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Coptidis rhizoma extract protects against cytokine-induced death of pancreatic β-cells through suppression of NF-κB activation

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Abbreviations: CRE, *Coptidis rhizoma* extract; GSIS, glucose-stimulated insulin secretion; NO, nitric oxide

Abstract

We demonstrated previously that *Coptidis rhizoma* extract (CRE) prevented S-nitroso-N-acetylpenicillamine-induced apoptotic cell death via the inhibition of mitochondrial membrane potential disruption and cytochrome c release in RINm5F (RIN) rat insulinoma cells. In this study, the preventive effects of CRE against cytokine-induced β-cell death was assessed. Cytokines generated by immune cells infiltrating pancreatic islets are crucial mediators of β-cell destruction in insulin-dependent diabetes mellitus. The treatment of RIN cells with IL-1β and IFN-γ resulted in a reduction of cell viability. CRE completely protected IL-1β and IFN-γ-mediated cell death in a concentration-dependent manner. Incubation with CRE induced a significant suppression of IL-1β and IFN-γ-induced nitric oxide (NO) production, a finding which correlated well with reduced levels of the iNOS mRNA and protein. The molecular mechanism by which CRE inhibited iNOS gene expression appeared to involve the inhibition of NF-κB activation. The IL-1β and IFN-γ-stimulated RIN cells showed increases in NF-κB binding activity and p65 subunit levels in nucleus, and IκBα degradation in cytosol compared to unstimulated cells. Furthermore, the protective effects of CRE were verified via the observation of reduced NO generation and iNOS expression, and normal insulin-secretion responses to glucose in IL-1β and IFN-γ-treated islets.

Keywords: cell death; *Coptidis rhizoma* extract; cytokines; drugs, Chinese herbal; insulin secreting cells; NF-kappa B; nitric oxide

Introduction

Type 1 diabetes mellitus is an autoimmune disease which induces a selective destruction of the insulin-producing β-cells of the Langerhans islets (Nossal *et al.*, 1992). A great deal of evidence supports the crucial role of infiltrating immune cells in and around the pancreatic islets at early stages of pathogenesis (Kanazawa *et al.*, 1984; Jorns *et al.*, 2005). In their activated state, T-lymphocytes and macrophages, which are primary cellular components of islet insulitis, secrete high levels of IL-1β and IFN-γ, respectively. IL-1β alone, or in combination with TNF-α or IFN-γ, induces an excess production of nitric oxide (NO) by the iNOS in the pancreatic islets (Cetkovic-Cvrlje and Eizirik, 1994; Corbett and McDaniel, 1995; Heitmeier *et al.*, 1997; Kwon *et al.*, 2003a). Nitric oxide is a short-lived and highly reactive radical, which inhibits the Krebs-cycle enzyme aconitase and electron transport chain complexes I and II, resulting in reduced glucose oxidation rates, ATP generation, and insulin production (Welsh *et al.*, 1991; Corbett *et al.*, 1992; Cunningham and Green, 1994). In fact, iNOS inhibitors, such as N^{w}-nitro-L-arginine methylester (L-NAME) and aminoguanidine, attenuate cytokine-induced β-cell dysfunction and islet degeneration (Southern *et al.*, 1990; Welsh *et al.*, 1991; Eizirik *et al.*, 1996).

The transcriptional NF-κB has been implicated as

a key IL-1β signaling mediator and has also been suggested as a regulator of the transcription of the iNOS gene (Eizirik *et al.*, 1996; Mandrup-Poulsen, 1996). NF-κB is initially located within the cytoplasm as an inactive form complexed with IκB, an inhibitory factor of NF-κB. A variety of inducers cause the dissociation of this complex, presumably via the phosphorylation of IκB, permitting NF-κB to be released from the complex. NF-κB is then translocated to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription (Baeuerle and Henkel, 1994; Baldwin, 1996; May and Ghosh, 1998).

Coptidis rhizoma is a rhizome of *Coptis chinensis Franch,* and a member of the *Ranunculaceae* family. It has been employed in Chinese medicine for the treatment of diabetes mellitus. A great deal of evidence suggests that it may also prove effective against inflammation (Iizuka *et al.*, 2000a), cancer cell proliferation (Iizuka *et al.*, 2000b, 2002), and the induction of novel growth-related genes, most notably *rcl* (Chan *et al.*, 2002). We recently reported that the pretreatment of RIN cells with *Coptidis rhizoma* extract (CRE) prevented S-nitroso-N-acetylpenicillamine-induced apoptotic cell death (Kwon *et al.*, 2005). In the present study, we have attempted to determine the feasibility of *Coptidis rhizoma* as a means for the prevention of IL-1β and IFN-γ-induced β-cell damage. CRE 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 anti-diabetic effects associated with *Coptidis rhizoma*.

Materials and Methods

Cell culture

RINm5F (RIN) cells were obtained from the American Type Culture Collection and grown at 37°C under a humidified, 5% CO_2 atmosphere in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B.

Preparation of *Coptidis rhizoma* extract

The plants were purchased by the Wonkwang Oriental Medical Hospital in Iksan, Chonbuk, South Korea, and were confirmed by Professor Ho-Joon Song. Voucher samples were preserved in the Herbarium of the Department of Physiology, at the School of Oriental Medicine, Wonkwang Univ. (Omcphy 2001-90). For extraction, 200 g of *Coptidis rhizoma* was ground and extracted with boiling water for 4 h. After 20 min of centrifugation at 3,000 × *g*, the supernatant was concentrated under reduced pressure to 200 ml and freeze-dried to 32.12 g. The sterile extract was then stored at -70°C.

MTT assay for cell viability

The viability of the cultured cells was determined via assays for the reduction of MTT to formazan, as previously described (Zhang *et al.*, 2005). In brief, after 48 h of incubation, cells (10^4/ well) in 96-well plates were washed twice with PBS. MTT (100 μg/0.1 ml of PBS) was added to each of the wells. Cells were then incubated for 1 h at 37°C, and DMSO (100 μl) was added in order to dissolve the formazan crystals. The absorbance was determined at 570 nm with a model Spectra MAX PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA).

5-Bromo-2-deoxyuridine (BrdU)-labeling cell proliferation assay

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Amersham Biosciences, Piscataway, NJ) was employed to measure the incorporation of BrdU during DNA synthesis, in accordance with the manufacturer's protocols. In brief, the cells were seeded overnight in 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson, Franklin Lakes, NJ) at a density of 10^5 cells per well in 100 μl of medium. The cells were treated for 48 h at a range of concentrations. BrdU (10 μM) was added to the culture medium for 2 h, the BrdU-labeled cells were fixed, and the DNA was denatured for 30 min in fixative solution at room temperature. The cells were incubated for 2 h with peroxidase-conjugated anti-BrdU antibody at room temperature and washed three times in washing solution. The immune complex was detected via the 3,3',5,5'-tetramethylbenzidine substrate reaction and absorbance was determined at 405 nm with a Spectra MAX PLUS spectrophotometer.

Nitrite measurement

Biologically generated NO is oxidized rapidly to nitrite and nitrate in aqueous solutions (Moncada *et al.*, 1991). Nitrite concentrations in the cell-free culture supernatant, therefore, were utilized as a reflection of NO production, and were measured via a colorimetric assay (Green *et al.*, 1982). 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, absorbance was determined at 540 nm using a Spectra MAX PLUS spectrophotometer. NO concentrations were determined from a linear standard curve obtained from serial dilutions of sodium nitrite in working medium.

RNA isolation and RT-PCR for iNOS

Total RNA was isolated from RIN cells with Trizol reagent (Life Technologies Ltd, UK). RNA was precipitated with isopropanol, dissolved in DEPC-treated distilled water, and maintained at -80°C until use. One microgram of total RNA was transcribed into cDNA in a 20 μl final volume of reaction buffer (10 mM Tris-HCl, pH 7.4, 50 mM $MgCl_2$, 1 mM each dNTP) and 2.4 μM oligo-d(T)16-primer, 1 U RNase inhibitor, and 2.5 U MulV reverse transcriptase via 10 min of incubation at 21°C and 15 min at 42°C. The reaction was halted via 5 min of incubation at 99°C. For rat iNOS PCR, aliquots of the synthesized cDNA were added to a 45 μl PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2 U *Taq* DNA polymerase, and 0.4 μM of each PCR primer; upstream primer, 5'-CCACAATAGTACAATACTACTTGG-3', downstream primer, 5'-ACGAGGTGTTCAGCGTGCTCCACG-3'. Amplification was initiated with 3 min of denaturation at 94°C followed by 26 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. After the last amplification cycle, the samples were incubated for 5 min at 72°C. β-actin PCR was conducted with 2.5 μl of an aliquot of synthesized cDNA using primers at a concentration of 0.15 μM; upstream primer, 5'-TGCCCATCTATGAGGGTTACG-3' downstream primer, TAGAAGCATTTGCGGTGCACG-3'. The obtained PCR products were analyzed on ethidium bromide-stained agarose (1.5%) gels.

Real-time PCR for iNOS in islets

Total RNA was isolated from batches of 30 islets using Trizol reagent (Life Technologies Ltd, UK). Real-time PCR was conducted using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, UK) in accordance with the manufacturer's instructions. Specific primers for the iNOS and β-actin genes were designed using LightCycler software 4 (Roche). The primer sequences were as follows: iNOS gene upstream primer, 5'-GTTCTTTGCTTCTGTGCTAAT-3' downstream primer, 5'-GTGTTTGCCTTATACTGTTCCA-3'; β-actin gene upstream primer, 5'-GTGCTATGTTGCTCTAGACT-3' downstream primer, 5'-CACAGGATTCCATACCCAAG-3'. The real-time PCR contained, in a final volume of 20 μl, 100 ng of reverse-transcribed total RNA, 500 nM of the forward and reverse primers, and 14 μl of 2 × SYBR green buffer (Roche). PCR amplification was initiated with a 10-min pre-incubation step at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 58°C (iNOS) or 63°C (β-actin) for 5 s, and an elongation step at 72°C for 10 s. The relative concentrations of the PCR products derived from the target gene (iNOS) were calculated using LightCycler System software. The results were expressed relative to the number of β-actin transcripts used as an internal control. All experiments were conducted 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, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The homogenates, containing 20 μg of protein, were separated via SDS-PAGE with 10% acrylamide resolving and 3% stacking gels, then transferred to nitrocellulose sheets in a Western blot apparatus (Bio-Rad, Hercules, CA). The nitrocellulose paper was blocked with 2% BSA, then incubated for 4 h with 1 μg/ml of primary antibodies for iNOS, IκBα, IκBβ, or p65 (Santa Cruz Biochemicals, Santa Cruz, CA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA) was employed as a secondary antibody. Protein expression levels were determined via analysis of the signals captured on nitrocellulose membranes, using a Chemi-doc image analyzer (Bio-Rad).

Preparation of nuclear extracts

Nuclear extracts were prepared as previously described (Park and Park, 2001). The cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.9), and pelleted at 12,000 × *g* for 30 s. The cell pellets were then suspended in ice-cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM KCl, 0.2 mM phenylmethylsulfonylfluoride and 0.5 mM dithiothreitol), vortexed for 10 s and then centrifuged for 5 min at 3,000 rpm. The packed cells were resuspended with ice-cold hypotonic lysis buffer in the presence of 50 μl of 10% Nonidet P-40, then incubated for 25 min on ice. The nuclear fractions were precipitated via 15 min of centrifugation at 4,000 rpm. IκBα and IκBβ in the cytoplasmic fractions were analyzed via Western blotting. The pelleted nuclei were then resuspended in 50-100 μl of low salt extraction buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride and 0.5 mM dithiothreitol) and added to equal volumes of high salt extraction buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl-

fluoride and 0.5 mM dithiothreitol) in a dropwise fashion, then incubated for 45 min under continuous shaking at 4°C. The samples were centrifuged for 20 min at 12,000 × *g*. Aliquots of the nuclear extracts were stored at -80°C. Protein concentrations were determined by the Bradford method (Bradford, 1976).

Electrophoretic mobility shift assay (EMSA)

NF-κB activation was assayed via a gel mobility shift assay using nuclear extracts from the control and treated cells (Kim *et al*., 2005). As a probe for the gel retardation assay, an oligonucleotide harboring the κ-chain binding site (κB, 5'-CCGGTTAACAG-AGGGGGCTTTCCGAG-3') was synthesized. The two complementary strands were annealed and labeled with [α-^{32}P] dCTP. 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(dI·dC) and 1 mM DTT) were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixtures were analyzed via electrophoresis on 4% polyacrylamide gel in 0.5 × Tris-borate buffer. The gels were dried and examined via 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 via collagenase digestion as previously described (Kim *et al*., 1994). After isolation, the islets were cultured overnight in RPMI-1640 (RPMI-1640 containing 2 mM L-glutamine, 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) under an atmosphere of 95% O_2 and 5% CO_2 at 37°C. Prior to each experiment, the islets were washed three times in RPMI-1640, counted, and cultured overnight.

Insulin secretion assay

The islets were cultured for 24 h with cytokines in the presence or absence of CRE. The islet were washed three times in Krebs-Ringer bicarbonate buffer (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 0.1% BSA, pH 7.4) containing 3 mM D-glucose, and insulin secretion assays were conducted in the presence of either 5.5 or 20 mM D-glucose. The insulin contents of the medium were determined via ELISA (Park *et al*., 1995).

Statistical analysis

Statistical analysis of the data was performed with ANOVA and Duncan's test. Differences with $P < 0.05$ were considered statistically significant.

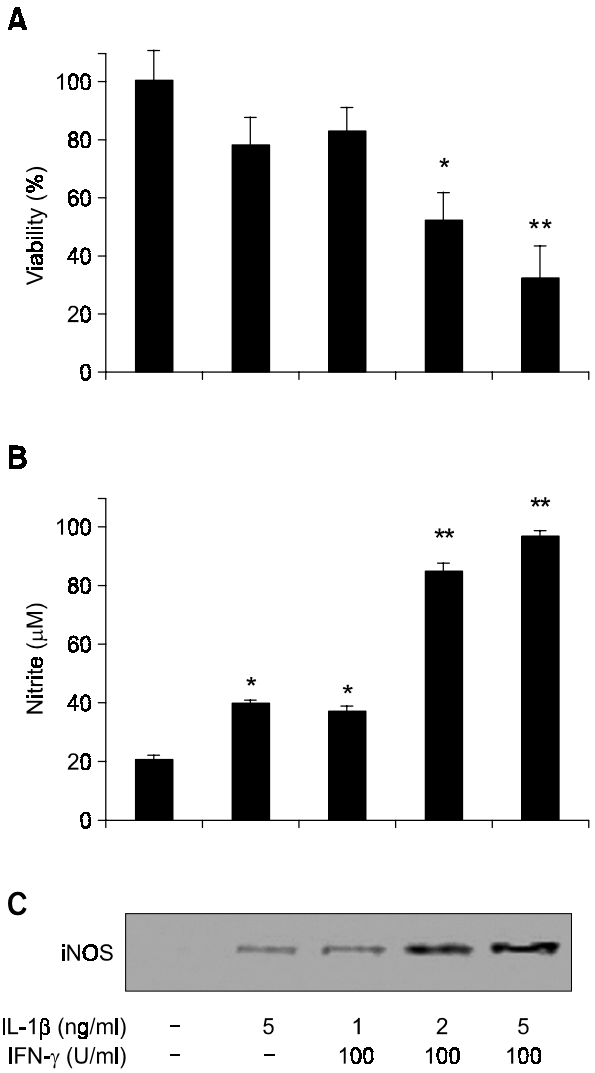

Figure 1. Cytokine-induced viability, NO production, and iNOS expression in RIN cells. (A) RIN cells (1×10^5) were incubated with IL-1β alone or combined with IFN-γ as indicated. The percentage of cells viable after these treatments was determined via MTT colorimetric assay and calculated as the A_{570} ratio of treated- and control cells (treated with 0.05% DMSO vehicle). (B) Cells (4×10^5) were treated for 48 h with the cytokines and the nitrite concentrations were assessed in the cell-free culture supernatants as a reflection of NO synthesis. The results of triplicate samples were expressed as the means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. untreated control. (C) Cells (5×10^6) were incubated with cytokines and Western blot analysis for iNOS was conducted.

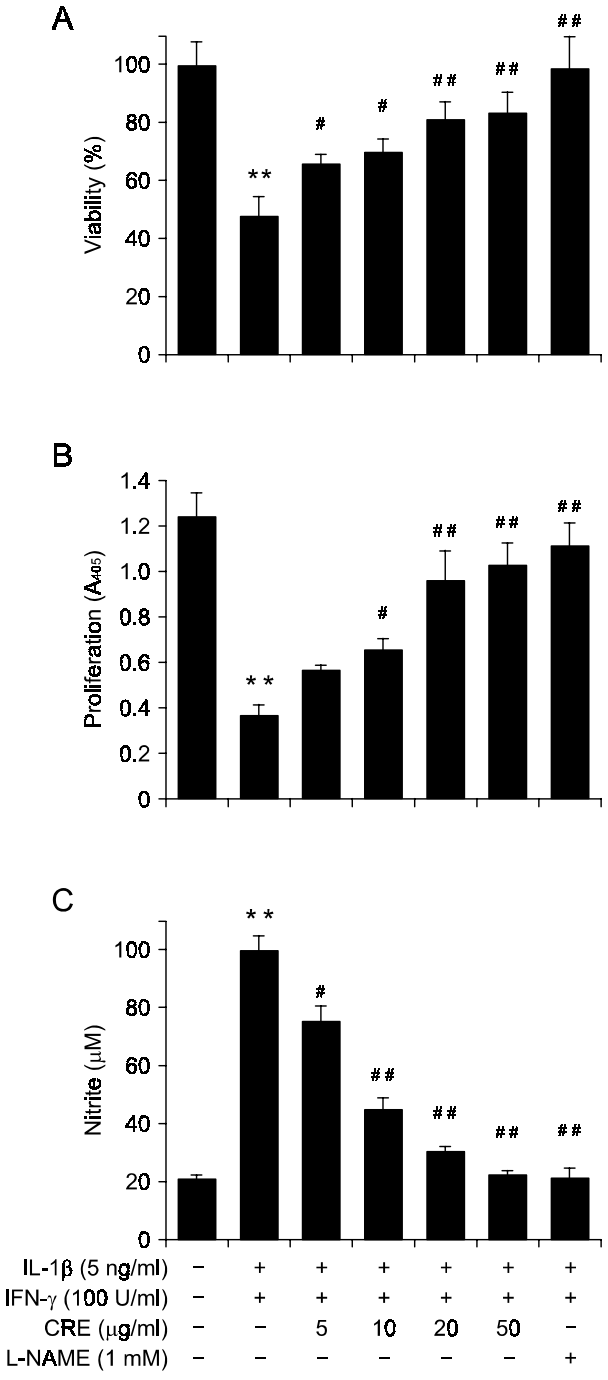

Figure 2. Prevention of cytokine-induced cell death by CRE. RIN cells (1×10^5) were incubated with cytokines in the presence or absence of CRE or L-NAME for 48 h as indicated. Cell viability (A), proliferation capacity (B), and NO production (C) were determined. Each value is expressed as the mean ± SEM of four independent experiments. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. IL-1β + IFN-γ.

Results

Prevention of cytokine-induced viability reduction by CRE

RIN cells from a rat pancreatic β-cell line were cultured to near confluence. A single treatment with IL-1β (5 ng/ml) induced a reduction in viability to 78.2 ± 9.7%, whereas the combination of IL-1β (5 ng/ml) and IFN-γ (100 U/ml) induced a significant reduction in cell viability, of 32.6 ± 10.8% (Figure 1A). By way of contrast, CRE increased the viability of IL-1β and IFN-γ-treated RIN cells in a concentration-dependent manner (Figure 2A). The protective effects of CRE on IL-1β and IFN-γ-induced cytotoxicity were confirmed further via the incorporation of BrdU into RIN cells. BrdU is a thymidine analogue that is incorporated into proliferating cells during DNA synthesis; thus, it is reflective of the proliferative potential of the cells. IL-1β and IFN-γ reduced the level of BrdU incorporation, hence proliferation, at 48 h incubations to 29.5 ± 3.2% of control levels (Figure 2B). Consistent with the data obtained in the MTT assay, CRE prevented the cytokine-mediated decrease in cell proliferation potential, coupled with an increase in its concentration (Figure 2B). CRE alone did not affect the viability, even at higher concentrations (1 mg/ml) (data not shown).

Effect of CRE on IL-1β and IFN-γ-induced NO production by RIN cells

It has been reported that the IL-1β and IFN-γ-mediated destruction of β-cells is induced by an increase in NO production (Eizirik *et al.*, 1996; Flodstrom *et al.*, 1996). The incubation of RIN cells with IL-1β and IFN-γ for 48 h resulted in significant nitrite (a stable oxidized product of NO) production by these cells (Figure 1B). The addition of L-NAME, an iNOS inhibitor, completely prevented the IL-1β and IFN-γ-induced reduction in viability, as had been expected (Figure 2). The presence of CRE attenuated cytokine-mediated nitrite production, a result that was correlated well with its increased viability (Figure 2). In order to determine whether CRE inhibits NO production via the suppression of iNOS gene expression, changes in iNOS mRNA and protein levels were investigated via RT-PCR and Western blot analysis, respectively. Cells treated with IL-1β alone induced a slight increase in iNOS mRNA (Figure 1C, lane 2) and 130 kDa iNOS protein expression (Figure 3, lane 2), whereas marked increases of iNOS mRNA and protein were observed in the cells treated with IL-1β and IFN-γ (Figure 1C, lane 5 and Figure 3A and 3B, lane 3). Cells pretreated with CRE at a concentration of 50

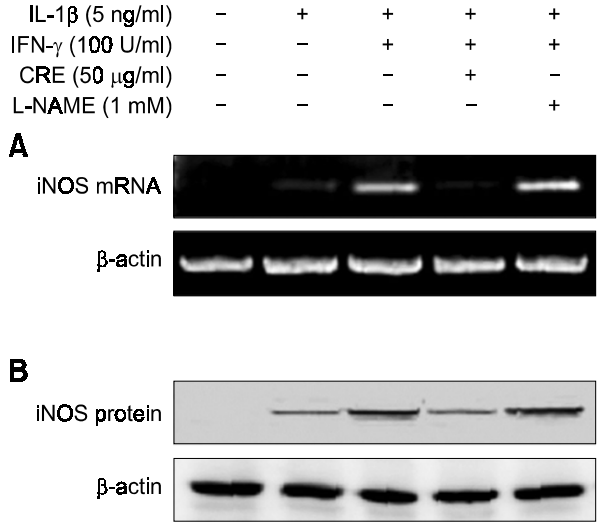

Figure 3. Inhibition of cytokine-induced iNOS expression by CRE. RIN cells (5×10^6) were incubated with IL-1β (5 ng/ml) and/or IFN-γ (100 U/ml) in the presence or absence of CRE (50 μg/ml) or L-NAME (1 mM) for 48 h. RT-PCR (A) and Western blot analysis (B) for iNOS were conducted. A representative result is presented among at least three separate experiments with similar results.

μg/ml showed suppressed iNOS expressions in both their mRNA and protein levels (Figure 3). These results show that IL-1β and IFN-γ-induced NO production were induced by iNOS expression in the RIN cells.

Effect of CRE on IL-1β and IFN-γ-induced NF-κB activation

NF-κB has been implicated in the transcriptional regulation of cytokine-induced iNOS expression. Thus, we attempted to determine the effects of CRE on the cytokine-stimulated translocation of NF-κB from the cytoplasmic compartment to the nucleus in RIN cells. The IL-1β and IFN-γ-stimulated RIN cells resulted in increases in NF-κB binding activity and p65 subunit levels within their nuclei (Figure 4A and 4B) and IκBα degradation in the cytosol (Figure 4B), as compared to unstimulated cells. IκBβ was not affected by treatments with IL-1β and IFN-γ (data not shown). IL-1β and IFN-γ-induced NF-κB activation and IκBα degradation were suppressed markedly by the addition of CRE, suggesting that CRE inhibits the expression of iNOS via the inhibition of NF-κB activation. The specificity of the DNA-protein interactions for NF-κB was demonstrated via competition assays using a 50-fold excess of unlabeled oligonucleotides (Figure 4A, lane 4).

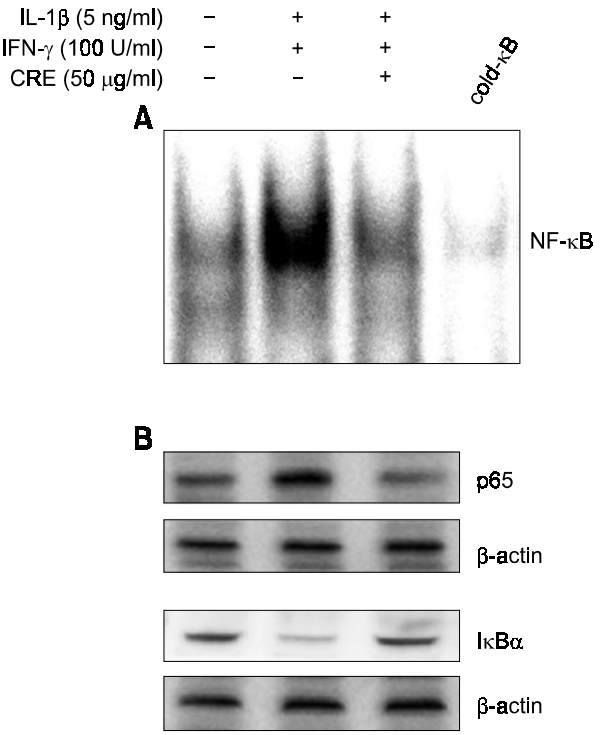

Figure 4. Inhibition of cytokine-stimulated translocation of NFκB to the nucleus and IκBα degradation by CRE. RIN cells (5×10^6) were treated with IL-1β (5 ng/ml) and IFN-γ (100 U/ml) in the presence or absence of CRE (50 μg/ml). After 30 min of incubation, NF-κB translocation was analyzed by EMSA (A), and p65 expression and IκBα degradation (B) were evaluated by Western blotting.

Reduction of NO production and preservation of glucose-stimulated insulin secretion by CRE in the presence of IL-1β and IFN-γ

We further assayed the preventive effects of CRE on cytokine-induced NO production, iNOS expression, and GSIS using rat pancreatic islets isolated from male Sprague-Dawley rats to support the physiological importance of the results observed in the cell line studies (Figure 5). 24 h of incubation of the rat islets with IL-1β and IFN-γ resulted in 3.4-fold and 7.6-fold increases in nitrite production and iNOS mRNA expression, respectively (Figure 5A). However, islets treated with IL-1β and IFN-γ in the presence of L-NAME produced control levels of nitrite. The incubation of islets with IL-1β and IFN-γ for 24 h also resulted in marked iNOS protein expression (Figure 5C) and a significant reduction of GSIS from 10.2 ± 0.6 ng/ml to 4.8 ± 1.6 ng/ml (Figure 5D). Pretreatment with CRE blocked the effects of IL-1β and IFN-γ on nitrite production and iNOS expression, and restored insulin secretion responses to glucose in a concentration-dependent manner. CRE alone had no effect on insulin-secre-

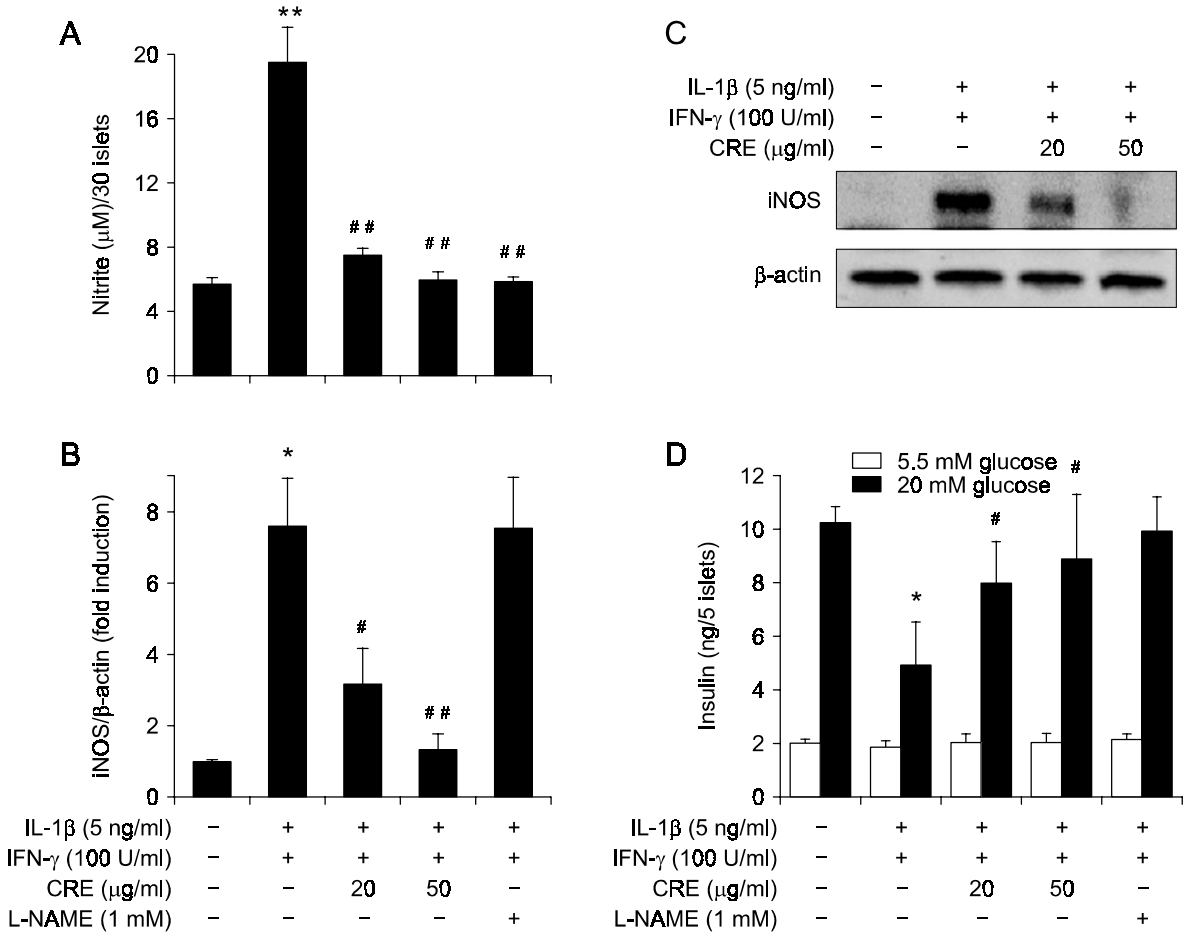

Figure 5. Effects of CRE on IL-1β and IFN-γ-induced NO production, iNOS expression, and glucose-stimulated insulin secretion. Rat islets (30 islets/500 μl) were treated with cytokines in the presence or absence of CRE or L-NAME for 24 h as indicated. After the incubation period, NO production (A) and iNOS mRNA (B) and protein (C) expressions were assessed. (D) For GSIS, five islets were utilized and assayed as described in the Materials and Methods section. Results of triplicate samples were expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. IL-1β + IFN-γ.

ting responses to glucose (data not shown).

Discussion

In this study, we have evaluated the preventative effects of CRE on IL-1β and IFN-γ-induced reductions of viability in β-cells. Rat insulinoma cells, pretreated with CRE, proved resistant to cytokine-induced cytotoxicity, as assessed by MTT and BrdU assays. The protective actions of CRE on cellular metabolism are associated with an inability of β-cells to express iNOS or generate NO in response to cytokines. The activation of the transcriptional regulator, NF-κB, was required for cytokine-induced iNOS expression by β-cells. We observed that CRE prevented cytokine-induced NF-κB nuclear translocation via the inhibition of IκBα degradation. These findings show that CRE provides functional protection against the damaging effects of cytokines by preventing the expression of iNOS.

Cytokines, as humoral mediators of inflammation, cause the destruction of pancreatic β-cells of various species, including rats and humans (Eizirik *et al*., 1996; Mandrup-Poulsen, 1996). It has been previously shown that, whereas IL-1β is a sufficient stimulus for the induction of iNOS mRNA expression and NO production in RIN cells, a combination of IL-1β and IFN-γ is required for the induction of NO formation by human islets (Cetkovic-Cvrlje and Eizirik, 1994; Eizirik *et al*., 1996; Heitmeier *et al*., 1997; Lortz *et al*., 2000). The increased toxicity of the cytokine combination compared with that of IL-1β alone (Figure 1) is attributable to a significantly higher rate of iNOS mRNA and protein expression (Figure 3) and subsequent NO production (Figure

1B). Data obtained in other cell types suggest that putative sites for the action of IFN-γ include the stabilization of iNOS mRNA (Vodovotz *et al*., 1993), the potentiation of IL-1β-induced NF-κB activation (Amoah-Apraku *et al*., 1995), and the induction of other nuclear transcription factors, including the interferon regulatory factor-1 (Flodstrom and Eizirik, 1997).

NF-κB governs both proinflammatory and anti-apoptotic responses according to the mode of insults in β-cells. NF-κB regulates the expressions of multiple proinflammatory genes that contribute to islet destruction, including Fas, iNOS, and cyclooxygenase-2 (Cetkovic-Cvrlje and Eizirik, 1994; Sorli *et al*., 1998; Darville and Eizirik, 2001). In addition, the promoters of other proinflammatory genes induced in β-cells, including chemokines and adhesion molecules, also possess binding elements for NF-κB (May and Ghosh, 1998). The importance of NF-κB in β-cell damage is underscored by the fact that the 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 (Kwon *et al*., 1998; Giannoukakis *et al*., 2000; Heimberg *et al*., 2001; Eldor *et al*., 2006). By way of contrast, aside from its proinflammatory functions, there is a wealth of data on the defensive and protective functions of NF-κB. NF-κB limited tissue damage in a cerulean-induced acute pancreatitis model, whereas the blockage of NF-κB activation exerted an adverse effect by causing increased tissue damage by increasing apoptosis (Steinle *et al*., 1999). NF-κB regulates apoptosis via its ability to control the expression of multiple antiapoptotic genes, including the inhibitor of apoptosis protein (IAP) and A20 (Karin and Lin, 2002; Liuwantara *et al*., 2006). Blockade of NF-κB via the use of an IκBα super-repressor also sensitized β-cells to TNF-α-mediated apoptosis (Chang *et al*., 2003). Thus, it remains to be determined 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, but with variable efficacy, depending on the species and the combination of the cytokines (Darville and Eizirik, 1998; Rabinovitch and Suarez-Pinzon, 1998). We have already reported on the protective effects of herbs and dietary supplements against cytokine- or cytotoxin-induced β-cell damage via the suppression of iNOS expression (Park and Park, 2001; Kwon *et al*., 2003a, b, 2005, 2006; Kim *et al*., 2007). Many other groups have also attempted to save β-cells from those insults by repressing either NF-κB activation or NF-κB-dependent iNOS expression (Scarim *et al*., 1998; Ho and Bray, 1999; Ho *et al*., 1999; Evans *et al*., 2002). With regard to this feature, the repression of NF-κB or its downstream iNOS expression are important targets for the protection of β-cells against a variety of diabetogenic agents. Indeed, we recently determined the protective effects of CRE against S-nitroso-N-acetyl-penicillamine-induced apoptotic cell death in RIN cells (Kwon *et al*., 2005). With the current study, we further demonstrated the importance of NO generation in β-cell damage, which was prevented by treatment with L-NAME, an iNOS inhibitor. Taken together, our results indicate that NO is an indispensable component of the cytokine- or cytotoxin-induced toxicity of β-cells, and the protective effect of CRE against IL-1β and IFN-γ-mediated killing is attributable to the inhibition of NO generation.

In addition, data in the relevant literature indicate that cytokine-induced NF-κB activation and iNOS induction can be suppressed in islets by antioxidants including pyrrolidine dithiocarbamate (Kwon *et al*., 1995). Moreover, NF-κB is a redox-sensitive transcription factor, as indicated by the fact that NF-κB activation can be induced by H_2O_2 or, conversely, NF-κB nuclear translocation is blocked by antioxidants including pyrrolidine dithiocarbamate (Schreck *et al*., 1991; Ferran *et al*., 1995). Interestingly, several studies have addressed the protective potential of antioxidants in the islets via the overexpression of free radical-scavenging enzymes (Kubisch *et al*., 1997; Benhamou *et al*., 1998; Hohmeier *et al*., 1998). The overexpression of manganese-superoxide dismutase (MnSOD) in an engineered β-cell induced selective protection against cytokine-induced cytotoxicity, in addition to a reduction of cytokine-induced NO generation (Hohmeier *et al*., 1998). Berberine, a major alkaloidal component of *Coptidis rhizoma* has been reported to exert antidiabetic effects in a variety of animal models of diabetes (Leng *et al*., 2004; Lee *et al*., 2006; Tang *et al*., 2006). One possible antidiabetic mechanism involves its ability to scavenge oxygen free radicals. Berberine-treated animals showed increases in superoxide dismutase and glutathione peroxidase activities, as compared with control rats (Tang *et al*., 2006). Therefore, we are unable to dismiss the possibility that oxygen free radicals may be involved in our system, and that the protective effects of CRE occur via the reduced generation of oxygen free radicals. The potential for CRE to interfere at the oxidative step of NF-κB activation is currently under examination.

In summary, we have demonstrated the profound inhibitory effect of CRE against cytokine-induced viability reduction in β-cells, using an insulinoma cell line and isolated pancreatic islets. CRE rescued

β-cells from cytokine-induced toxicity and completely restored function. No other reports have yet been published regarding the relation of CRE with the prevention of β-cell damage against cytokine toxicity. The results of this study will provide valuable information not only into the mechanisms underlying autoimmune β-cell destruction, but also into the development of drugs designed to combat Type 1 diabetes.

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References

Amoah-Apraku B, Chandler LJ, Harrison JK, Tang SS, Ingelfinger JR, Guzman NJ. NF-κB and transcriptional control of renal epithelial-inducible nitric oxide synthase. Kidney Int 1995;48:674-82

Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141-79

Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649-83

Benhamou PY, Moriscot C, Richard MJ, Beatrix O, Badet L, Pattou F, Kerr-Conte J, Chroboczek J, Lemarchand P, Halimi S. Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets. Diabetologia 1998;41:1093-100

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54

Cetkovic-Cvrlje M, Eizirik DL. TNF-α and IFN-γ potentiate the deleterious effects of IL-1β on mouse pancreatic islets mainly via generation of nitric oxide. Cytokine 1994;6:399-406

Chan CP, But PP, Ho JW. Induction of *rcl*, a novel growth-related gene by *Coptidis rhizoma* in rat H4IIE cells. Life Sci 2002;70:1691-9

Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS. Nuclear factor κB protects pancreatic β-cells from tumor necrosis factor-α-mediated apoptosis. Diabetes 2003;52:1169-75

Corbett JA, Wang JL, Sweetland MA, Lancaster JR Jr, McDaniel ML. Interleukin 1β induces the formation of nitric oxide by β-cells purified from rodent islets of Langerhans. Evidence for the β-cell as a source and site of action of nitric oxide. J Clin Invest 1992;90:2384-91

Corbett JA, McDaniel ML. Intraislet release of interleukin 1 inhibits β cell function by inducing β cell expression of inducible nitric oxide synthase. J Exp Med 1995;181:559-68

Cunningham JM, Green IC. Cytokines, nitric oxide and insulin secreting cells. Growth Regul 1994;4:173-80

Darville MI, Eizirik DL. Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. Diabetologia 1998;41:1101-8

Darville MI, Eizirik DL. Cytokine induction of Fas gene expression in insulin-producing cells requires the transcription factors NF-κB and C/EBP. Diabetes 2001;50:1741-8

Eizirik DL, Flodstrom M, Karlsen AE, Welsh N. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. Diabetologia 1996;39:875-90

Eldor R, Yeffet A, Baum K, Doviner V, Amar D, Ben-Neriah Y, Christofori G, Peled A, Carel JC, Boitard C, Klein T, Serup P, Eizirik DL, Melloul D. Conditional and specific NF-κB blockade protects pancreatic beta cells from diabetogenic agents. Proc Natl Acad Sci USA 2006;103:5072-7

Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev 2002;23:599-622

Ferran C, Millan MT, Csizmadia V, Cooper JT, Brostjan C, Bach FH, Winkler H. Inhibition of NF-κB by pyrrolidine dithiocarbamate blocks endothelial cell activation. Biochem Biophys Res Commun 1995;214:212-23

Flodstrom M, Welsh N, Eizirik DL. Cytokines activate the nuclear factor κB (NF-κB) and induce nitric oxide production in human pancreatic islets. FEBS Lett 1996;385:4-6

Flodstrom M, Eizirik DL. Interferon-γ-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. Endocrinology 1997;138:2747-53

Giannoukakis N, Rudert WA, Trucco M, Robbins PD. Protection of human islets from the effects of interleukin-1β by adenoviral gene transfer of an IκB repressor. J Biol Chem 2000;275:36509-13

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal Biochem 1982;126:131-8

Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL. Inhibition of cytokine-induced NF-κB activation by adenovirus-mediated expression of a NF-κB super-repressor prevents β-cell apoptosis. Diabetes 2001;50:2219-24

Heitmeier MR, Scarim AL, Corbett JA. Interferon-γ increases the sensitivity of islets of Langerhans for inducible nitric-oxide synthase expression induced by interleukin 1. J Biol Chem 1997;272:13697-704

Ho E, Bray TM. Antioxidants, NF-κB activation, and diabetogenesis. Proc Soc Exp Biol Med 1999;222:205-13

Ho E, Chen G, Bray TM. Supplementation of N-acetylcysteine inhibits NF-κB activation and protects against alloxan-induced diabetes in CD-1 mice. Faseb J 1999;13:1845-54

Hohmeier HE, Thigpen A, Tran VV, Davis R, Newgard CB. Stable expression of manganese superoxide dismutase (MnSOD) in insulinoma cells prevents IL-1β-induced cytotoxicity and reduces nitric oxide production. J Clin Invest 1998;101:1811-20

Iizuka N, Miyamoto K, Hazama S, Yoshino S, Yoshimura K, Okita K, Fukumoto T, Yamamoto S, Tangoku A, Oka M. Anticachectic effects of *Coptidis rhizoma*, an anti-inflammatory herb, on esophageal cancer cells that produce interleukin 6. Cancer Lett 2000a;158:35-41

Iizuka N, Miyamoto K, Okita K, Tangoku A, Hayashi H, Yosino S, Abe T, Morioka T, Hazama S, Oka M. Inhibitory effect of *Coptidis Rhizoma* and berberine on the proliferation of human esophageal cancer cell lines. Cancer Lett 2000b;148:19-25

Iizuka N, Hazama S, Yoshimura K, Yoshino S, Tangoku A, Miyamoto K, Okita K, Oka M. Anticachectic effects of the natural herb *Coptidis rhizoma* and berberine on mice bearing colon 26/clone 20 adenocarcinoma. Int J Cancer 2002;99: 286-91

Jorns A, Gunther A, Hedrich HJ, Wedekind D, Tiedge M, Lenzen S. Immune cell infiltration, cytokine expression, and β-cell apoptosis during the development of type 1 diabetes in the spontaneously diabetic LEW.1AR1/Ztm-iddm rat. Diabetes 2005;54:2041-52

Kanazawa Y, Komeda K, Sato S, Mori S, Akanuma K, Takaku F. Non-obese-diabetic mice: immune mechanisms of pancreatic β-cell destruction. Diabetologia 1984;27 Suppl:113-5

Karin M, Lin A. NF-κB at the crossroads of life and death. Nat Immunol 2002;3:221-7

Kim EK, Kwon KB, Lee JH, Park BH, Park JW, Lee HG, Jhee EC, Yang JY. Inhibition of cytokine-mediated nitric oxide synthase expression in rat insulinoma cells by scoparone. Biol Pharm Bull 2007;30:242-6

Kim HR, Rho HW, Park BH, Park JW, Kim JS, Kim UH, Chung MY. Role of Ca^{2+} in alloxan-induced pancreatic β-cell damage. Biochim Biophys Acta 1994;1227:87-91

Kim SK, Kim JH, Kim BS, Chung HY, Lee YY, Park CS, Lee YS, Lee YH, Chung IY. Identification and functional characterization of an alternative spice variant within the fourth exon of human nanog. Exp Mol Med 2005;37:601-7

Kubisch HM, Wang J, Bray TM, Phillips JP. Targeted overexpression of Cu/Zn superoxide dismutase protects pancreatic β-cells against oxidative stress. Diabetes 1997;46: 1563-6

Kwon G, Corbett JA, Rodi CP, Sullivan P, McDaniel ML. Interleukin-1β-induced nitric oxide synthase expression by rat pancreatic β-cells: evidence for the involvement of nuclear factor κB in the signaling mechanism. Endocrinology 1995; 136:4790-5

Kwon G, Corbett JA, Hauser S, Hill JR, Turk J, McDaniel ML. Evidence for involvement of the proteasome complex (26S) and NFκB in IL-1β-induced nitric oxide and prostaglandin production by rat islets and RINm5F cells. Diabetes 1998;47:583-91

Kwon KB, Kim JH, Lee YR, Lee HY, Jeong YJ, Rho HW, Ryu DG, Park JW, Park BH. *Amomum xanthoides* extract prevents cytokine-induced cell death of RINm5F cells through the inhibition of nitric oxide formation. Life Sci 2003a;73:181-91

Kwon KB, Ryu DG, Shin MK, Shin BC, Hwang WJ, Lee YR, Park JW, Park BH. *Fructus Benincasae Recens* extract prevents cytokine-induced nitric oxide formation and cytotoxicity of RINm5F cells. Immunopharmacol Immunotoxicol 2003b;25:615-25

Kwon KB, Kim EK, Lim JG, Shin BC, Han SC, Song BK, Kim KS, Seo EA, Ryu DG. Protective effect of *Coptidis Rhizoma* on S-nitroso-N-acetylpenicillamine (SNAP)-induced apoptosis and necrosis in pancreatic RINm5F cells. Life Sci 2005; 76:917-29

Kwon KB, Kim EK, Jeong ES, Lee YH, Lee YR, Park JW, Ryu DG, Park BH. *Cortex cinnamomi* extract prevents streptozotocin- and cytokine-induced β-cell damage by inhibiting NF-κB. World J Gastroenterol 2006;12:4331-7

Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB. Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. Diabetes 2006;55:2256-64

Leng SH, Lu FE, Xu LJ. Therapeutic effects of berberine in impaired glucose tolerance rats and its influence on insulin secretion. Acta Pharmacol Sin 2004;25:496-502

Liuwantara D, Elliot M, Smith MW, Yam AO, Walters SN, Marino E, McShea A, Grey ST. Nuclear factor-κB regulates β-cell death: a critical role for A20 in beta-cell protection. Diabetes 2006;55:2491-501

Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J, Lenzen S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. Diabetes 2000;49:1123-30

Mandrup-Poulsen T. The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia 1996;39:1005-29

May MJ, Ghosh S. Signal transduction through NF-κB. Immunol Today 1998;19:80-8

Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109-42

Nossal GJ, Herold KC, Goodnow CC. Autoimmune tolerance and type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1992;35 Suppl 2:S49-59

Park BH, Rho HW, Park JW, Cho CG, Kim JS, Chung HT, Kim HR. Protective mechanism of glucose against alloxan-induced pancreatic β-cell damage. Biochem Biophys Res Commun 1995;210:1-6

Park BH, Park JW. The protective effect of *Amomum xanthoides* extract against alloxan-induced diabetes through the suppression of NF-κB activation. Exp Mol Med 2001;33: 64-8

Rabinovitch A, Suarez-Pinzon WL. Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 1998;55:1139-49

Scarim AL, Heitmeier MR, Corbett JA. Heat shock inhibits cytokine-induced nitric oxide synthase expression by rat and human islets. Endocrinology 1998;139:5050-7

Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. Embo J 1991;10:2247-58

Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, Maclouf J, Robertson RP. Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. Proc Natl Acad Sci USA 1998;95:1788-93

Southern C, Schulster D, Green IC. Inhibition of insulin secretion by interleukin-1β and tumour necrosis factor-α via an L-arginine-dependent nitric oxide generating mechanism. FEBS Lett 1990;276:42-4

Steinle AU, Weidenbach H, Wagner M, Adler G, Schmid RM. NF-κB/Rel activation in cerulein pancreatitis. Gastroenterology 1999;116:420-30

Tang LQ, Wei W, Chen LM, Liu S. Effects of berberine on diabetes induced by alloxan and a high-fat/high-cholesterol diet in rats. J Ethnopharmacol 2006;108:109-15

Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C. Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. J Exp Med 1993; 178:605-13

Welsh N, Eizirik DL, Bendtzen K, Sandler S. Interleukin-1β-induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. Endocrinology 1991;129: 3167-73

Zhang SU, Park KW, Oh S, Cho HJ, Cho HJ, Park JS, Cho YS, Koo BK, Chae IH, Choi DJ, Kim HS, Lee MM. NF-κB decoy potentiates the effects of radiation on vascular smooth muscle cells by enhancing apoptosis. Exp Mol Med 2005;37:18-26