

Inhibitory effect of *Artemisia capillaris* extract on cytokine-induced nitric oxide formation and cytotoxicity of RINm5F cells

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Abstract. Cytokines produced by immune cells infiltrating pancreatic islets are important mediators of β -cell destruction in insulin-dependent diabetes mellitus. Cytokines stimulate an inducible form of nitric oxide synthase (iNOS) expression and nitric oxide (NO) production, leading to insulin insufficiency. In the present study, the effects of *Artemisia capillaris* extract (ACE) on cytokine-induced β -cell damage were examined. Treatment of RINm5F (RIN) rat insulinoma cells with interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ) induced cell damage. ACE completely protected IL-1 β and IFN- γ -mediated cytotoxicity in a concentration-dependent manner. Incubation with ACE resulted in a significant reduction in IL-1 β and IFN- γ -induced NO production, a finding that correlated well with reduced levels of the iNOS mRNA and protein. The molecular mechanism by which ACE inhibited iNOS gene expression appeared to involve the inhibition of NF- κ B activation. The IL-1 β and IFN- γ -stimulated RIN cells showed increases in NF- κ B binding activity and p65 subunit levels in the nucleus, and I κ B α degradation in cytosol compared to unstimulated cells. Furthermore, ACE restored the cytokine-induced inhibition of insulin release from isolated islets. These results suggest that ACE protects β -cells by suppressing NF- κ B activation.

Introduction

Type 1 diabetes mellitus is an autoimmune disease causing selective destruction of insulin-producing β -cells of the

Langerhans islets (1). Evidence supports a crucial role for infiltrated immune cells in and around pancreatic islets early in pathogenesis (2,3). In an activated state, T-lymphocytes and macrophages, the primary cellular components of islet insulinitis, release high levels of interleukin (IL)-1 β and interferon (IFN)- γ , respectively. IL-1 β alone, or in combination with tumor necrosis factor (TNF)- α or IFN- γ , causes the production of excess nitric oxide (NO) by the inducible form of nitric oxide synthase (iNOS) in pancreatic islets (4-6). Nitric oxide is a short-lived and highly reactive radical, which inhibits the Krebs-cycle enzyme aconitase and the electron transport chain complexes I and II leading to decreased glucose oxidation rates, ATP generation and insulin production (7-9). Heitmeier *et al* (10) demonstrated that IL-1 β -induced inhibition of insulin secretion and nitrite production by rat islets was completely prevented by N^w-nitro-L-arginine methylester (L-NAME) and amino-guanidine.

The transcriptional nuclear factor κ B (NF- κ B) has been implicated as a key signaling mediator for IL-1 β and has been proposed to regulate transcription of the iNOS gene (5,11). NF- κ B is initially located in the cytoplasm as an inactive form complexed with I κ B, an inhibitory factor of NF- κ B. Various inducers cause dissociation of this complex, presumably by the phosphorylation of I κ B, allowing NF- κ B to be released from the complex. NF- κ B then translocates to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription (12,13).

Artemisia capillaris is the young plant of *Artemisia capillaris* T_{HUNB} and belongs to the family of *Compositae*. It has been used to remove fever, to dispel dampness, and to relieve jaundice in traditional oriental medicine. Hong *et al* reported that ACE inhibited lipopolysaccharide-induced inflammatory response through suppression of NF- κ B activation (14). To our knowledge, the antidiabetic effects of *Artemisia capillaris* have not yet been reported. In the present study, we investigated the feasibility of *Artemisia capillaris* as a means of preventing IL-1 β and IFN- γ -induced β -cell destruction. *Artemisia capillaris* extract inhibited IL-1 β and IFN- γ -induced NF- κ B activation, iNOS expression, NO formation, glucose-stimulated insulin secretion (GSIS)

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and the cell death of β -cells, demonstrating the beneficial effects of *Artemisia capillaris* as an antidiabetic agent.

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) supplemented with 10% fetal bovine serum and 2 mM glutamine, 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 2.5 μ g/ml of amphotericin B.

Preparation of *Artemisia capillaris* extract (ACE). For extraction, 200 g of *Artemisia capillaris* was ground and extracted with boiling water for 4 h. After centrifugation at 3,000 \times g for 20 min, the supernatant was concentrated under reduced pressure to 200 ml and freeze dried to 23.4 g. The sterile extract was stored at -70°C.

MTT assay for cell viability. The viability of cultured cells was determined by assaying for the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously (15). In brief, after 48 h of incubation, cells (10⁴/well) in 96-well plates were washed twice with PBS. MTT (100 μ g/0.1 ml of PBS) was added to each well. Cells were incubated at 37°C for 1 h, and DMSO (100 μ l) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with a model SpectraMax 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 used to measure the incorporation of BrdU during DNA synthesis according to the manufacturer's protocol. Briefly, cells were seeded overnight in 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson, Franklin Lakes, NJ) at a density of 10⁵ cells per well in 100 μ l of medium. Cells were treated with a range of concentrations for 48 h. BrdU (10 μ M) was 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. Cells were incubated with peroxidase-conjugated anti-BrdU antibody for 2 h at room temperature and washed three times with washing solution. The immune complex was detected by the 3,3',5,5'-tetramethylbenzidine substrate reaction and the absorbance was measured at 405 nm with a SpectraMax Plus spectrophotometer.

Nitrite measurement. Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions (16). Nitrite concentrations in the cell-free culture supernatant, therefore, served as a reflection of NO production and were measured using a colorimetric assay (17). Following a 48-h incubation, 100- μ l aliquots of the culture supernatants were incubated with 100 μ l 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 was measured at 540 nm using a

Table I. Sequences and accession numbers for primers, forward and reverse, used in real-time PCR.

| Gene | Sequence for primers | Accession no. |
|----------------|---------------------------------|---------------|
| β -actin | Forward: GTGCTATGTTGCTCTAGACT | NM 007393 |
| | Reverse: CACAGGATTCCATACCCAAG | |
| iNOS | Forward: GTTCTTTGCTTCTGTGCTAAT | D 44591 |
| | Reverse: GTGTTTGCCTTATACTGTTCCA | |

SpectraMax Plus spectrophotometer. Concentrations of NO were determined from a linear standard curve obtained from serial dilutions of sodium nitrite in working medium.

RNA isolation and real-time PCR for iNOS. 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 μ g of RNA was reverse transcribed with SuperScript II reverse transcriptase (Life Technologies Ltd). The cDNA was diluted 10-fold prior to PCR amplification. Real-time PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, UK) according to the manufacturer's instructions. Specific primers for each gene (Table I) were designed using LightCycler software 4. The real-time PCR contained, in a final volume of 20 μ l, 100 ng of reverse transcribed total RNA, 500 nM of the forward and reverse primers, and 14 μ l of 2X SYBR-Green buffer (Roche). PCR amplification began with 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 μ l of ice-cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin). The homogenates containing 20 μ g of protein were separated by SDS-PAGE with 10% acrylamide resolving and 3% stacking gels, and were 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 μ g/ml of primary antibodies for iNOS, IkB α , IkB β , or p65 (Santa Cruz Biochemicals, Santa Cruz, CA). Horseradish peroxidase-conjugated IgG (Zymed, San Francisco, CA) was used as secondary antibody. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a ChemiDoc image analyzer (Bio-Rad).

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (18). Cells were immediately

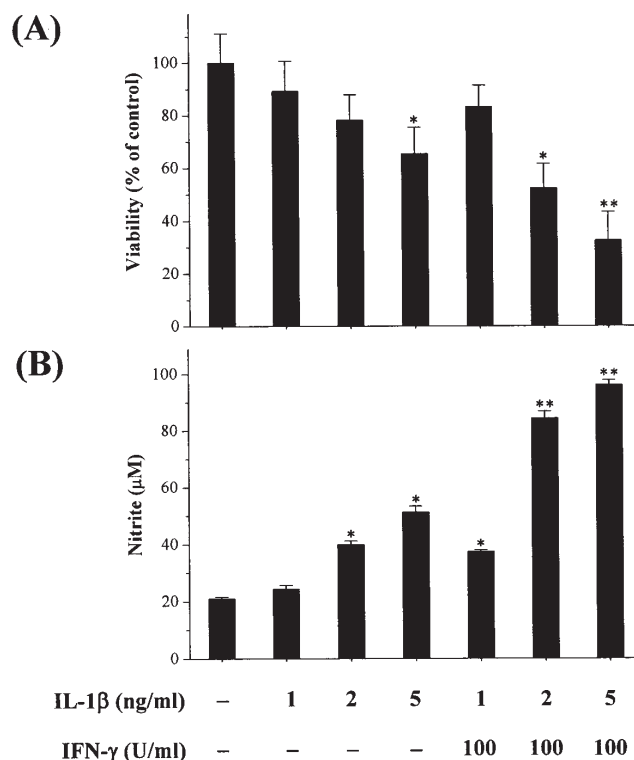


Figure 1. Cytokine-induced viability, and NO production in RIN cells. (A) RIN cells (1×10^5) were incubated with IL-1 β alone or in combination with IFN- γ as indicated. The percentage of cells viable after these treatments was determined by the MTT colorimetric assay and calculated as a ratio of A_{570} of treated and control cells (treated with 0.05% DMSO vehicle). (B) Cells (4×10^5) were treated with the cytokines for 48 h and the nitrite concentration was measured in the cell-free culture supernatants as a reflection of NO synthesis. Results of triplicate samples were expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. untreated control.

washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.9), and pelleted at 12,000 \times g for 30 sec. 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 phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol), vortexed for 10 sec and then centrifuged at 3,000 rpm for 5 min. The packed cells were re-suspended with ice-cold hypotonic lysis buffer in the presence of 50 μ l of 10% Nonidet P-40 and then incubated on ice for 25 min. The nuclear fractions were precipitated by centrifugation at 4,000 rpm for 15 min. I κ B α and I κ B β were analyzed in the cytoplasmic fractions by Western blotting. The pelleted nuclei were re-suspended in 50-100 μ l 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 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 MgCl₂, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, and 0.5 mM dithiothreitol) in a drop-wise fashion, and then incubated under continuous shaking at 4°C for 45 min. The samples were centrifuged for 20 min at 12,000 \times g. Aliquots of the nuclear extracts were stored at -80°C. Protein concentration was determined by the method of Bradford (19).

Electrophoretic mobility shift assay (EMSA). The activation of NF- κ B was assayed by a gel mobility shift assay using

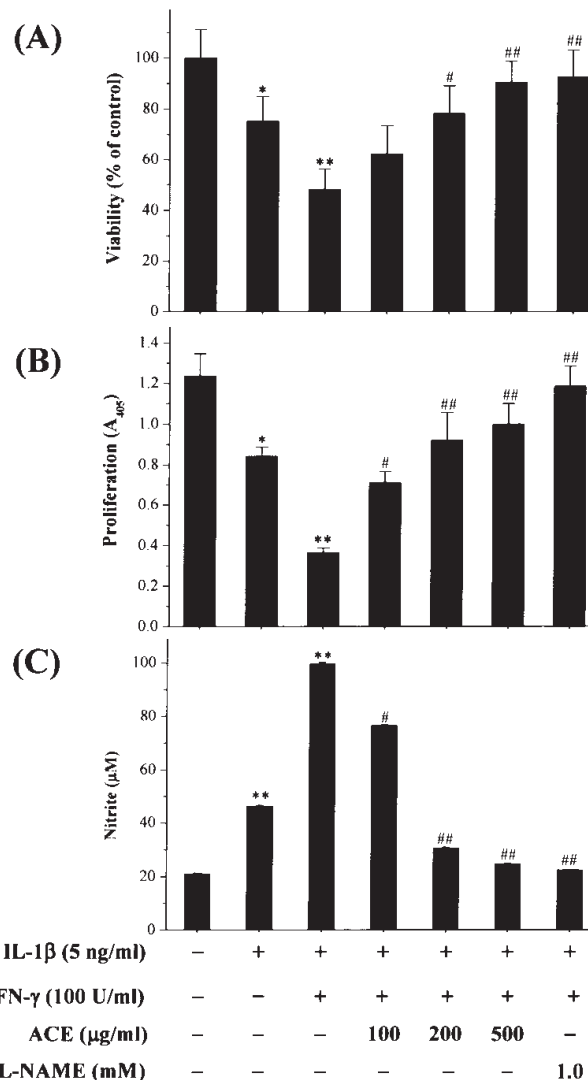


Figure 2. Prevention of cytokine-induced cell death and NO production by ACE. RIN cells were incubated with cytokines in the presence or absence of ACE or L-NAME for 48 h as indicated. The cell viability (A), proliferation capacity (B) and NO production (C) were determined. Each value is the mean \pm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

nuclear extracts from control and treated cells. As a probe for the gel retardation assay, an oligonucleotide containing the κ -chain binding site (κ B, 5'-CCGGTTAACAGAGGGGGCTT TCCGAG-3') was synthesized. The two complementary strands were annealed and labeled with [α -³²P] dCTP. Labeled oligonucleotides (10,000 cpm), 10 μ g 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 μ l. The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κ B oligonucleotide.

Isolation of islets. Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion as described

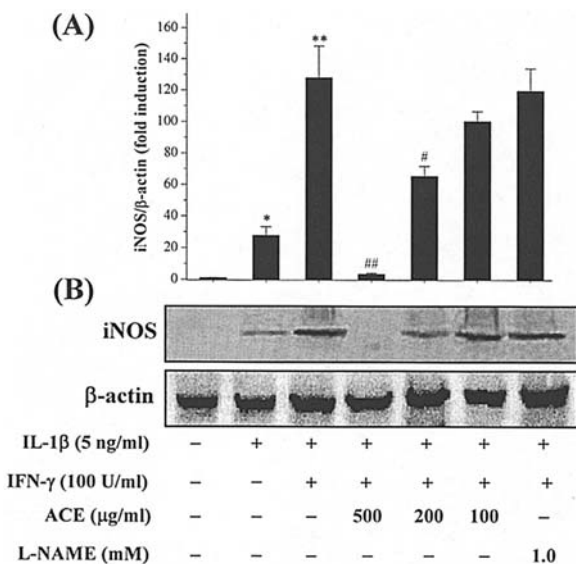


Figure 3. Inhibition of cytokine-induced iNOS expression by ACE. RIN cells (5×10^6) were incubated with IL-1 β (5 ng/ml) and/or IFN- γ (100 U/ml) in the presence or absence of ACE (500 μ g/ml) or L-NAME (1 mM) for 48 h. Real time-PCR (A) and Western blot analysis (B) for iNOS were performed as described in 'Materials and methods'. A representative result was determined from among at least three separate experiments with similar results. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. IL-1 β + IFN- γ .

previously (20). Following isolation, the islets were cultured overnight in RPMI-1640 (RPMI-1640 containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin) in an atmosphere of 95% O₂ and 5% CO₂ at 37°C. Prior to each experiment, the islet cells 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 ACE. The islets were 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 insulin secretion assays were performed in the presence of either 5.5 or 20 mM D-glucose. The insulin content of the medium was determined by ELISA (21).

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

Results

Prevention of cytokine-induced cell death by ACE. We initially evaluated the optimal conditions to observe the effect of cytokines on the viability of β -cells. The rat pancreatic β -cell line RIN was cultured to near confluence. Using a single-dose IL-1 β or a combination of IL-1 β and IFN- γ , we treated RIN cells with or without ACE for 48 h, at which time the cells were harvested and their viability was analyzed. A single treatment with IL-1 β decreased the viability in a dose-dependent manner, whereas a combination of IL-1 β and IFN- γ

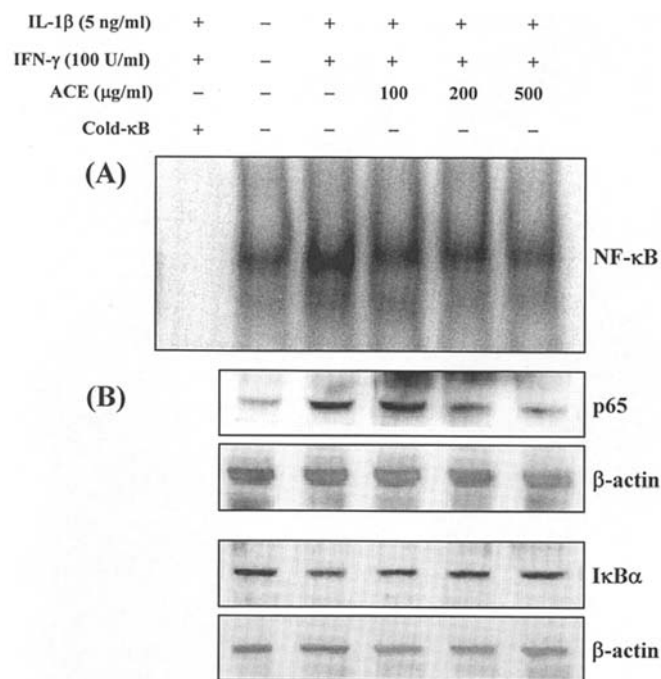


Figure 4. Inhibition of cytokine-stimulated translocation of NF κ B to the nucleus and I κ B α degradation by ACE. RIN cells (5×10^6) were treated with IL-1 β (5 ng/ml) and IFN- γ (100 U/ml) in the presence or absence of ACE. Following a 30-min incubation, NF- κ B translocation was analyzed by EMSA (A), and the p50 expression and I κ B α degradation (B) were determined by Western blotting.

(100 U/ml) caused a significant reduction in cell viability (Fig. 1A). In contrast, ACE increased the viability of IL-1 β and IFN- γ -treated RIN cells in a concentration-dependent manner (Fig. 2A). The protective effect of ACE on IL-1 β and IFN- γ -induced cytotoxicity was further confirmed using BrdU incorporation in RIN cells. BrdU is a thymidine analog that is incorporated into proliferating cells during DNA synthesis; thus it reflects the proliferative potential of the cells. IL-1 β and IFN- γ reduced the level of BrdU incorporation, hence proliferation, at 48-h incubations to $29.5 \pm 1.8\%$ of control levels (Fig. 2B). Similar to the MTT assay data, ACE restored the cell proliferation potential with an increasing concentration (Fig. 2B). ACE alone did not affect the viability even at a higher concentration (1 mg/ml) (data not shown).

Effect of ACE on IL-1 β and IFN- γ -induced NO production by RIN cells. It has been reported that IL-1 β and IFN- γ -mediated destruction of β -cells is caused by an increase in NO production (6,11,22). Incubation of RIN cells with IL-1 β and IFN- γ for 48 h resulted in a significant production of nitrite (a stable oxidized product of NO) by these cells (Fig. 1B). Cells preincubated with L-NAME (1 mM) exhibited complete resistance against IL-1 β and IFN- γ -mediated killing as expected (Fig. 2). Comparatively, ACE (500 μ g/ml) increased the viability and decreased the nitrite production of IL-1 β and IFN- γ -treated RIN cells to $90.3 \pm 8.3\%$ and $24.7 \pm 0.2 \mu$ M, respectively (Fig. 2). To examine whether ACE inhibited NO production via suppression of iNOS gene expression, the changes in iNOS mRNA and protein were investigated by real time-PCR and Western blot analysis, respectively. Cells treated with IL-1 β alone slightly increased iNOS mRNA

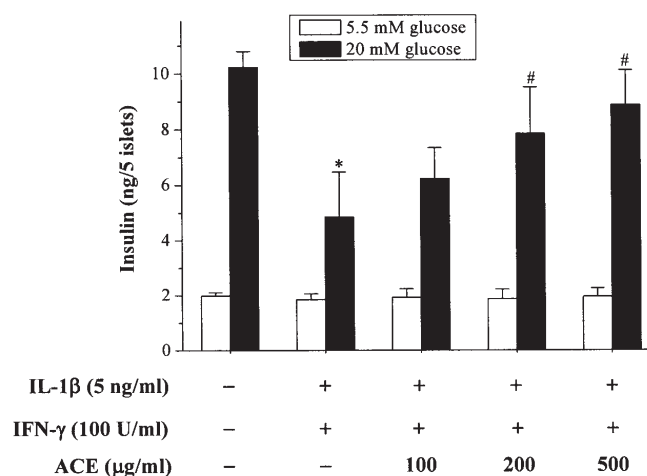


Figure 5. Effects of ACE on IL-1 β and IFN- γ -induced inhibition of glucose-stimulated insulin secretion. Rat islets (5 islets/500 μ l) were treated with IL-1 β (5 ng/ml) and IFN- γ (100 U/ml) in the presence or absence of ACE for 24 h. Following the incubation period, GSIS was assayed as described in 'Materials and Methods'. Results of triplicate samples were expressed as mean \pm SEM. *P<0.05 vs. untreated control; #P<0.05 vs. IL-1 β + IFN- γ .

and 130-kDa iNOS protein expression, whereas marked increases of iNOS mRNA and protein were observed in cells treated with IL-1 β and IFN- γ (Fig. 3). Cells pretreated with ACE at a concentration of 500 μ g/ml showed suppressed expression of iNOS in both their mRNA and protein levels in a concentration-dependent manner (Fig. 3). These results indicate that IL-1 β and IFN- γ -induced NO production was caused by the iNOS expression in the RIN cells.

Effect of ACE on IL-1 β and IFN- γ -induced NF- κ B activation. NF- κ B is implicated in the transcriptional regulation of cytokine-induced iNOS expression. Thus, we studied the effect of ACE on cytokine-stimulated translocation of NF- κ B from the cytoplasmic compartment to the nucleus in RIN cells. The IL-1 β and IFN- γ -stimulated RIN cells showed increases in NF- κ B binding activity and p65 subunit levels in their nucleus (Fig. 4A and B) and I κ B α degradation in the cytosol (Fig. 4B) when compared to unstimulated cells. I κ B β was not affected by IL-1 β and IFN- γ treatment (data not shown). IL-1 β and IFN- γ -induced NF- κ B activation and I κ B α degradation were markedly suppressed by the addition of ACE, which suggests that ACE inhibits iNOS expression through the inhibition of NF- κ B activation. Specificity of the DNA-protein interactions for NF- κ B was demonstrated by competition assays using a 50-fold excess of unlabeled oligonucleotide (Fig. 4A, lane 1).

Preservation of GSIS by ACE in the presence of IL-1 β and IFN- γ . We assayed GSIS using rat pancreatic islets isolated from the male Sprague-Dawley rat to support the physiological importance of the results observed in the cell line studies (Fig. 5). After a 24-h exposure to IL-1 β and IFN- γ , insulin secretion in response to 20 mM glucose was observed. Control islets secreted insulin at a concentration of 10.2 ± 0.6 ng/ml, whereas insulin secretion from IL-1 β and IFN- γ -treated cells was significantly decreased to 4.8 ± 1.6 ng/ml (P<0.05). Pretreatment with ACE blocked the IL-1 β and

IFN- γ effect and restored islet insulin secretion to near control levels. ACE alone did not affect insulin release compared to control (data not shown).

Discussion

Cytokines, as humoral mediators of inflammation, confer destruction to pancreatic β -cells of various species, including the rat and the human (5,11). It has been previously shown that, whereas IL-1 β is a sufficient stimulus for induction of iNOS mRNA expression and NO production in RIN cells, a combination of IL-1 β and IFN- γ is required to induce NO formation by human islets (4,5,10,23). The increased toxicity of the cytokine combination compared with that of IL-1 β alone (Fig. 1) was due to a significantly higher rate of iNOS mRNA and protein expression (Fig. 3) and subsequent NO production (Fig. 1B). Data obtained in other cell types suggest that putative sites for IFN- γ action include stabilization of iNOS mRNA (24), potentiation of IL-1 β -induced NF- κ B activation (25), and induction of other nuclear transcription factors, such as interferon regulatory factor-1 (26).

The central role of NO in cytokine-mediated β -cell toxicity prompted us to examine whether the protective effect of ACE was associated with modulation of NO levels. We found that ACE abrogated NO production in response to cytokines. Taken together with our current data showing that pharmacologic suppression of NO production by L-NAME, an iNOS inhibitor, also protects RIN cells from cytokine toxicity, our results establish the suppression of NO production as a critical mechanism by which ACE protects β -cells. The mechanism by which ACE suppresses cytokine-induced NO production was shown to be via the inhibition of cytokine-induced iNOS mRNA and protein expression (Fig. 3). Expression of iNOS protein in islets is regulated by *de novo* transcription of the iNOS gene (4-6), indicating that ACE regulates iNOS expression at the level of gene transcription.

Since NF- κ B is the major transcription factor responsible for *de novo* activation of iNOS transcription by cytokines, we examined the effect of ACE on NF- κ B activation. We found that ACE suppressed the activation of the transcription factor NF- κ B in RIN cells. We have previously shown that herbs and dietary supplements protect β -cells from cytokine or cytotoxin toxicity through suppression of iNOS expression (6,18,27,28). Many other groups have also tried to save β -cells from these insults by repressing either NF- κ B activation or NF- κ B-dependent iNOS expression (29-32). Thus, repression of NF- κ B or its downstream iNOS expression are important targets for protecting β -cells. NF- κ B is a ubiquitous transcription factor constitutively expressed in the cytoplasm in an inactive form associated with an inhibitory protein termed I κ B (12,13). Cellular activation by inflammatory stimuli such as cytokines results in the phosphorylation and subsequent degradation of I κ B α , thus allowing NF- κ B to translocate into the nucleus and activate target genes such as iNOS (12,13). Therefore, we examined the effect of ACE on I κ B α degradation. Our data demonstrate that ACE interferes with NF- κ B activation at the level of I κ B α degradation after cytokine stimulation.

In summary, we demonstrated the potent inhibitory effect of ACE on cytokine-induced β -cell damage using an

insulinoma cell line and isolated pancreatic islets. ACE rescued β -cells from cytokine toxicity, reversed impairment, and completely restored function. No other report has yet documented the application of ACE in the prevention of β -cell damage against cytokine toxicity. The results of this study will provide valuable information for both elucidating the mechanisms of autoimmune β -cell destruction and for developing drugs to combat type 1 diabetes.

Acknowledgements

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