Radix asari extract protects pancreatic β cells against cytokine-induced toxicity: Implication of the NF-κB-iNOS signaling cascade

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Abstract. In this study, we assessed the preventive effects of Radix asari extract (RAE) against cytokine-induced β-cell destruction. Cytokines secreted by immune cells that have infiltrated pancreatic islets are crucial mediators of β-cell destruction in insulin-dependent diabetes mellitus. Treatment of RINm5F (RIN) cells with interleukin (IL)-1ß and interferon (IFN)-γ resulted in a reduction of cell viability and proliferation. However, treatment of RIN cells with RAE protected the IL-1ß and IFN-γ-mediated viability and proliferation reduction in a concentration-dependent manner. Incubation with RAE also resulted in significant suppression of IL-1ß and IFN-γ-induced nitric oxide (NO) production, and this reduction was correlated with reduced levels of mRNA and protein associated with the inducible form of NO synthase (iNOS). The molecular mechanism by which RAE inhibited iNOS gene expression appeared to involve the inhibition of NF-κB activation as a result of RAE's suppression of IL-1ß and IFN-γ-induced IκBα degradation. The protective effects of RAE were verified via the observation of reduced NO generation and iNOS expression, as well as the observation of normal insulin-secretion responses to glucose in IL-1ß and IFN-γ-treated rat islets. These results suggest that RAE protects β cells from cytokine toxicity by suppression of NF-κB activation.

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Introduction

Type 1 diabetes mellitus is characterized by autoimmune destruction of insulin-producing β cells in the pancreas by infiltrated immune cells in and around pancreatic islets (1-3). In an activated state, T-lymphocytes and macrophages, the primary components of islet insulitis, release high levels of interleukin (IL)-1ß and interferon (IFN)-γ, respectively. IL-1ß, alone or in combination with tumor necrosis factor (TNF)-α or IFN-γ, produces excess nitric oxide (NO) via the inducible form of nitric oxide synthase (iNOS) in pancreatic islets, which leads to apoptosis in rat and human pancreatic β cells (4-7). Nitric oxide is a short-lived and highly reactive radical that inhibits both aconitase, which is involved in the Krebs cycle, and the electron transport chain complexes I and II. This inhibition leads to decreased rates of glucose oxidation, ATP generation and insulin production (8-10). Additionally, it has been reported that the iNOS inhibitors Nω-nitro-L-arginine methylester (L-NAME) and aminoguanidine attenuate cytokine-induced β-cell dysfunction and islet degeneration (10-12).

The transcriptional nuclear factor κB (NF-κB) has been implicated as a key signaling mediator for IL-1ß toxicity, and it has been proposed that NF-κB regulates transcription of the iNOS gene (11,13). In non-stimulated cells, NF-κB is located in the cytoplasm in an inactive form that is complexed with IκB, an inhibitory factor of NF-κB. When cells are induced by various stimuli, IκB is phosphorylated by an IκB kinase complex and then degraded in the proteasome, which causes NF-κB to be released from the complex and translocated into the nucleus where it interacts with its DNA recognition sites to mediate gene transcription (14-16).

Radix asari is a rhizome that is found in Asarum sieboldii Miq., Asarum heterotropoides var. seoulense (Nakai) Kitag., or Asarum maculatum Nakai, all of which are members of the Aristolochiaceae family. Radix asari has traditionally been used to dispel colds, to alleviate pain and to relieve nasal obstruction. It has also been reported that Radix asari blocked anaphylaxis in a murine model of peanut hypersensitivity (17). However, no known studies regarding the antidiabetic
effects of *Radix asari* have been conducted. Therefore, in this study, the feasibility of using *Radix asari* to prevent IL-1β and IFN-γ-induced β-cell damage was investigated. *Radix asari* extract (RAE) inhibited IL-1β and IFN-γ-induced NF-κB activation, iNOS expression, NO formation, glucose-stimulated insulin secretion (GSIS) and viability reduction in RIN cells and islets, which may explain the beneficial effects of *Radix asari* in type 1 diabetes.

**Materials and methods**

**Cell culture.** RINm5F (RIN) cells were purchased from the American Type Culture Collection and grown at 37°C under a humidified 5% CO₂ atmosphere in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B.

**Preparation of *Radix asari* extract.** *Radix asari* was purchased from Wonkwang Oriental Medical Hospital in Iksan, Jeonbuk, Korea, and certified by Ho-Joon Song, keeper of the herbarium at the School of Oriental Medicine, Wonkwang University. Reference samples were preserved in the herbarium. For extraction, 200 g of *Radix asari* was ground in a herbarium. For extraction, 200 g of *Radix asari* was ground and extracted by placing it in boiling water for 3 h. Next, the sample was centrifuged at 3,000 x g for 20 min, and then the supernatant was concentrated to 200 ml under reduced pressure. The concentrated supernatant was then freeze-dried and stored at -70°C until used.

**MTT assay for cell viability.** The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (18). After treatment with cytokines, the cells, which were in 96-well plates, were washed twice with PBS, and then MTT (100 μg/100 μl of PBS) was added to each well. Next, the cells were incubated at 37°C for 1 h, and then DMSO (100 μl) was added to dissolve the formazan crystals. The absorbance at 570 nm was then measured using a Spectra Max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

**5-Bromo-2-deoxyuridine (BrdU) labeling cell proliferation assay.** The incorporation of BrdU during DNA synthesis was determined using a cell proliferation enzyme-linked immunosorbant assay (BrdU kit; Amersham Biosciences, Piscataway, NJ), according to the manufacturer's protocol. Briefly, cells were seeded at a density of 10⁵ cells per well in 100 μl of medium and incubated overnight in 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson, Franklin Lakes, NJ). Next, cells were treated with varying concentrations of RAE for 3 h, followed by treatment with IL-1β (5 ng/ml) and IFN-γ (100 U/ml) for 48 h. BrdU (10 μM) was then added to the culture medium for 2 h, the BrdU-labeled cells were fixed, and the DNA was denatured in fixative solution for 30 min at room temperature. The cells were then incubated with peroxidase-conjugated anti-BrdU antibody for 2 h at room temperature and then washed three times with washing solution. The immune complex was then detected by reacting the cells with 3,3′,5,5′-tetramethylbenzidine substrate and measuring the absorbance at 405 nm using a Spectra Max Plus spectrophotometer.

**Nitrite measurement.** Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions (19), therefore, nitrite concentrations in cell-free culture supernatant can be used to determine NO production. In this study, NO production in the cell-free supernatant was measured using a colorimetric assay following a previously described method (20). Briefly, following 48 h of incubation, 100 μl aliquots of the culture supernatants were incubated with 100 μl of a 1:1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid at room temperature. After 5 min, the absorbance at 540 nm was measured using a Spectra Max Plus spectrophotometer. Concentrations of NO were then determined using a linear standard curve obtained from serial dilutions of sodium nitrite in working medium.

**RNA isolation and real-time PCR.** Total RNA was isolated from RIN cells using Trizol reagent (Life Technologies Ltd., UK). RNA was precipitated with isopropanol, dissolved in DEPC-treated distilled water and kept at -80°C until use. One microgram of total RNA was added to 20 μl (final volume) of reaction buffer (10 mM Tris-HCl, pH 7.4, 50 mM MgCl₂, 1 mM each dNTP) that also contained 2.4 μM oligo-d(T)16-primer, 1 unit RNase inhibitor, and 2.5 units MuLV reverse transcriptase. The RNA was then transcibed into cDNA by incubating the reaction mixture for 10 min at 21°C, and then 15 min at 42°C. The reaction was stopped by incubation at 90°C for 5 min. Real-time PCR was then performed using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., UK) according to the manufacturer's instructions. Specific primers for the iNOS and β-actin genes were designed using the LightCycler software 4 (Roche). The following primer sequences were used: iNOS gene upstream primer, 5'-GTGGTTGCTCTTGCTGATAATT-3' and downstream primer, 5'-GTGGTTGCTCTTGATACTTTCA-3'; β-actin gene upstream primer, 5'-GTGGCTATGTGATCTCAGT-3' and downstream primer, 5'-CACAGATTTCATACCCAGG-3'. Real-time PCR was conducted using a 20-μl reaction mix containing 100 ng of reverse transcribed total RNA, 500 nM of the forward and reverse primers, and 14 μl of 2X SYBR-Green buffer (Roche). PCR amplification consisted of a 10-min preincubation step at 95°C, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 58°C (iNOS) or 63°C (β-actin) for 5 sec, and elongation at 72°C for 10 sec. The relative concentrations of PCR product derived from the target gene (iNOS) were calculated using the LightCycler System software. Results were expressed relative to the number of β-actin transcripts used as an internal control. All experiments were performed in triplicate.

**Western blot analysis.** Cells were homogenized in 100 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenates containing 20 μg of protein were separated by SDS-PAGE using a 10% acrylamide resolving gel and a 3%
stacking gel, and then transferred to nitrocellulose sheets in a Western blot apparatus (Bio-Rad, Hercules, CA). The nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 h with 1 μg/ml of primary antibodies for iNOS, IκBα, IκBβ, proliferating cell nuclear antigen (PCNA), or p50 (Santa Cruz Biochemicals, Santa Cruz, CA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA) was used as the secondary antibody. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA).

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (21). Cells were washed twice, immediately scraped into 1.5 ml of ice-cold PBS (pH 7.9), and then pelleted at 12,000 x g for 30 sec. Next, the cell pellets were suspended in ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM MgCl₂, 0.2 mM KCl, 0.2 mM PMSF, and 0.5 mM dithiothreitol), vortexed for 10 sec and then incubated on ice for 15 min. The packed cells were then resuspended in ice-cold hypotonic lysis buffer in the presence of 50 μl of 10% Nonidet P-40 and incubated on ice for 25 min. Next, the nuclear fraction was precipitated by centrifugation at 13,000 x g for 1 min at 4°C. The supernatants (cytosolic extracts) were collected and stored at -80°C for later use. The nuclear pellet was re-suspended in 50-100 μl of low salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol), added to an equal volume of high salt extraction buffer (200 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol) in a dropwise fashion, and then incubated under continuous shaking at 4°C for 45 min. The sample was then centrifuged for 20 min at 12,000 x g, and the nuclear extract was aliquoted and stored at -80°C. The concentration of protein in the nuclear extract was determined using the Bradford method (22).

Electrophoretic mobility shift assay (EMSA). A gel mobility shift assay using nuclear extracts obtained from control and treated cells was conducted to determine if activation of NF-κB had occurred. The gel retardation assay was probed using an oligonucleotide containing the κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′). Briefly, the two complementary strands were annealed and labeled with [α-32P]dCTP. A 20-μl reaction mixture containing labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly(dl-dC), 1 mM dithiothreitol) was then incubated for 30 min at room temperature. Next, the reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5X Tris-borate buffer followed by drying and examination of the gel by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

Isolation of islets. Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion, as described previously (23). Following isolation, the islets were cultured overnight in RPMI-1640 medium containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 5% CO₂ at 37°C. Prior to each experiment, the islets were washed three times in RPMI-1640, counted and then cultured overnight.

Insulin secretion assay. The islets were cultured for 24 h with cytokines in the presence or absence of RAE. The islets were then washed three times in Krebs-Ringer bicarbonate buffer (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, and 0.1% bovine serum albumin, pH 7.4) containing 3 mM D-glucose, and then insulin secretion assays were performed in the presence of either 5.5 or 20 mM D-glucose. The insulin content of the medium was then determined by ELISA (24).

Statistical analysis. Statistical analysis of the data was performed using the Student's t-test and ANOVA. Differences of p<0.05 were considered statistically significant.

Results

Prevention of cytokine-induced viability reduction by RAE. RIN cells from a rat pancreatic B cell line were cultured to near confluence. Treatment with a combination of IL-1β (5 ng/ml) and IFN-γ (100 U/ml) caused a significant reduction in cell viability of 36.8±2.3%. In contrast, pretreatment with RAE increased the viability of IL-1β and IFN-γ-treated RIN cells in a concentration-dependent manner (Fig. 1A). The protective effect of RAE on IL-1β and IFN-γ-induced cytotoxicity was further confirmed by measuring the level of BrdU incorporation into the RIN cells. BrdU is a thymidine analog that is incorporated into proliferating cells during DNA synthesis; therefore its incorporation reflects the proliferative potential of the cells. Treatment with a combination of IL-1β and IFN-γ for 48 h caused a significant reduction in BrdU incorporation compared to that of the control (Fig. 1B). Pretreatment with RAE prevented the cytokine-mediated decrease in cell proliferation potential as its concentration was increased (Fig. 1B), which is similar to the results obtained from the MTT assay. Treatment with RAE alone did not affect the viability, even at higher treatment concentrations (10 mg/ml) (data not shown).

Effect of RAE on IL-1β and IFN-γ-induced NO production and iNOS expression by RIN cells. It has been reported that IL-1β and IFN-γ-mediated destruction of B cells is caused by increases in NO production and iNOS expression (11,25). RIN cells that were incubated with IL-1β and IFN-γ for 48 h showed significant production of nitrite (a stable oxidized product of NO) (Fig. 2A). Furthermore, addition of L-NAME, an inhibitor of iNOS, completely prevented the IL-1β and IFN-γ-induced reduction in viability, as expected (Fig. 1). Additionally, the presence of RAE diminished the cytokine-mediated nitrite production of RIN cells (Fig. 2A), and this reduction was well correlated with their increased viability. To examine whether RAE inhibits NO production via suppression of iNOS gene expression, changes in iNOS mRNA and protein levels were investigated by real-time PCR and Western blot analysis, respectively. Cells treated
with IL-1β and IFN-γ exhibited marked increases of iNOS mRNA (Fig. 2B) and 130 kDa iNOS protein (Fig. 2C) expression, whereas cells pretreated with RAE showed suppressed expression of iNOS, and this suppression occurred in a concentration-dependent manner (Fig. 2B and C). These results indicate that the cytoprotective effect of RAE against IL-1β and IFN-γ is due to the suppression of iNOS expression.

**Effect of RAE on IL-1β and IFN-γ-induced NF-κB activation.**

NF-κB has been implicated in the transcriptional regulation of cytokine-induced iNOS expression. Therefore, we investigated the effect of RAE on cytokine-stimulated translocation of NF-κB from the cytosolic compartment to the nucleus in RIN cells. The IL-1β and IFN-γ-stimulated RIN cells showed increased NF-κB binding activity, as well as increased levels of the p50 subunit present in their nuclei (Fig. 3A and B). Furthermore, the IL-1β and IFN-γ-stimulated RIN cells showed increased iκBα degradation in the cytosol (Fig. 3C) when compared to that of unstimulated cells. Additionally, iκBα was not affected by IL-1β and IFN-γ treatment (data not shown). Finally, IL-1β and IFN-γ-induced NF-κB activation and iκBα degradation were suppressed by the addition of RAE in a concentration-dependent manner, which suggests that RAE inhibits NO production and iNOS expression through the inhibition of iκBα degradation and NF-κB activation. The specificity of the DNA-protein interactions for NF-κB was demonstrated by competition assays using a 50-fold excess of unlabeled oligonucleotide (Fig. 3A, lane 7).

**Reduction of NO production and iNOS expression, and preservation of glucose-stimulated insulin secretion by RAE in the presence of IL-1β and IFN-γ.** We assayed the preventive effects of RAE on cytokine-induced NO production, iNOS expression and GSIS in rat pancreatic islets isolated from male Sprague-Dawley rats to support the physiological importance of the results observed in the cell line studies. Incubation of rat islets for 24 h with IL-1β and IFN-γ resulted in 4-fold and 7-fold increases in nitrite production (Fig. 4A) and iNOS mRNA expression (Fig. 4B), respectively. However, islets treated with IL-1β and IFN-γ in the presence of RAE showed levels of nitrite production and iNOS mRNA similar to those of the controls. The incubation of islets with IL-1β and IFN-γ for 24 h also resulted in marked expression of iNOS protein (Fig. 4C), as well as a significant reduction
Additionally, pretreatment of islets with RAE blocked the effects of IL-1ß and IFN-γ on iNOS expression, and restored the insulin secretion responses to glucose to the levels observed in the control islets. In addition, treatment with RAE alone did not affect the insulin secretion responses to glucose (data not shown).

Discussion

In this study, we examined the preventative effects of RAE on IL-1ß and IFN-γ-induced toxicity in β cells. MTT and BrdU assays demonstrated that RIN cells that had been pretreated with RAE were resistant to cytokine-induced cytotoxicity. Furthermore, the protective actions of RAE on cellular metabolism were associated with an inability of β cells to express iNOS or produce NO in response to cytokines. Additionally, activation of the transcriptional regulator, NF-κB, was required for cytokine-induced iNOS expression by β cells. We also showed that pretreatment with RAE prevented cytokine-induced NF-κB nuclear translocation by inhibiting IkBα degradation.

Exposure of human or rodent β cells to cytokines as humoral mediators of inflammation causes functional impairment of β cells, ultimately leading to cell death (11,13). It has previously been shown that a combination of IL-1ß and IFN-γ is required to induce NO formation by human islets (4,6,11,26), and that the toxicity of this cytokine combination is due to a significantly higher rate of iNOS mRNA and protein expression and subsequent NO production in pancreatic β cells (21,27). Studies conducted on other cell types suggest that the putative mechanisms of IFN-γ action include stabilization of iNOS mRNA (28), potentiation of IL-1ß-induced NF-κB activation (29), and induction of other nuclear transcription factors, such as interferon regulatory factor-1 (30).
It has been reported that NF-κB can govern both pro-inflammatory and antiapoptotic responses according to the modes of insults to the β cells. NF-κB regulates the expression of multiple proinflammatory genes that contribute to islet destruction, such as Fas, iNOS and cyclooxygenase-2 (4,31,32). In addition, the promoters of other proinflammatory genes induced in β cells, including chemokines and adhesion molecules, also possess binding elements for NF-κB (16). The importance of NF-κB in β-cell damage is underscored by the fact that inhibition of NF-κB activation or translocation prevents IL-1β and IFN-γ-induced β-cell dysfunction and death in both in vitro and in vivo models (24,33-35). In contrast, the defensive and protective role of NF-κB is also reported. Activation of NF-κB limited tissue damage in the cerulean-induced acute pancreatitis model, however, when NF-κB activation was blocked, more tissue damage occurred as a result of increased apoptosis (36). NF-κB regulates apoptosis by controlling the expression of multiple antiapoptotic genes, including the inhibitors of apoptosis protein (IAP) and A20/tumor necrosis factor (TNF)-induced protein 3 (TNFAIP3) (37,38). Blockage of NF-κB via the use of an IκBα super-repressor also sensitized β cells to TNF-α-mediated apoptosis (39). Recently, Kim et al reported that NF-κB had a proapoptotic function in β cells incubated with a combination of IL-1β and IFN-γ, and that it showed an antiapoptotic effect in β cells treated with TNF-α and IFN-γ (40). Therefore, it remains unclear whether NF-κB activation in the pancreas is protective or detrimental.

Previous reports have provided evidence that chemical inhibitors of NO generation protected insulin-secreting cells against cytokine-mediated toxicity, however, the efficacy of this protection varied depending on the species and the combination of cytokines used (41,42). We previously reported that herbs and dietary supplements had protective effects against cytokine- or cytokinin-induced β-cell damage that occurred through the suppression of iNOS expression (7,21,27,43,44). Numerous other studies have also attempted to demonstrate that β cells could be protected from cytokine and cytotoxin-induced damage by repressing either NF-κB activation or NF-κB-dependent iNOS expression (45-48). It was recently reported that lentiviral vector-based iNOS-specific shRNA efficiently suppressed the cytokine-mediated induction of iNOS expression and the accumulation of nitrite, thereby providing significant protection against the cytotoxic effects of cytokine exposure in β cells (49). Similarly, repression of NF-κB or its downstream iNOS expression is important for protecting β cells from various kinds of diabetogenic agents. In the current study, we further demonstrated the importance of NO generation in β-cell damage, which was prevented by L-NAME, an inhibitor of iNOS. Taken together, these results indicate that NO is an indispensable component of cytokine- or cytotoxin-induced toxicity of β cells, and the protective effect of RAE against IL-1β and IFN-γ-mediated cell death is due to the inhibition of NO generation.

In summary, we demonstrated that RAE has an inhibitory effect on cytokine-induced toxicity in β cells using an insulinoma cell line and isolated pancreatic islets. RAE rescued β cells from cytokine toxicity and completely restored their function. To our knowledge, this is the first report regarding the relationship of Radix asari extract and the prevention of β-cell damage as a result of cytokine toxicity. The results of this study provide valuable information that will help elucidate the mechanisms involved in autoimmune β-cell destruction, as well as provide insight into the development of drugs for the treatment of type 1 diabetes.

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