

Protective effects of fermented ginseng on streptozotocin-induced pancreatic β -cell damage through inhibition of NF- κ B

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Abstract. Ginseng (*Panax ginseng* C.A. Meyer) is widely used in Asian countries as a traditional medicine for the treatment of various diseases. It is known to have anti-inflammatory effects, although the mechanism is not clear. In this study, preventive effects of fermented ginseng (FG) against streptozotocin (STZ)-induced pancreatic β -cell death was assessed in RINm5F insulinoma cells. FG markedly inhibited the production of nitrite in a dose-dependent manner. The decrease in nitrite production was found to correlate with reduced inducible nitric oxide (iNOS) protein and mRNA levels. To characterize the anti-inflammatory mechanism of FG at the transcriptional level, we examined effects of FG on the activity of nuclear factor- κ B (NF- κ B). FG reduced a translocation of the NF- κ B subunit and NF- κ B-dependent transcriptional activity. FG blocked signaling upstream of NF- κ B activation, such as degradation of inhibitor factor- κ B α (I κ B α) and phosphorylations of extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK). These results suggest that FG protects against STZ-induced pancreatic β -cell damage by downregulation of iNOS, cyclooxygenase-2 (COX-2), and tumor necrosis factor- α (TNF- α) gene expressions by blocking NF- κ B and mitogen-activated protein kinase activities.

Introduction

Type 1 diabetes is an autoimmune disease, induced by selective destruction of insulin producing β -cells of the Langerhans islets (1). The mechanism underlying β -cell destruction is not clear (2). In the STZ-induced diabetic model, hyperglycemia arises from the irreversible destruction of pancreatic β -cells through free radicals and nitric oxide (NO), causing degranulation

and reduction of insulin secretion (3). Many effects of STZ on pancreatic islets are similar to those exerted by interleukin-1 β (IL-1 β) and some studies indicate that IL-1 β -induced islet injury as well as STZ-induced islet cytotoxicity are mediated by NO production (4,5).

NO is a free radical messenger molecule that readily diffuses through plasma lemma to exert its biological activity in a variety of cells (6). Depending on the cell type, NO is produced in an enzymatic reaction catalyzed by one of the three isoforms of NO synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (7). The third form of NOS, iNOS, is generally not present in resting cells, but is induced by various cytokines, such as IL-1 β , TNF- α and interferon- γ (IFN- γ) (8,9). Similarly, it is now known that there are two distinct isoforms of cyclooxygenase (COX), COX-1 and COX-2. COX-2 is induced by several stimuli, and is responsible for the production of pro-inflammatory prostaglandins at the inflammatory site (10). A large amount of the pro-inflammatory mediator can stimulate many proteins and enzymes crucial to inflammatory reactions, such as the NF- κ B and MAPKs pathways (11). Several studies have shown that mitogen-activated protein kinases (MAPKs) play critical roles for the activation of NF- κ B and subsequently, regulate COX-2 as well as iNOS-NO expression (12,13).

Ginseng (*Panax ginseng* C.A. Meyer) has been used for remedies in traditional Chinese medicine. Numerous studies demonstrate that ginseng improves the immune response in diabetic patients (14-16). To develop an anti-diabetic agent with better efficacy from ginseng radix, fermented ginseg (FG) was developed. Recently, FG was reported to possess hypoglycemic activity (17). In the present study, we investigated the protective effect of FG on STZ-induced β -cell dysfunction in the rat insulinoma cell line (RINm5F), and observed that FG prevented STZ-induced pancreatic β -cell damage, and suppressed iNOS, COX-2, and TNF- α gene expressions via downregulation of the MAPKs and inactivation of NF- κ B.

Materials and methods

Antibodies and chemicals. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, USA). Antibodies against ERK, phospho-ERK, JNK, phospho-JNK, I κ B α , phospho-I κ B α ,

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NF- κ B oligonucleotide probe, iNOS, COX-2, and β -actin were from Santa Cruz Biotechnology (Santa Cruz, USA). Reverse transcriptase, Taq polymerase and MTS solution were supplied by Promega (Mannheim, Germany). The polyvinylidene difluoride blotting membrane was from Millipore (Massachusetts, USA) and ECL-reagent and protein and RNA extraction kits were from Intron Biotechnology Inc. (Beverly, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise noted.

Preparation of FG. FG extract was obtained from ILHWA Co. Ltd. (Guri, Korea) which prepared the extract with its patented protocol. Briefly, the dried ginseng (1 kg) was extracted from 5 l of ethanol and concentrated with a SpeedVac. Then the dry ginseng extract was incubated with an enzyme solution containing β -galactosidase (1.5%, Sigma, USA) at 50°C for 72 h and the chilled solution was adjusted with citric acid to pH 5.6, and finally incubated the solution with cellulose (Sigma, USA) for 48 h at 37°C until diol ginsenoside peaks disappeared in UPLC chromatogram.

Analysis of ginsenosides in ginseng extracts. An Acquity liquid chromatograph (Waters, USA) equipped with gradient pump, autosampler, and diode array detection and an Acquity UPLC BEH C18 reversed-phase column (100x1.0 mm, i.d., 1.7 μ m) were used. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). The gradient elution was used as follows: 0-3 min, 5% B; 10 min, 15% B; 12 min, 30% B; 15 min, 35% B; 20 min 60% B. The column temperature was kept constant at 35°C and the flow rate was 0.5 ml/min.

Cell culture. Cells were purchased from the American Type Culture Collection. Rat islet insulinoma cell line was cultured in RPMI medium 1640 (Gibco BRL) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 2.5 μ g/ml of amphotericin B in a humidified atmosphere containing 5% CO₂ at 37°C.

MTS cell proliferation assay. The cell viability was detected by MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium) assay in which cells were incubated with MTS solution and absorbance was recorded at 490 nm. In the MTS assay, absorption is directly proportional to the number of viable cells. RINm5F cells seeded on 96-well plates at 1x10⁵ cells/well were incubated and treated with indicated concentrations of FG for 24 h. MTS reagent was then added and cells were incubated at 37°C for 1 h. At the end of the incubation period, absorbance was recorded at 490 nm (Multiskan Ascent, Thermo Labsystems, Finland).

Nitrite measurement. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reagent. Briefly, 100 μ l of culture medium was mixed with 100 μ l of Griess reagent [equal volumes of 1% (w/v) naphthylethylenediamine-HCl] and incubated at room temperature for 10 min. The absorbance at 550 nm was then measured using a microplate reader. Fresh culture medium was used as a blank in all experiments. The amount of nitrite in the test samples was calculated from a sodium nitrite standard curve.

Western blot analysis. RINm5F cells (1x10⁶ cells/dish) were pretreated with various concentrations of FG (30, 60, 90 μ g/ml) for 3 h, and then treated with STZ (10 mM) for 24 h. After 24 h, cells were harvested, and total protein extracts were prepared using a protein extraction kit and insoluble protein was removed by centrifugation at 13,000 rpm for 15 min. Supernatant was collected from the lysates and protein concentrations were determined using a Bio-Rad protein assay reagent according to the manufacturer's instruction. For Western blotting, 40 μ g of protein was separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20 mM Tris-HCl, 154 mM glycine, and 20% methanol. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TTBS) and incubated with specific antibodies and revealed with horseradish-peroxidase-conjugated secondary antibodies. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham) and then exposed to X-ray film.

RNA extraction and RT-PCR. Total RNA was isolated using an Easy-Blue total RNA extraction kit according to the manufacturer's instruction. Single-strand cDNA synthesis was performed as described previously (18), using 5 μ g of RNA, oligo-dT primers and reverse transcriptase in a total volume of 50 μ l. PCR reactions were performed in a total volume of 20 μ l comprising 2 μ l of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer and 1 unit of Taq polymerase. Oligonucleotide primer sequences used in PCR amplification were as follows, iNOS sense 5'-ATGGCTTGC CCCTGGAAFT-3', anti-sense 5'-GTACTTGGGATGCTCC ATGGTCA-3'; COX-2 sense 5'-ATGCTCTTCCGAGCTGT GCT-3', anti-sense 5'-TTACAGCTCAGTTGAACGCCT TTT-3'; TNF- α sense 5'-TACAGGCTTGTCACCTCGAATT-3'; anti-sense 5'-ATGAGCACAGAAAGCATGATC-3'; CPN sense 5'-ATGGTCAACCCACCGTG-3', anti-sense 5'-TTAG AGTTGTCCACAGTTCCGAGA-3'. For iNOS, COX-2 and CPN, PCR was performed at 95°C for 5 min, 95°C for 30 sec, followed by 57°C for 30 sec, and 72°C for 30 sec, and amplified for 30 cycles. The RT-PCR products were electrophoresed on 1% agarose gels and visualized by 0.5 μ g/ml ethidium bromide staining and scanning densitometry was performed with I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea). CPN was amplified as a control gene.

Electrophoretic mobility shift assay (EMSA). RINm5F cells (1x10⁶ cells/dish) were pretreated with various concentrations of FG (30, 60, 90 μ g/ml) for 3 h, and then 10 mM STZ was added to the culture media and after 4 h, cells were harvested, and nuclear extract was prepared in nuclear extraction kit (Fermentas, Burlington, Canada). IkB α and p-IkB α in the cytoplasmic fractions were analyzed via Western blotting. Nuclear extract (10 μ g) was mixed with the double-stranded NF- κ B oligonucleotide 5'-AGTTGAGGGGACTTTCC CAGGC-3' end-labeled by [γ -³²P]-dATP (underlying indicates a κ B consensus sequence or a binding site for NF- κ B/cRel homodimeric and heterodimeric complex). The binding reactions were performed at 37°C for 30 min in 30 μ l of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM

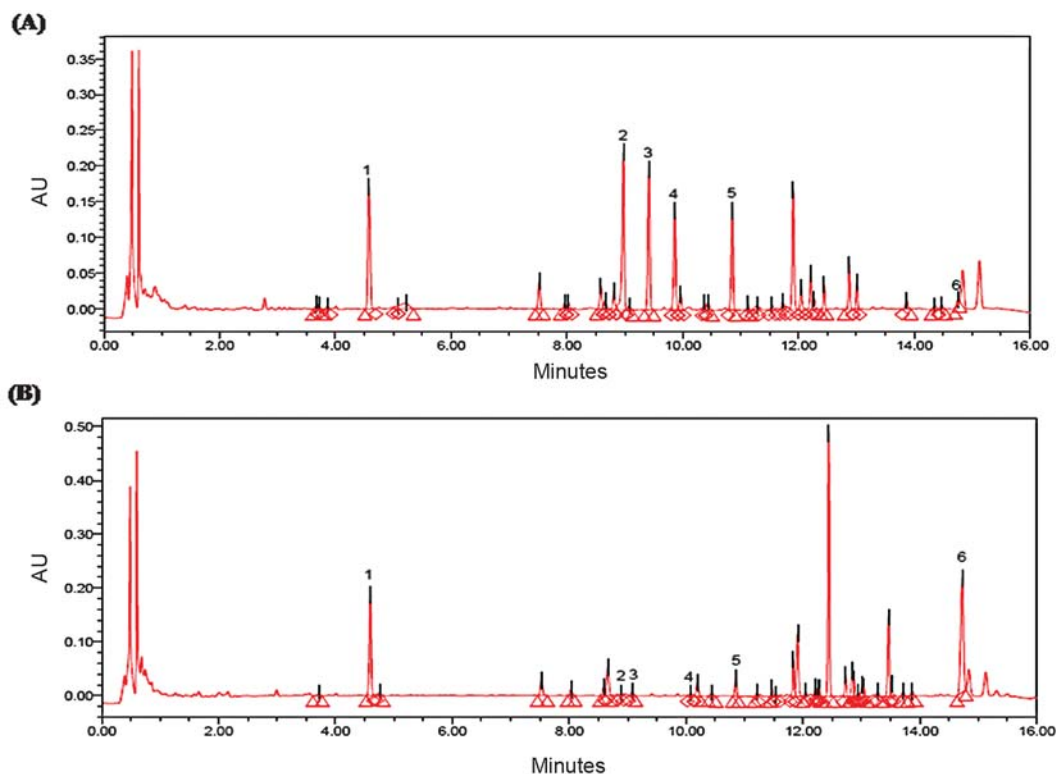


Figure 1. (A) UPLC profiles of ginseng radix and (B) fermented ginseng. 1, Rg1; 2, Rb1; 3, Rc; 4, Rb2; 5, Rd; 6, compound K.

NaCl, 1 mM EDTA, 4% glycerol, 1 μ g of poly (dI-dC) and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA/nuclear protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5x TBE buffer. The gels were vacuum dried for 1 h at 80°C and exposed to X-ray film at -70°C for 24 h.

Statistical analysis. Data are expressed as mean values $\pm$ SD and comparisons of data were done by unpaired Student's t test. Mean values were considered significantly different when $p < 0.05$.

Results

UPLC analysis. UPLC chromatograms of untreated and β -galactosidase treated ginseng radix extracts are shown in Fig. 1. The saponin peaks in untreated ginseng radix, ginsenoside Rb1, Rc, Rb2 and Rd were decreased during the enzyme treatment. After 72 h of β -galactosidase treatment, these four ginsenosides were difficult to identify in the chromatogram (Fig. 1B). On the other hand, compound K (peak 6) appeared during the enzyme process.

FG has no effect on the viability of RINm5F cells. To investigate whether FG affects the viability of RINm5F cells, a colorimetric proliferation assay was performed. FG did not affect cell viability at concentrations employed in this study (data not shown).

Effect of FG on cell proliferation. The potential protective effects of FG on cell survival in STZ-treated cells were evaluated. RINm5F cells were cultured to near confluence, then cells

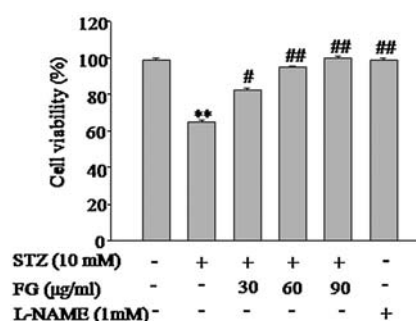


Figure 2. Prevention of STZ-induced cell death by FG. RINm5F cells (1×10^5) were pretreated with the indicated concentrations of FG or L-NAME (1 mM) for 3 h, and STZ (10 mM) were added for 24 h. The percentages of viable cells after treatment were determined by MTS assay. Each value is expressed as the mean $\pm$ SE of three independent experiments. ** $p < 0.01$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs STZ.

were treated with FG (30, 60, 90 μ g/ml) for 3 h, prior to the addition of STZ (10 mM). After 24 h, cell proliferation was determined by MTS assay. As shown in Fig. 2, STZ caused a significant reduction in cell viability to $64 \pm 1\%$ when compared to control. FG restored the viability of STZ-treated RINm5F cells in a dose-dependent manner.

Effects of FG on nitrite production and iNOS expression. To assess the effect of FG on STZ-induced nitrite production in RINm5F cells, the cells were treated with STZ (10 mM) for 24 h in the absence or presence of FG (30, 60, 90 μ g/ml). The amount of nitrite, a stable metabolite of NO, was determined as an indicator of NO production in the culture medium. STZ

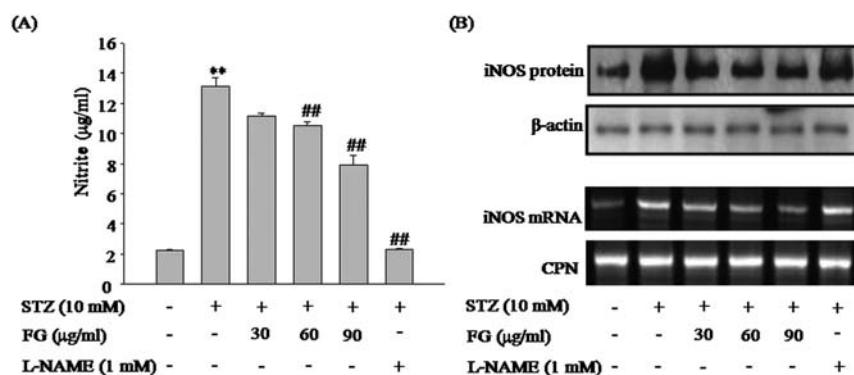


Figure 3. Effects of FG on STZ-induced NO production and iNOS expressions in RINm5F cells. (A) RINm5F cells (5×10^5) were pretreated with the indicated concentrations of FG or L-NAME (1 mM) for 3 h, and STZ (10 mM) were added. After 24 h incubation, nitrite concentration was measured in the cell-free supernatants. (B) RINm5F cells (1×10^6) were cultured in 6-well plates and pretreated with the indicated concentrations of FG or L-NAME (1 mM) for 3 h, after which STZ were added. Following 24 h incubation, iNOS protein and mRNA expression were analyzed by Western blotting and RT-PCR. Nitrite production results of triplicate samples were expressed as mean $\pm$ SE. ** $p < 0.01$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs STZ.

induced significant NO production compared to the naive control. The addition of 1 mM N[omega]-nitro-L-arginine methylester (L-NAME), an iNOS inhibitor, completely prevented the production of NO (Fig. 3) and STZ-mediated cytotoxicity as expected (Fig. 2). Pretreatment with FG was found to significantly inhibit STZ-induced nitrite production in a dose-dependent manner (Fig. 3A). Cells treated with STZ markedly increased iNOS protein and mRNA expression, whereas cells treated with FG showed suppressed expression of iNOS in both protein and mRNA levels in a dose-dependent manner (Fig. 3B).

Effects of FG on COX-2 and TNF- α expression. Inhibition of COX-2 was shown to preserve β -cell function and increase basal insulin secretion. To evaluate the effect of FG on STZ-induced expression of COX-2 genes in RINm5F cells, expressions of COX-2 protein and mRNA level were monitored by Western blot analysis and RT-PCR, respectively. As shown in Fig. 4, COX-2 protein and mRNA expressions were markedly increased in cells treated with STZ, whereas cells pretreated with FG showed suppressed expressions of the protein and mRNA level of COX-2. To further assess the potential protective effect of FG, TNF- α expression was also examined by RT-PCR. As shown in Fig. 4B, FG treatment inhibited TNF- α mRNA expression in a dose-dependent manner.

Effect of FG on NF- κ B activation. NF- κ B is an important transcription factor that, together with its inhibitor (I κ B), participates in the activation of genes involved in immune responses (19). In order to elucidate the mechanism of FG, EMSA was performed to examine its effect on STZ-induced NF- κ B activation. Treatment of RINm5F cells with STZ was found to increase NF- κ B DNA binding, and pretreating cells with FG, reduced NF- κ B DNA binding in a dose-dependent manner (Fig. 5A). NF- κ B activation usually occurs after degradation of I κ B α (20). To determine whether this I κ B α degradation was related to I κ B α phosphorylation, we examined the effect of FG on STZ-induced p-I κ B α by Western blot analysis. The result showed that FG also reduced STZ-induced I κ B α phosphorylation in a dose-dependent manner (Fig. 5B).

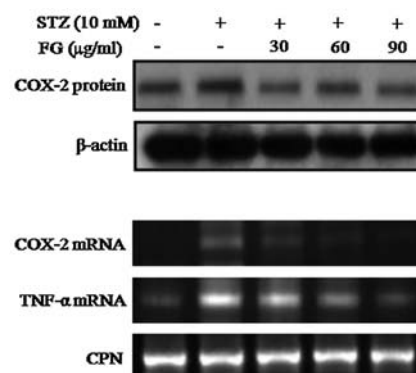


Figure 4. Effects of FG on STZ-induced COX-2 and TNF- α expressions in RINm5F cells. RINm5F cells (1×10^6) were cultured in 6-well plates and pretreated with the indicated concentrations of FG for 3 h, and STZ (10 mM) were added. After 24 h incubation, COX-2 and TNF- α expression were measured by (A) Western blotting and (B) RT-PCR.

Effects of FG on ERK1/2 and JNK phosphorylation. To investigate whether the inhibition of NF- κ B activation and NO production by FG is mediated through the MAPK pathway, we examined the effect of FG on STZ-induced phosphorylation of ERK1/2 and JNK by using Western blot analysis. Cells were pretreated with FG for 3 h and then treated with 10 mM STZ for 4 h. As shown in Fig. 6, STZ-stimulated RINm5F cells had marked strong increases in the levels of phosphorylated ERK1/2 and JNK. However, pretreatment with FG significantly suppressed STZ-induced phosphorylations of ERK1/2 and JNK (Fig. 5).

Discussion

Medicinal plants have long been used for therapeutic purposes, and many of the currently available drugs are directly or indirectly derived from plants. Ginseng is a well known medical plant used in traditional oriental medicine for several thousand years. The pharmacological properties of ginseng are mainly attributed to ginsenosides, which are the active components found in the extracts of different species of ginseng. To develop an anti-diabetic agent with better efficacy from ginseng radix,

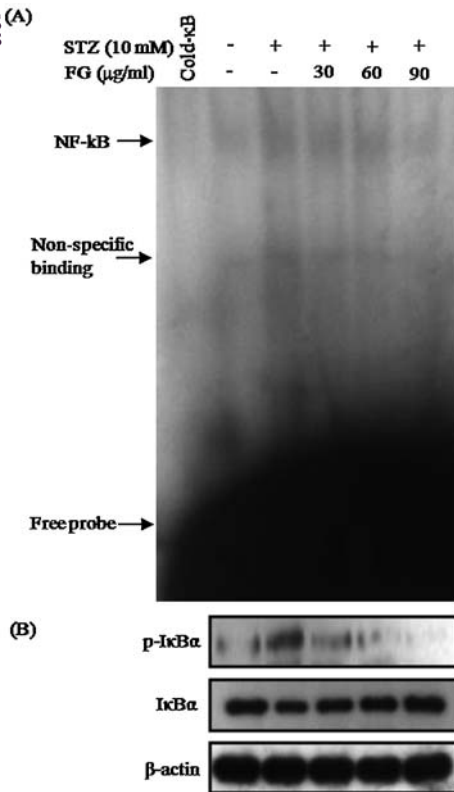


Figure 5. Effects of FG on the STZ-induced translocation of NF- κ B from cytosol to the nucleus and I κ B α degradation. RINm5F cells (1×10^6) were cultured in 6-well plates and pretreated with the indicated concentrations of FG for 3 h, and STZ (10 mM) were added. After 4 h incubation, NF- κ B translocation was analyzed by the electrophoretic mobility shift assay. Protein levels of p-I κ B α , I κ B α in the cytosolic extract were determined by Western blotting.

ginseng ethanol extract was treated with β -glucosidase to enrich compound K (Fig. 1). Recently, ginsenosides, Rb1, Rg1, Rg3, Rh2, Re, compound K have been widely documented in many studies and shown to have anti-oxidative, anti-inflammatory and anti-proliferative effects (21,22). However, the active component responsible for anti-inflammatory activity in the pancreatic β -cell has yet to be identified.

In the present study, we demonstrated that FG has a protective effect against STZ-induced β -cell death in RINm5F cells. Whereas STZ induced nitrite generation in pancreas β -cells, was attenuated by FG. Much evidence supports the hypothesis that high-output NO by iNOS provokes deleterious consequences in inflammatory diseases (3-5,23). The ability of FG to attenuate STZ-induced β -cell damage was demonstrated by modulating the activity of immune response.

NO is a short-lived and highly reactive radical (24). Besides its direct toxicity, NO reacts with superoxide to form peroxynitrite, which has much stronger oxidant activity and mediates β -cell destruction in type 1 diabetes (25). Many studies documented that the iNOS inhibitor L-NAME attenuates cytokine-induced β -cell dysfunction and islet degeneration (24-26). In our experiment, L-NAME prevented STZ-induced NO production and β -cell dysfunction. Pretreatment with FG was found to significantly inhibit STZ-induced NO production in a dose-dependent manner (Fig. 3A). This result suggests that the protective effect of FG against STZ-induced β -cell damage is due to the inhibition of nitrite production.

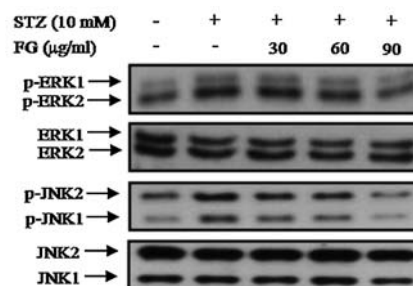


Figure 6. Effects of FG on STZ-induced phosphorylations of MAPKs in RINm5F cells. RINm5F cells (1×10^6) were cultured in 6-well plates and pretreated with the indicated concentrations of FG for 3 h, and STZ (10 mM) were added. After 4 h incubation, the whole cell lysates were analyzed by immunoblot analysis using various antibodies against the activated MAPKs.

NO is regulated mainly by the transcriptional factor NF- κ B (26). NF- κ B regulates the expressions of multiple pro-inflammatory genes that contribute to β -cell destruction such as iNOS, COX-2 and TNF- α (27,28). Therefore, we examined the DNA-binding activity of p65 to confirm the inhibition of the expressions of iNOS, COX-2 and TNF- α (Figs. 3B and 4). Our results suggested that DNA binding activity of p65 is inhibited in a dose-dependent manner by FG (Fig. 5A). In unstimulated cells, NF- κ B is located in the cytosol as an inactive complex bound to I κ B; this complex is phosphorylated, subsequently degraded and then dissociates to activate NF- κ B (29). In this study, our results demonstrate that FG prevented STZ-induced degradation and resynthesis of I κ B α protein (Fig. 5B). Therefore, these results suggest that FG inhibits STZ-induced expressions of iNOS, COX-2 and TNF- α and thus inhibit NO production through inactivation of NF- κ B by reducing I κ B α phosphorylation and degradation.

One of the most extensively investigated intracellular signaling cascades involved in pro-inflammatory responses is the MAPK pathway. The MAPK pathway is important for the activation of NF- κ B (11-13). To further investigate the molecular mechanism underlying NF- κ B inactivation and NO inhibition by FG, protective effects of FG on STZ-induced phosphorylation of ERK1/2 and JNK were examined by Western blot analysis. The data demonstrated that FG significantly inhibited the phosphorylation of ERK1/2 and JNK (Fig. 6). This result suggests that the mechanism by which pretreatment with FG inhibits STZ-induced NF- κ B activation is due to the downregulation of the phosphorylation of ERK1/2 and JNK.

In summary, our results suggest that FG prevented STZ-induced pancreas β -cell damage and inhibited NO production in STZ-induced RINm5F through suppression of iNOS, COX-2 and TNF- α expression via downregulation of the MAPK signal pathway and inactivation of NF- κ B. These data indicate that FG has a beneficial effect when used to prevent the progress of type 1 diabetes.

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