Inhibition of Cytokine-Mediated Nitric Oxide Synthase Expression in Rat Insulinoma Cells by Scoparone

Eun Kyung Kim, Kang Beom Kwon, Ju Hyung Lee, Byung Hyun Park, Jin Woo Park, Hern Koo Lee, Eun Chung Jhee, and Jeong Yeh Yang

Department of Biochemistry, Preventive Medicine, and Immunology, Medical School, Chonbuk National University; Jeonju, 561–756, Korea: Department of Physiology, School of Oriental Medicine, Won-Kwang University; Iksan, 570–749, Jeonbuk, Korea: and Department of Biochemistry, Dental School, Chonbuk National University; Jeonju, 561–756, Korea. Received May 15, 2006; accepted October 16, 2006

Cytokines produced by immune cells infiltrating pancreatic islets are important mediators of β-cell destruction in insulin-dependent diabetes mellitus. Scoparone (6,7-dimethoxycoumarin) is known to have a wide range of pharmacological properties in vitro. In this study, the effects of scoparone on cytokine-induced β-cell dysfunction were examined. Presence of scoparone significantly protected interleukin-1β (IL-1β) and interferon-γ (IFN-γ)-mediated cytotoxicity of RINm5F, a rat insulinoma cell line, and preserved glucose-stimulated insulin secretion in rat pancreatic islets. Scoparone also resulted in a significant reduction in IL-1β and IFN-γ-induced nitric oxide (NO) production, a finding that correlated well with reduced levels of the inducible form of NO synthase (iNOS) mRNA and protein. The molecular mechanism by which scoparone inhibited iNOS gene expression appeared to involve the inhibition of NF-κB activation. These results revealed the possible therapeutic value of scoparone for the prevention of diabetes mellitus progression.

Key words scoparone; nuclear factor (NF)-κB; inducible form of nitric oxide synthase (iNOS); cytokine

Autoimmune diabetes is characterized by a local inflammatory reaction in and around the pancreatic islets, followed by selective destruction of insulin-producing β-cells. Much evidence supports an effector role for cytokines in mediating β-cell destruction. Incubation of rat islets with interleukin-1β (IL-1β) alone, or in combination with tumor necrosis factor (TNF-α) and interferon-γ (IFN-γ) results in the expression of the inducible form of nitric oxide synthase (iNOS), increased production of nitric oxide (NO), and potent inhibition of glucose-stimulated insulin secretion. Heitmeier et al. demonstrated that IL-1β-induced inhibition of insulin secretion and nitric oxide production by rat islets were completely prevented by Nω-nitro-L-arginine methylester (L-NAME) and aminoguanidine.

One cellular target activated in response to IL-1β is the transcriptional regulator nuclear factor κB (NF-κB). NF-κB is normally bound to inhibitory κB (1κB) in the cytosol; this binding prevents its movement into the nucleus. Various stimuli induce the phosphorylation of 1κB, which releases NF-κB and translocates to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription.

Scoparone (6,7-dimethoxycoumarin), a derivative of coumarin (1,2-benzopyrone) is known to have potent immunosuppressive, vascular relaxant and lipid lowering properties. In this study, we examined the feasibility of scoparone as a means of preventing IL-1β and IFN-γ-induced β-cell destruction. Scoparone inhibited IL-1β and IFN-γ-induced NF-κB activation, iNOS expression, NO formation, glucose-stimulated insulin secretion (GSIS), and cell death of RIN cells, which may explain the beneficial effects of scoparone as an anti-diabetic agent.

MATERIALS AND METHODS

Cell Culture RIN, clone 5F (RINm5F), is an insulinoma cell line derived from the NEDH rat islet cell tumor. Cells were purchased from the American Type Culture Collection and grown at 37 °C under a humidified, 5% CO 2 atmosphere in RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10000 units/ml of penicillin, 10 mg/ml of streptomycin, and 2.5 μg/ml of amphotericin B.

Cell Viability Assay 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 as described previously. After treatment, cells in 96-well plates were washed twice with PBS. MTT (100 μg/100 μl of PBS) was added to each well. Cells were then incubated at 37 °C for 1 h, and DMSO (100 μl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a model Spectra MAX PLUS (Molecular Devices).

Cell Proliferation Assay A cell proliferation enzyme-linked immunosorbent assay, BrdU kit (Amersham Biosciences, U.K.) was used to measure the incorporation of 5-bromo-2-deoxyuridine (BrdU) during DNA synthesis according to the manufacturer’s protocol. Briefly, cells were seeded overnight in black 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson) at a density of 10000 cells per well in 100 μl medium. Cells were treated with the desired concentrations of agents for 24 h. BrdU (10 μM) was added to the culture medium for 2 h and BrdU-labeled cells were fixed and DNA was denatured in fixative solution for 30 min at room temperature. Cells were incubated with peroxidase-conjugated anti-BrdU antibody for 2 h at room temperature and washed 3 times with washing solution. The immune complex was detected by the 3,3′,5,5′-tetramethylbenzidine.
substrate reaction. Absorbance was measured at 405 nm with a model Spectra MAX PLUS.

**Nitrite Measurement** Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions.\(^{18}\) Nitrite concentration in the cell free culture supernatant, therefore, served as a reflection of NO production and was measured using a colorimetric assay.\(^{19}\) Following 24 h incubation at 37 °C in a humidified 95% air/5% CO\(_2\) atmosphere, nitrite concentration was measured in the cell free culture supernatant. Briefly, 100 μl aliquots of the culture supernatants dispersed into 96-well microtiter plates (flat bottom) 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 measured at 540 nm using a spectrophotometer. Concentrations were determined from a linear standard curve obtained from serial dilutions of sodium nitrite in working medium.

**Western Blotting** 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, 10 μg/ml aprotinin). The homogenates containing 20 μg of protein were separated by SDS-PAGE with 10% resolving and 3% acrylamide stacking gel,\(^{20}\) and transferred to nitrocellulose sheets (Schleicher & Schuell) in a Western blot apparatus (Bio-Rad) run at 50 V for 2 h. The nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 h with 1 μg/ml anti-mouse macrophage iNOS rabbit IgG (Transduction Laboratories). The binding of antibody was detected with anti-rabbit IgG conjugated to an alkaline phosphatase (Sigma). Immunoblots were developed using a BCIP/NBT solution (Pierce).

**RNA Isolation and RT-PCR Analysis for iNOS** Total cellular RNA was prepared using Trizol solution (Gibco-BRL) according to the manufacturer’s instructions. After the preparation of cDNA with oligo d(T)\(_{16}\) as a reverse transcriptase primer from the extracted RNA, amplification with PCR was performed using GeneAmp kit (Perkin Elmer) according to the manufacturer’s instructions. The oligonucleotide primers used for PCR are as follows: iNOS upstream 5’-CCA CAA TAG TAC AAT ACT AC-3’, downstream 5’-ACG AGG TGT TCA GGC TG-3’. β-Actin upstream 5’-TGC CCA TCT ATG AGG GTT ACG-3’, downstream 5’-TAG AAG CAT TTG CGG TGC ACG-3’.\(^{21}\) Each cDNA amplification used the product of about 1 μg of total RNA. The reaction was cycled 30 times through 30 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C. Fifty percent of reaction mixture was analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. To check the reproducibility of the results, each experiment was carried out more than three times.

**Electrophoretic Mobility Shift Assay** The nuclear extracts were prepared from the cells by the method of Kwon et al.\(^{21}\) To inhibit endogenous protease activity, 1 mM phenylmethylsulfonyl fluoride was added. Protein contents in nuclear extracts were measured using the Bradford method.\(^{22}\) As a probe for the gel retardation assay, NF-κB specific oligonucleotide (κB, 5’-CGG GTT AAT ACA AGA GGG GGC TTT CCG AG-3’) was synthesized. The two complementary strands were annealed and labeled with [α-\(^{32}\)P]dCTP. Labeled oligonucleotides (10000 cpm), 10 μg of nuclear extracts, and binding buffer (10 mM Tris–HCl, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly(dI·dC), and 1 mM DTT, pH 7.6) were incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5X Tris–borate buffer.

**Isolation of Islets** Islets were isolated from male Sprague–Dawley rats by collagenase digestion as described previously.\(^{23}\) Following isolation, islets were cultured overnight in RPMI-1640 (RPMI-1640 containing 2 mM l-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) under an atmosphere of 95% and 5% CO\(_2\) at 37 °C. Prior to each experiment, islets were washed three times in RPMI-1640, counted and then cultured overnight.

**Insulin Secretion Assay** Islets were cultured for 24 h with the cytokines in the presence or absence of scoparone. The islets 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% bovine serum albumin, pH 7.4) containing 5.5 mM d-glucose, and insulin secretion was assayed in the presence of either 5.5 or 20 mM d-glucose. Medium insulin content was determined by ELISA (Linco Research, Inc.).\(^{24}\)

**Statistical Analysis** Statistical analysis of the data was performed with Student’s t-test, and differences with p<0.05 were considered statistically significant.

**RESULTS**

RINm5F cells, an insulinoma cell line derived from the rat islet cell tumor were cultured to near confluence. After treatment of cells with high concentrations of IL-1β (5 ng/ml) alone or IL-1β and IFN-γ (100 U/ml) for 24 h, the viability of cultured cells was determined by MTT method (Fig. 1B). Treatment with IL-1β alone decreased the cell viability to 82±1.5%, whereas the combination of IL-1β and IFN-γ caused even further decrease (59±0.8%). The addition of scoparone (50 μg/ml) in the presence of both IL-1β and IFN-γ significantly attenuated the cytokine-induced cytotoxicity to 82±2.2% of viable cells. Scoparone alone at this concentration did not show any cytotoxicity (data not shown). The protective effect of scoparone on IL-1β and IFN-γ-induced cytotoxicity was further confirmed using BrdU incorporation in RIN cells. BrdU, a thymidine analog, is incorporated into proliferating cells during its DNA synthesis. BrdU incorporation therefore reflects the proliferative potential of the cells. IL-1β and IFN-γ reduced the level of BrdU incorporation and hence proliferation of the RIN cells in 24 h incubation was 26.4±1.9% of the control level (Fig. IC). Similar to MTT data, scoparone restored BrdU incorporation to near the control level.

It is well known that IL-1β-mediated destruction of β-cells is caused by activation of iNOS expression and production of NO, and potentiated by the presence of IFN-γ and/or TNF-α.\(^{4–7,25}\) Incubation of RIN cells with IL-1β and IFN-γ for 24 h led to the accumulation of nitrites in the culture medium (Fig. 2). RIN cells stimulated with IL-1β alone showed slight increase of nitrite production, but IL-1β and IFN-γ showed a synergistic effect, producing 63±3.2 μM nitrite. The presence of scoparone diminished the IL-1β and IFN-γ-induced nitrite production.
formation by the cells in a dose-dependent manner. Near complete inhibition of nitrite formation was observed at 100 μg/ml concentration. Scoparone alone did not produce nitrite even at 100 μg/ml concentration (data not shown). l-NAME, an inhibitor of iNOS showed almost complete inhibition against IL-1β and IFN-γ-mediated NO production, which was very consistent with its protective effect on the cytotoxicity (Figs. 1, 2).

To examine whether scoparone inhibits NO production via suppression of iNOS gene expression, lysate of RIN cells treated with cytokines was subjected to RT-PCR and immunoblot analysis (Fig. 3). Cells treated with IL-1β alone slightly increased iNOS mRNA (Fig. 3A, lane 2) and protein expression (Fig. 3B, lane 2), whereas marked increase of iNOS mRNA and 130 kDa iNOS protein were observed in cells treated with both IL-1β and IFN-γ (Figs. 3A, B, lane 3). Treatment of cells with IL-1β and IFN-γ in the presence of 50 μg/ml of scoparone showed decreased levels of iNOS at both mRNA and protein levels (Figs. 3A, B, lane 4). However, l-NAME did not affect the expression of iNOS (Figs. 3A, B, lane 5).

Because NF-κB is implicated in the transcriptional regulation of cytokine-induced iNOS expression, we studied the effect of scoparone on cytokine-stimulated translocation of NF-κB from cytosol to the nucleus in RIN cells. The nuclear extract from IL-1β alone or IL-1β and IFN-γ stimulated RIN cells showed an increased translocation of NF-κB, whereas NF-κB binding activity was not seen in unstimulated cells (Fig. 4). The binding activity was markedly suppressed by the addition of scoparone, implying that scoparone inhibits iNOS expression and NO production through the inhibition of NF-κB activation. These results indicate that cytokine-induced iNOS protein is inhibited by scoparone at a transcriptional level. To provide further evidence of protective effects of scoparone on IL-1β and IFN-γ cytotoxicity, we assayed GSIS. Static incubation of islets at 20 mM glucose for 1 h resulted in a 4.3-fold increase in insulin secretion by control cells (Fig. 5). When islets were incubated with IL-1β and IFN-γ, it resulted in a 63.2% inhibition of GSIS following 24 h of incubation. When pretreated with scoparone for 3 h, the GSIS was restored to levels not significantly different from control.
Glucose-stimulated insulin secretion and metabolism are associated with an inability of assays. The protective actions of scoparone on cellular mechanisms were examined as described in Materials and Methods. Results of triplicate samples were expressed as mean ± S.E.M. *p < 0.05 vs. untreated control; #p < 0.05 vs. IL-1β + IFN-γ.

**DISCUSSION**

In the current study, we examined the preventive effects of scoparone on IL-1β and IFN-γ-induced β-cell damage. Rat insulinoma cells, pretreated with scoparone, are resistant to cytokine-induced cytotoxicity as assessed by MTT and BrdU assays. The protective actions of scoparone on cellular metabolism are associated with an inability of β-cells to express iNOS or produce NO in response to cytokines. Activation of the transcriptional regulator NF-κB is required for cytokine-induced iNOS expression by β-cells. We showed that scoparone prevented cytokine-induced NF-κB nuclear translocation. These findings indicate that scoparone provides functional protection against the damaging effect of cytokine by preventing iNOS expression. In addition, this study provides the first evidence that scoparone prevents cytokine-induced expression of iNOS by inhibiting nuclear translocation of NF-κB.

The protective actions of the scoparone are associated with the inhibition of NF-κB activation and nuclear localization. Scoparone may prevent NF-κB activation by either inhibiting IκB degradation, stimulating IκB expression, or both. Although our studies do not discriminate these possibilities, the net result of each event is the sequestration of NF-κB in the cytoplasm and inhibition of the subsequent NF-κB-dependent mRNA transcription including iNOS. In this point, repression of NF-κB or its downstream events are important targets for protecting β-cells from various kinds of insults. We earlier reported the protective effect of A. xanthoids extracts against alloxan- and cytokine-induced β-cell damage through suppression of NF-κB activation. Many other groups have also tried to save β-cells from cytokine or cytotoxic-induced damage by repressing either NF-κB activation or NF-κB dependent gene expression. Actually, iNOS is not the only one induced by NF-κB. Heat shock protein 70, heme oxygenase, and Mn-superoxide dismutase are also induced by NF-κB activation and those are all stress response proteins known to be induced by cytokines, heat shock and oxidative stress.

There is considerable *in vitro* data suggesting that NO secreted by β-cells could be directly involved in their own demise. IL-1β plays an important role in the NO production in the β-cells, especially in the presence of IFN-γ. Alone, IFN-γ does not modulate islet function or viability; many lines of evidence support a supportive role for IFN-γ in the IL-1β-induced β-cell damage model. The mechanism by which IFN-γ increases the sensitivity of β-cells for iNOS expression and NO production in response to IL-1β seems to be associated with an increase in the stability of iNOS mRNA. In our current study, IFN-γ also increased the sensitivity of RIN cells to the effects of IL-1β on iNOS expression, NO production and β-cell cytotoxicity through affecting the nuclear translocation of NF-κB. The generation of oxygen free radicals is another important way of cytokine- and other diabetogenic drug-mediated toxicity in β-cells. Experimental evidence indicates that oxygen free radicals are generated in cytokine-stimulated and alloxan-treated β-cells, and overexpression of antioxidant enzymes protects β-cells from such insults. Huang et al. reported the free radical scavenging effect of scoparone in alloxan treated mononuclear cells and aortic rings. Alloxan-stimulated O₂⁻ production and vasoconstriction from intact vascular rings were abolished by pretreatment with scoparone. Thus, we cannot exclude the possibility that oxygen free radicals are involved in our system and that the protective effect of scoparone occurs through the reduced generation of oxygen free radicals. Scoparone also elevated the basal level of cGMP, which results in decreased phosphorylation of myosin light chain, then dilatation. NO produced by the eNOS activation can dilate the vessel wall and scoparone may potentiate this vasodilative effect of NO. Paradoxically, scoparone has an inhibitory effect on NO generation through iNOS activation under our experimental conditions. Recently, Jang et al. showed interesting results which are somehow similar to ours. In there work, scoparone inhibited IFN-γ and LPS-induced NO and PGE₂ generation in murine macrophage RAW 264.7 cell line by suppressing iNOS and
COX-2 protein expression. Although the authors did not show any evidence of NF-kB repression, they strongly suggested there were inhibitory effects by scoparone on iNOS activated NO generation.

Excessive NO production by overexpression of iNOS gene is seen in acute and chronic disease states.\(^{43-45}\) Thus, down-regulation of iNOS gene expression may be an effective therapeutic strategy for preventing inflammatory reactions. In this study, scoparone prevented the cytotoxic action of cytokines by inhibiting NF-κB. The result of this study will provide valuable information not only on the elucidation of mechanisms of autoimmune β-cell destruction, but on the development of drugs to combat Type 1 diabetes.

Acknowledgements This work was supported by the Korea Science and Engineering Foundation (KOSEF-R08-2003-000-10222-0), and partly by Chonbuk National University (JYY).

REFERENCES