 activity was significantly reduced by 38.4%.

Activation of apoptotic signaling can be induced by the mitochondria pathway and the death receptor pathway (1). Fig. 2B shows that IL-1β treatment did not alter the levels of Bax and Bcl-2, indicating that IL-1β-induced INS-1 cell apoptosis was not mediated by the mitochondria pathway. PVAE administration also did not affect the levels of Bax and Bcl-2 in INS-1 cells (Fig. 2B). However, IL-1β stimulation significantly increased the levels of Fas and FasL in INS-1 cells compared with untreated cells. PVAE administration significantly attenuated IL-1β-increased Fas and FasL (Fig. 2C). The data indicate that PVAE attenuates IL-1β-induced apoptosis in INS-1 cells, in part, by regulating the Fas/FasL apoptotic signaling pathway.

**PVAE decreases IL-1β-increased phosphorylation of JNK in INS-1 cells.** It is well known that the JNK signaling pathway plays a critical role in activating apoptosis (20). We examined...
whether the anti-apoptotic effect of PVAE involves blunting JNK activation. We observed that IL-1β treatment significantly increased JNK phosphorylation by 5.7-fold compared with untreated cells (Fig. 3). In the presence of PVAE, however, IL-1β-treated levels of phosphorylated JNK was significantly attenuated (Fig. 3A). However, IL-1β treatment did not increase the levels of p38 and ERK phosphorylation in the INS-1 cells (Fig. 3B and C). PVAE administration also did not affect IL-1β-mediated changes in phosphorylation of p38 and ERK in INS-1 cells (Fig. 3B and C). The data suggest that the anti-apoptotic effect of PVAE is mediated, in part, by attenuating IL-1β-activated JNK signaling.

Figure 3. PVAE attenuates IL-1β-increased JNK phosphorylation in INS-1 cells. INS-1 cells were treated with IL-1β for 30 min in the presence or absence of PVAE. The levels of phospho-JNK (A), phospho-ERK (B) and phospho-p38 (C) were examined by western blot analysis. There were three replicates in each group. *p<0.05 compared with indicated groups; **p<0.01 compared with the indicated groups.

Figure 4. PVAE prevents IL-1β-induced NF-κB activation. INS-1 cells were treated with IL-1β for 30 min in the presence or absence of PVAE. The cells were harvested and the nuclear and cytoplasmic proteins were isolated. (A) NF-κB binding activity was measured by EMSA with the nuclear proteins. (B) The levels of phosphorylated-IκB were examined by western blot analysis. There were three replicates in each group. *p<0.05 compared with indicated groups; **p<0.01 compared with the indicated groups.

**PVAE attenuates IL-1β-increased NF-κB activation in INS-1 cells.** IL-1β is an important inflammatory mediator of diabetes and stimulates NF-κB activation which controls inflammatory cytokine gene expression (21). We examined the effect of PVAE on IL-1β-stimulation of NF-κB activation in INS-1 cells. It was found that IL-1β treatment significantly increased NF-κB binding activity (Fig. 4A) by 61.1% and IκBα phosphorylation (Fig. 4B) by 2.5-fold compared with untreated cells. In contrast, PVAE administration significantly
attenuated IL-1β-increased levels of phosphorylated IkBα and NF-κB binding activity, respectively.

**PVAE attenuates IL-1β-increased inflammatory cytokine expression by INS-1 cells.** Activation of NF-κB stimulates inflammatory cytokine expression (16). We examined whether PVAE attenuates IL-1β-increased pro-inflammatory cytokine expression (Fig. 5). As expected, IL-1β stimulation significantly increased the levels of TNF-α (Fig. 5A) and IL-6 (Fig. 5B) in the supernatants of cultured INS-1 cells by 59.4% and 1.1-fold, respectively. However, PVAE administration significantly attenuated IL-1β-increased expression of both TNF-α and IL-6 in INS-1 cells. The data indicate that PVAE exerts an anti-inflammatory effect.

**Discussion**

A significant finding in the present study was that PVAE significantly prevented IL-1β-induced pancreatic β cell apoptosis and attenuated IL-1β-increased NF-κB activation, resulting in decreases in IL-1β-increased TNF-α and IL-6 production in INS-1 cells. Our data indicate that PVAE may be a novel treatment for diabetic patients.

*Prunella vulgaris* has been used as a traditional medicine for treatment of herpetic keratitis (22). Recent studies have reported that *Prunella vulgaris* extracts have anti-oxidant and anti-microbial properties (7,8). We previously reported that administration of PVAE to rats significantly reduced post-prandial hyperglycemia in ICR mice (11). PVAE treatment also regulated glucose transport gene expression (SGLT-1, GLUT-2, and Na^+-K^+-ATPase) in Caco-2 cells (23). It has been demonstrated that innate immune and inflammatory responses play an important role in the pathophysiologic mechanisms leading to the development of diabetes. For example, inflammatory cytokines, such as TNF-α, IL-1β and IL-6, induce pancreatic β cell apoptosis and promote inflammatory responses in pancreatic islets, resulting in damage of β cells (24). Therefore, anti-inflammatory responses by pharmacological approaches will prevent inflammatory cytokine-induced cytotoxicity in pancreatic β cells.

We observed in the present study that PVAE administration significantly prevented IL-1β-induced pancreatic β cell (INS-1) cytotoxicity and apoptosis. The protective effect of PVAE involved the attenuation of the IL-1β-increased Fas/Fasl-mediated apoptotic signaling pathway. Our observation was supported by numerous studies. For example, Jacobsen et al reported that IL-1β induced insulin-producing cell apoptosis through the Fas-mediated apoptotic signaling pathway (25). We also observed that PVAE administration significantly attenuated IL-1β-induced increases in JNK phosphorylation in INS-1 cells. Activation of MAPK, including JNK, stimulates pancreatic β cell apoptosis via downregulation of both Fas/Fasl and JNK-mediated apoptotic signaling pathways. Thus, PVAE maintains the function of β cells by influencing β cell proliferation and death.

Activation of the NF-κB signaling pathway contributes to dysfunction of pancreatic islet β cells (28). A number of pro-inflammatory cytokines can activate NF-κB to regulate both the survival and death of β cells. Thus, inflammatory cytokines and chemokines play an important role in the development of the inflammatory infiltrate in insulinitis in the early stages of diabetes (21). IL-1β has been reported to induce NF-κB activation in INS-1 cells (28). We observed that PVAE administration significantly protected INS-1 cells from IL-1β-induced NF-κB activation. Moreover, PVAE prevented IL-1β-increased levels of IL-6 and TNF-α in INS-1 cells. Recent studies have shown that the PVAE has an anti-inflammatory effect. Choi et al reported that PVAE suppressed phorbol myristate acetate (PMA)-induced NF-κB activation (16). When considered as a whole, PVAE exhibited a significant anti-inflammatory effect via the inhibition of NF-κB activation.

In summary, our data suggest that PVAE has a protective effect on IL-1β-induced pancreatic β cell apoptosis and cytoxicity. The mechanisms involve attenuation of IL-1β-activated Fas/Fasl and JNK apoptotic signaling pathways and prevention of IL-1β-activated NF-κB, resulting in decreases in inflammatory cytokine (TNF-α and IL-6) production. Thus, PVAE may be a novel treatment for diabetic patients.

**Figure 5.** PVAE prevents IL-1β-increased TNF-α and IL-6 expression in INS-1 cells. INS-1 cells were treated with IL-1β for 48 h in the presence or absence of PVAE. The supernatants were harvested and the levels of TNF-α (A) and IL-6 (B) were measured by ELISA. There were three replicates.

"p<0.05 compared with indicated groups; "p<0.01 compared with indicated groups.

![Graph A](image1.png)

![Graph B](image2.png)
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References