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Inhibitory effects of epicatechin on interleukin-1β-induced inducible nitric oxide synthase expression in RINm5F cells and rat pancreatic islets by down-regulation of NF-κB activation

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Abstract

Cytokines that are released by infiltrating inflammatory cells around the pancreatic islets are involved in the pathogenesis of type 1 diabetes mellitus. Specifically, interleukin-1β (IL-1β) stimulates inducible nitric oxide synthase (iNOS) expression and nitric oxide overproduction, leading to the β-cell damage. In activating this pathway, nuclear factor-κB (NF-κB) plays a crucial role, and many of the IL-1β-sensitive genes contain NF-κB binding sites in their promoter regions. We have recently shown that epicatechin, which is a flavonoid, had a protective effect on pancreatic β-cells in both streptozotocin-treated rats and islets. In the present study, the effects of epicatechin on IL-1β-induced β-cell damage were examined. RINm5F cells and islets were pretreated with epicatechin and next incubated with IL-1β. The released nitrite, iNOS protein and mRNA expression levels were then measured. IκBα protein, nuclear translocation of NF-κB, and NF-κB DNA binding activity were also determined. Following the transient transfection of an iNOS promoter into the cells, the iNOS promoter activity was measured. ATP- or D-glucose-induced insulin release was measured in RINm5F cells and islets, respectively. Epicatechin significantly reduced IL-1β-induced nitrite production, iNOS protein and mRNA expressions, and it also inhibited IL-1β-induced IκBα protein degradation, NF-κB activation, and iNOS promoter activity. Epicatechin partly restored the IL-1β-induced inhibition of insulin release. These results suggest that epicatechin inhibits the IL-1β-induced iNOS expression by down-regulating NF-κB activation, and protecting β-cells from IL-1β.

Keywords: Epicathechin; Interleukin-1β; iNOS; NF-κB; RINm5F; Islet

1. Introduction

Autoimmune insulitis, the infiltration of inflammatory cells within and around the pancreatic islets, is the primary characteristic feature of type 1 (insulin-dependent) diabetes mellitus [1]. Proinflammatory agents, such as cytokines and free radicals, are involved in the insulitis. It has been especially noted that interleukin-1β (IL-1β) induces the expression of inducible nitric oxide synthase (iNOS) in β-cells, and this leads to the overproduction of nitric oxide (NO) that mediates the inhibition of insulin secretion and the cytotoxicity of β-cells [2,3]. The time-dependent inhibitory action of IL-1β on the islet function correlates well with the time-dependent expression of iNOS and the production of NO [4]. NOS inhibitors prevent the inhibitory actions of IL-1β on the islet oxidative metabolism, insulin secretion, and the islet secretory function [5].

Abbreviations: IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NF-κB, nuclear factor-κB; STZ, streptozotocin; DMEM, Dulbeccos Modified Eagle Medium; HBSS, Hanks balanced salt solution; IFN-γ, interferon-γ; DIG, digoxigenin; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; ATP, adenosine 5′-triphosphate disodium salt; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; KRB, Krebs–Ringer bicarbonate

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Recently, pancreatic β-cells was proved to be the primary source of cytokine-induced free radical formation in streptozotocin-induced diabetic rats [6].

The inhibitory actions of IL-1β on islets are associated with the expression of various genes activated by nuclear factor-κB (NF-κB), a transcription factor. The long exposure of islets to IL-1β stimulates various NF-κB-mediated genes including iNOS and cyclooxygenase-2 [7,8]. For NF-κB activation, the phosphorylation and proteolytic degradation of IκB is requisite. MG132, a proteasome complex inhibitor, and TLCK, a serine protease inhibitor, inhibited both the IL-1β-induced activation of NF-κB and the degradation of IκBα in islets and RINm5F cells [7].

Catechins are flavonoids that exert antioxidant and anticarcinogenic activities [9,10]. Since the beginning of 1980s, the effects of epicatechin on diabetes mellitus have been studied. Epicatechin has been reported to protect pancreatic β-cells and to stimulate β-cell regeneration in alloxan-induced diabetes mellitus [11,12]. In another study, however, the protective effect of epicatechin was not observed [13].

Recently, we found that epicatechin inhibited the deleterious effects of streptozotocin (STZ) on the pancreatic β-cells. Epicatechin inhibited STZ-induced hyperglycemia and β-cell destruction in the rat pancreas, and it blocked STZ-induced NO production and inhibition of insulin release from the isolated islets [14].

Thus, the aim of this study was to investigate the effect of epicatechin on IL-1β-induced β-cell damage, focusing on epicatechin's effect on the iNOS expression. The inhibitory effects of epicatechin on IL-1β-induced NF-κB activation, iNOS mRNA and protein expression, and the subsequent NO production were observed in both RINm5F cells and the isolated islets.

2. Materials and methods

2.1. Materials

FBS, RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), OPTI-MEM, and Hank's balanced salt solution (HBSS) were purchased from Gibco BRL. Ficoll separating solution was from Biochrom AG. Recombinant human interleukin-1β (IL-1β) and human interferon-γ (IFN-γ) were from R&D systems. BCA protein assay kit was from Perbio Science. Griess Reagent System, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit, pGEM-T Easy Vector, pGL3 Luciferase Reporter Vector, CMV-driven β-galactosidase expression vector, and T4 polynucleotide kinase were from Promega. Top-Pfu^{TM} DNA polymerase was from Bio-online. Anti-mouse iNOS antibody was from BD Transduction Laboratories. Western Blotting Luminol Reagent, NF-κB oligonucleotides probe, antibodies to p50, p65 and c-Rel, and anti-rabbit IκBα antibody were from Santa Cruz Biotechnology. RNA STAT-60 was from TEL-TEST. AccuPrep genomic DNA extraction kit was from Bioneer. First Strand cDNA Synthesis Kit for RT-PCR, alkaline phosphatase-conjugated anti-digoxigenin (DIG) antibody, CDP-Star, and FuGENE 6 transfection reagent were from Roche Diagnostics. Rat insulin RIA Kit was from LINCO Research. All other reagents, including lipopolysaccharide (LPS, *Escherichia coli* 026:B6), (−)-epicatechin, N^G-monomethyl-L-arginine (L-NMMA), peroxidase-conjugated anti-mouse antibody and adenosine 5′-triphosphate disodium salt (ATP) were purchased from Sigma. Epicatechin was dissolved in DMSO at 100 mM stock solution and added to the culture medium. The final concentration of DMSO did not exceed 0.2%.

2.2. Animals and cell culture

Male Sprague–Dawley rats weighing 300 g (Daehan Biolink Company) were used. The animals were kept under specific pathogen-free conditions and had free access to a standard commercial diet (Samyang Oil Feed Co). All experimental procedures performed on the animals were conducted with the approval of the ethics committee of The Catholic University of Korea and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80−23, revised 1996). RINm5F (CRL-11605) cells, a rat insulinoma cell line, were obtained from American Type Tissue Collection. RINm5F cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and maintained at 37 °C in humidified air containing 5% CO_2. Cells were subcultured weekly and passages 19–30 were used exclusively. Cells were plated at a concentration of 2×10^5 per mL. At 70% confluency, the cells were incubated with serum-free RPMI medium for 16 h prior to the incubation with IL-1β and other chemicals.

2.3. Isolation of pancreatic islets

Following fasting overnight, pancreatic islets was isolated using collagenase digestion according to a modified method of Kim et al. [15]. Under ether anesthesia, the common bile duct was clamped at the opening to the duodenum and cannulated. The pancreas was distended by the infusion of HBSS containing collagenase (0.8 mg/mL, type V) and excised out quickly. The distended pancreas was incubated for 30 min at 37 °C. Then, the collagen-digested pancreas was washed with HBSS supplemented with 5.6 mM D-glucose, and 5% FBS and passed through a screen (mesh size, 520 μm) to remove exocrine tissues. Filtered tissue was centrifuged at $800 \times g$ for 15 min at 4 °C on a discontinuous Ficoll gradient (1.037, 1.069, 1.085, and 1.100 mg/mL). Islets were aspirated from the two interfaces between the 1.037, 1.069, and

1.085 density layers. Islet viability was confirmed by trypan blue exclusion. Freshly isolated islets were aliquoted into sterile 6-well plates (150 islets/well) and cultured in RPMI-1640 medium containing 5.6 mM D-glucose.

2.4. Proliferation assay

Epicatechin (0.1, 0.3, and 1 mM) or vehicle was added to the culture medium 48 h after plating of cells. Following another 48-h incubation, a proliferation assay was conducted according to the manufacturer's directions. To calculate the absorbance values at each dose, the mean absorbance of two blank wells containing epicatechin without cells in culture medium was subtracted from that of the each well containing cells.

2.5. Measurement of NO as nitrite

RINm5F cells or islets were pretreated with varying concentrations of epicatechin (0.1, 0.3, and 1 mM) for 1 h, next incubated with IL-1β (100 pg/mL) for an additional 24 h. This concentration of IL-1β was chosen based on the experiments performed to determine the lowest concentration that will induce the significant NO production in RINm5F cells (Fig. 1A). Dose-response data were best fitted with the following logistic equation using Origin 7.0 software (Microcal Software, Inc.). $Y = 1/[1 + IC_{50}/F]^n$ where IC_{50} is the concentration of epicatechin resulting in 50% inhibition, F is the concentration of epicatechin and n is the Hill coefficient. We fixed the concentration of epicatechin at 0.8 mM based on the IC_{50} values and the concentration used in our previous study [14]. The inhibitory effects of epicatechin and L-NMMA (10 and 100 μM), an NOS inhibitor, on IL-1β-induced NO production were compared. Released nitrite, a stable product of NO in aqueous medium, was measured using Griess Reagent System as described previously [14]. Briefly, the culture medium was mixed with an equal volume of sulfanilamide solution (1% in 5% phosphoric acid) and of *N*-1-naphtylethylenediamine dihydrochloride solution (0.1% in water). The absorbance was measured at 540 nm on MR700 Microplate Reader (Dynatech Laboratories Inc.). Nitrite concentrations were then determined from a calibration curve of standard $NaNO_2$ concentrations against absorbance. Data are expressed as mM per mg protein to adjust for the differences in the cell mass among the groups.

2.6. Western blot analysis

Following the pretreatment of epicatechin (0.8 mM) or L-NMMA (10 and 100 μM) for 1 h, RINm5F cells and islets were incubated with IL-1β (100 pg/mL) for an additional 8 and 16 h, respectively. Western blot analysis was performed essentially as described previously [16].

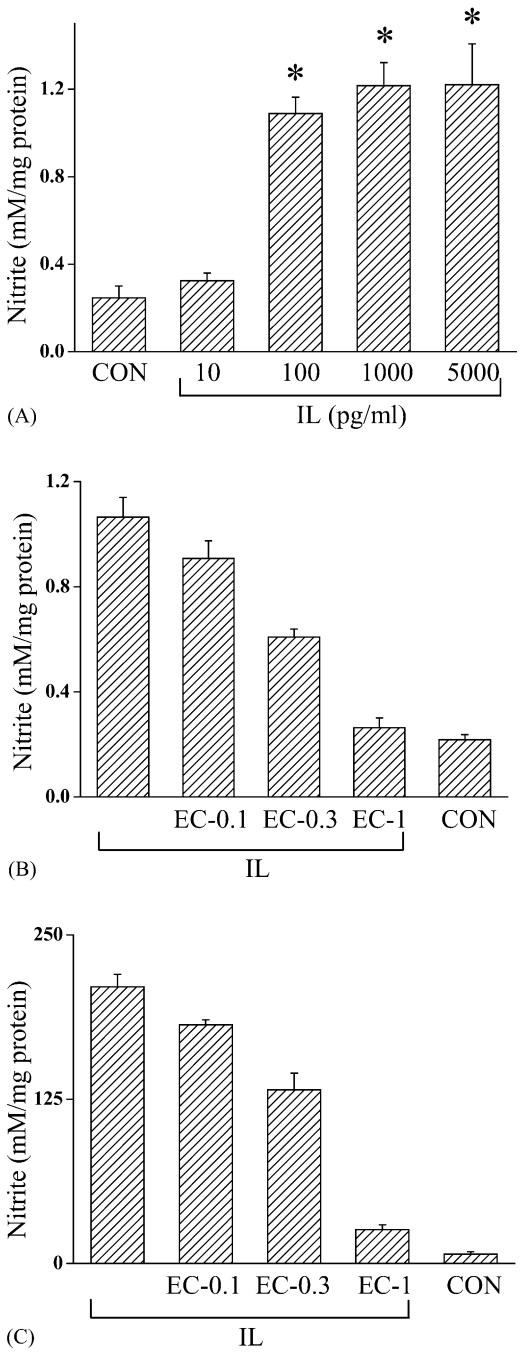

Fig. 1. Dose-dependent effects of epicatechin (EC) on IL-1β-induced nitrite formation in RINm5F cells and rat islets. (A) RINm5F cells were treated with varying doses of IL-1β (10, 100, 1000, and 5000 pg/mL) for 24 h. The culture medium (50 μl) was mixed with an equal volume of sulfanilamide solution, followed by the addition of an equal volume of *N*-1-naphtylethylenediamine dihydrochloride solution. Nitrite production was measured at 540 nm-absorbance using MR700 Microplate Reader. B and C, RINm5F cells (B) or islets (C) were pretreated with indicated doses of EC (0.1, 0.3, and 1 mM) for 1 h, and next incubated with IL-1β (100 pg/mL) for an additional 24 h. Nitrite production was measured as the same way as mentioned above. Data are expressed as mean ± S.D., $^*P < 0.05$ versus CON (ANOVA/Bonferroni's test). CON: control without any treatment; IL, IL-1β.

RINm5F cells and islets were rinsed with ice-cold phosphate-buffered saline (PBS, pH 7.4), harvested, and solubilized with LIPA buffer (25 mM Tris–HCl, pH 7.4, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL aprotinin, and 5 μg/mL leupeptin). After centrifugation at 12,000 × *g* for 30 min at 4 °C, the soluble fraction was collected, and protein content was determined by BCA. Thirty microgram of total protein was separated on an 8% SDS–PAGE and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk in TTBS (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, and 0.1% Tween 20), the membrane was incubated with anti-mouse iNOS IgG (0.5 μg/mL of TTBS) at 4 °C overnight. After washing, the membrane was probed with peroxidase-conjugated anti-mouse IgG (0.5 μg/mL of TTBS). The membrane was then washed with TTBS, and the signal was visualized by enhanced chemiluminescence system. For the measurement of IκBα protein, RINm5F cells were pretreated with epicatechin (0.8 mM) for 30 min, and then incubated with IL-1β (100 pg/mL) for an additional 30 min. Western blot analysis was performed according to the same procedure as mentioned above. Equal loading and transfer of samples were verified by Ponseau S staining or the band intensity of β-actin (42 kDa) or β-tubulin (52 kDa).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR), and northern blot analysis

To determine the peak induction time of iNOS mRNA in response to IL-1β in RINm5F cells, RT-PCR was conducted. The cells were treated with IL-1β (100 pg/mL) for indicated time periods. The cells were harvested, and total RNA was isolated using STAT-60 reagent according to manufacturer's instruction. RT-PCR was carried out using 1st Strand cDNA Synthesis kit, in which 1 μg of total RNA was used as template. The primers used for the amplification of a 689-bp fragment of rat iNOS coding region were designed based on GenBank/EMBL (accession number: U03699) as follows: forward, from +611, 5′-GGTCCAACCTGCAGGTCTTC-3′; reverse, from +1300, 5′-GGTCCATGATGGTCACATTC-3′. Amplification of a 451-bp fragment of rat GAPDH gene was carried out as an internal control. One tenth of RT reaction was amplified in a thermal cycler (GeneAmp PCR cycler 9700, Perkin-Elmer) as following temperature profiles: 5 min at 95 °C; 30 cycles of 30 s at 52 °C, 1 min at 72 °C, and 30 s at 95 °C; and 2 min at 72 °C. For gene expression determination, a positive control for iNOS (RNA isolated from RAW-264.7 macrophage cell line activated with LPS and IFN-χ) was included in the RT-PCR analysis. Based on the result of RT-PCR, RINm5F cells were pretreated with epicatechin (0.8 mM) for 30 min, and then incubated with IL-1β (100 pg/mL) for an additional 6 h. Northern blot analysis was performed as follows. Equal amounts of total RNA (10 μg) were electrophoresed through a 1% agarose gel, then transferred overnight to a positively charged nylon membrane by capillary reaction, and secured to the membrane by UV cross-linking. The membrane was hybridized with DIG-labeled iNOS cDNA probe. iNOS mRNA level was detected by chemiluminescence using CDP-Star as substrate. The ribosomal 18S and 28S bands verified equal loading of samples.

2.8. Immunofluorescent staining

RINm5F cells were grown onto 25 mm round cover glass (Fisher Scientific) at a density of 5×10^4 cells/cover glass. To study the localization of p65 NF-κB subunit, quiescent cells were pretreated with epicatechin (0.8 mM) for 10 min, and then incubated with IL-1β (100 pg/mL) for 30 min. The immunofluorescent staining was performed according to a modified method described by Ryu et al. [17]. The cells were washed, fixed in 2% paraformaldehyde in PBS for 15 min, and were then treated with 0.1% Triton X-100 for 30 s to permeabilize nuclear membranes. Following blocking nonspecific reaction with normal donkey serum, the cells were incubated with anti-rabbit p65 IgG (400 ng/mL) for 16 h at 4 °C. Subsequently, FITC-conjugated goat anti-rabbit IgG was applied on the cells as a secondary antibody. FITC fluorescence was visualized by using 490 nm filter on a Zeiss Axiophot photomicroscope (Carl Zeiss). Negative controls were stained with non-immune serum or with the secondary antibody alone.

2.9. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

RINm5F cells were treated with IL-1β (100 pg/mL) for 30 min following 10-min pretreatment of epicatechin (0.8 mM). Nuclear extracts were isolated according to a modified procedure described by Dignam et al. [18]. The cells were rinsed with PBS, suspended in hypotonic buffer A (10 mM KCl, 10 mM HEPES, pH 7.9, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.2 mM Na_3VO_4, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 0.5% Nonidet P-40) for 15 min on ice. Nuclei were pelleted by centrifugation at 12,000 × *g* for 15 s at 4 °C. Then, the pellets were suspended in hypertonic buffer B (400 mM NaCl, 20 mM HEPES, pH 7.9, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM Na_3VO_4, 1 μg/mL leupeptin, 1 μg/mL aprotinin) for 30 min on ice. The nuclear proteins in the supernatant were recovered after centrifugation at 12,000 × *g* for 10 min and stored at −80 °C. For the DNA binding activity of NF-κB, the oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a κB binding sites (underlined) was used. Two complementary oligonucleotides were end-labeled with [χ-^{32}P]dATP using T4 polynucleotide kinase. Nuclear extracts (10 μg) were incubated with the ^{32}P-labeled probe (30,000 cpm) in the

binding reaction buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 1 μg poly (dI–dC), and 5% glycerol) for 20 min at 4 °C. The reaction product was resolved on a 6% non-denaturing polyacrylamide gel in Tris/borate/EDTA buffer. The gel was dried and exposed at −80 °C. For supershift experiments, 0.2 μg of specific antibodies against NF-κB proteins p50, p65, or c-Rel was applied to the reaction mixture 30 min before the addition of ^{32}P-labeled probe.

2.10. Transient transfection and iNOS promoter luciferase expression assay

Rat genomic DNA was prepared from RINm5F cells using AccuPrep genomic DNA extraction kit according to the manufacturer's instruction. The iNOS promoter region between nucleotides −1173 and +33 was amplified by PCR on 250 ng genomic DNA with Top-PfuTM DNA polymerase. Primers, designed according to the published sequence [19], were as follows: forward, from −1173, 5′-CCGGTACCAAGGCAAGCACTTTGACGACTC-3′ with *KpnI* site (underlined); reverse, from +33, 5′-CCG-CTCGAGAGAGTCTCAGTCTTCAACTCCCTG-3′ with *XhoI* site (underlined). The PCR product was purified from agarose gel, digested, and cloned into *KpnI* and *XhoI* sites of pGL3-Basic vector to obtain piNOS-Luc plasmid construct. The resultant construct was verified by DNA sequencing. Transient transfection was performed by lipofection with FuGENE6 system according to the manufacturer's instruction. RINm5F cells were plated 24 h before the transfection at a density of 2×10^5 cell/well in 12-well plates. piNOS-Luc (0.5 μg) and pCMV/β-gal (0.1 μg, as an internal control) plasmids were incubated with 2 μl of FuGENE6 solution in 50 μl of OPTI-MEM for 15 min and overlaid onto cells. Following overnight incubation, the cells were pretreated with epicatechin (0.8 mM) for 30 min, and then IL-1β (100 pg/mL) was added for an additional 8 h. Subsequently, the cells were solubilized in 1× reporter lysis buffer and cell lysates were prepared by repeated freezing and thawing. Luciferase and β-galactosidase activities were measured with a luminometer (TD20/20, Turner Designs Instrument) and MR700 Microplate Reader (Dynatech Laboratories Inc.), respectively. Transfection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity obtained from the same sample.

2.11. Measurement of insulin release

Insulin release was monitored under static incubation conditions as described previously [14]. Following the treatment of IL-1β (100 pg/mL) for 24 h with the pretreatment of epicatechin (0.8 mM) for 1 h, RINm5F cells and islets were washed with Krebs–Ringer bicarbonate (KRB) buffer (25 mM HEPES, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM D-glucose, and 0.1% bovine serum albumin). Following the treatments of ATP (RINm5F cells, 100 μM) for 30 min or of D-glucose (islets, either 5.6 (to study basal insulin secretion) or 20 mM (to study glucose-induced insulin secretion)) for 1 h, KRB buffer was aspirated and the insulin content was measured radioimmunochemically using rat anti-insulin antibody. ^{125}I-labeled insulin was used as a tracer, and rat insulin was used as the standard. Data are expressed as μg per mg protein to adjust for the differences in the cell mass among the groups.

2.12. Statistical analysis

All data obtained from each experiment were expressed as mean ± S.D. The data were analyzed using one-way ANOVA with Prism software (GraphPad Software Inc.). Statistical comparisons among the groups were done by Bonferroni's multiple range *t*-test after the ANOVA. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Epicatechin has no effect on the viability in RINm5F cells

To investigate whether epicatechin affects the viability of RINm5F cells, a colorimetric proliferation assay was performed. Epicatechin did not affect the cell viability at all concentrations used. Given that the control value was 100%, the values of epicatechin (0.1, 0.3, and 1 mM) treatment were 105, 100, and 103%, respectively. The vehicle (DMSO) was without effect (data not shown).

3.2. Epicatechin inhibits IL-1β-induced NO production

We initially evaluated the optimal conditions to observe the effect of IL-1β on nitrite production in RINm5F cells. As shown in Fig. 1A, IL-1β significantly induces nitrite production at a concentration of 100 pg/mL or above. Accordingly, the single concentration of IL-1β was set at 100 pg/mL in the following experiments. Epicatechin at varying concentrations (0.1, 0.3, and 1 mM) inhibited dose-dependently IL-1β-induced nitrite production in both RINm5F cells (Fig. 1B) and islets (Fig. 1C). The IC_{50} values of epicatechin in RINm5F cells and islets were 0.32 and 1.03 mM, respectively. In inhibiting nitrite formation, epicatechin showed as much propensity as L-NMMA in both RINm5F cells (Fig. 2A) and islets (Fig. 2B).

3.3. Epicatechin inhibits IL-1β-induced iNOS protein

To examine whether the epicatechin-induced decrease of NO production was mediated through iNOS protein synthesis, Western blot analysis was performed. Epicatechin inhibited the IL-1β-induced iNOS protein (∼130 kDa)

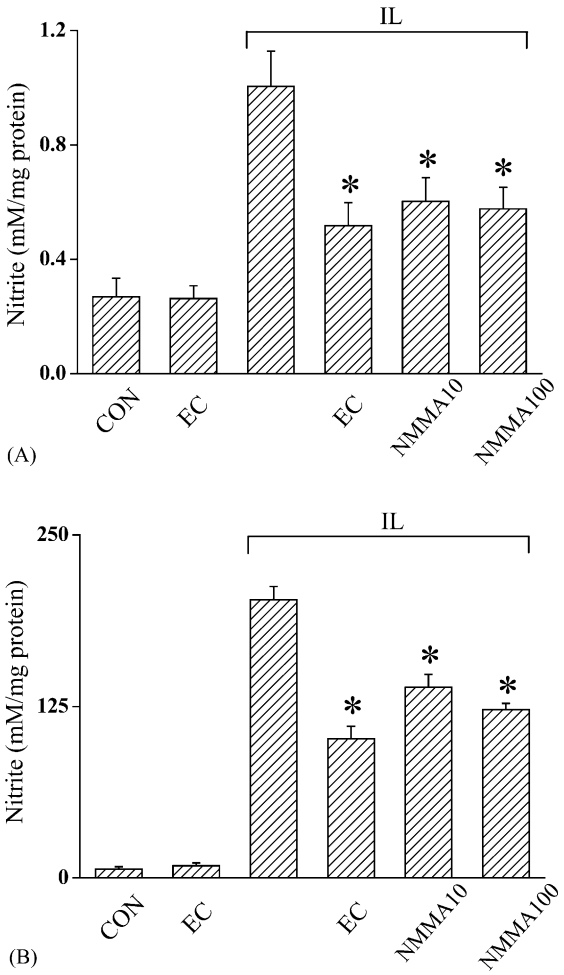

Fig. 2. The effects of epicatechin (EC) or N^G-monomethyl-L-arginine (NMMA) on IL-1β-induced nitrite formation in RINm5F cells and rat islets. A and B, RINm5F cells (A) or islets (B) were pretreated with EC (0.8 mM) or NMMA (10 and 100 μM) for 1 h, and then incubated with IL-1β (100 pg/mL) for an additional 24 h. The culture medium (50 μl) was mixed with an equal volume of sulfanilamide solution, followed by addition of an equal volume of *N*-1-naphtylethylenediamine dihydrochloride solution. Nitrite production was measured at 540 nm-absorbance using MR700 Microplate Reader. Data are expressed as mean ± S.D., $^*P < 0.05$ versus treatment with IL-1β alone (ANOVA/Bonferroni's test). CON: control without any treatment; IL, IL-1β.

expression in both RINm5F cells (Fig. 3A) and islets (Fig. 3B). However, L-NMMA, which had inhibited the IL-1β-induced nitrite production, failed to inhibit IL-1β-induced iNOS protein. Rather, L-NMMA had a tendency to slightly increase the band density of the IL-1β-induced iNOS protein.

3.4. Epicatechin inhibits IL-1β-induced iNOS mRNA expression

To determine whether epicatechin inhibits iNOS at the mRNA level, we next examined the iNOS mRNA expression in RINm5F cells. The results from RT-PCR showed that iNOS mRNA expression peaked at 3 and 6 h after IL-

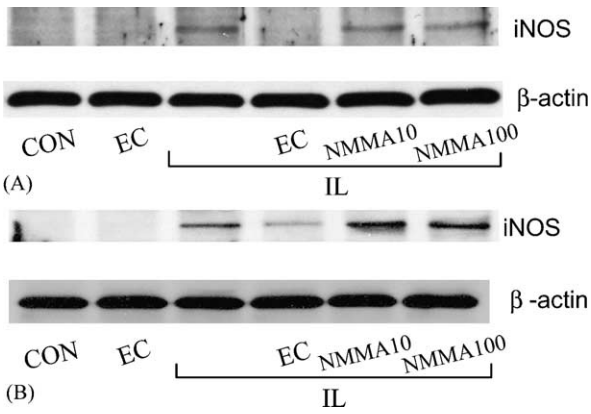

Fig. 3. The effects of epicatechin (EC) or N^G-monomethyl-L-arginine (NMMA) on IL-1β-induced inducible nitric oxide synthase (iNOS) protein in RINm5F cells and rat islets. Following the pretreatment of EC (0.8 mM) or NMMA (10 and 100 μM) for 1 h, RINm5F cells and islets were incubated with IL-1β (100 pg/mL) for an additional 8 and 16 h, respectively. Total cellular proteins (30 μg) were separated and analyzed on an 8% SDS–PAGE and Western blot analysis as described in Section 2. Equal loading of protein was verified by probing the same blot for β-actin. Results are representative of three independent experiments. CON: control without any treatment; IL, IL-1β.

1β treatment (Fig. 4A). Following pretreatment of epicatechin, the cells were exposed to IL-1β for 6 h. By Northern blot analysis, epicatechin significantly inhibited, although not completely, the IL-1β-induced iNOS mRNA expression (Fig. 4B).

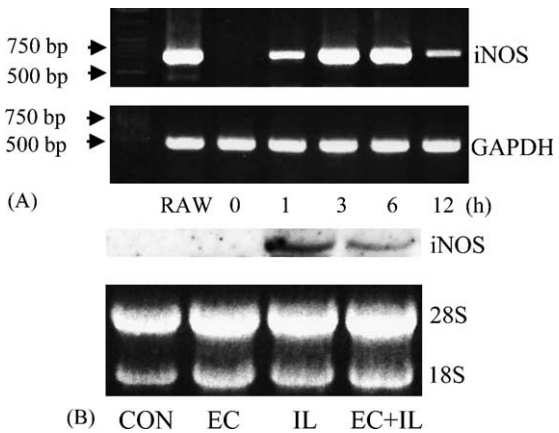

Fig. 4. The effect of epicatechin (EC) on IL-1β-induced inducible nitric oxide synthase (iNOS) mRNA expression in RINm5F cells. A, RINm5F cells were treated with IL-1β (100 pg/mL) for different time periods (1, 3, 6, and 12 h). The levels of iNOS and GAPDH (as an internal control) mRNAs were measured by reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were 1% agarose gel and stained with ethidium bromide. For gene expression determination, a positive control for iNOS [RNA isolated from RAW 264.7 macrophage cell (RAW) activated with LPS and IFN-χ] was included in the RT-PCR analysis. B, RINm5F cells were pretreated with epicatechin (EC, 0.8 mM), next incubated with IL-1β (100 pg/ml) for an additional 6 h. The expressions of iNOS mRNA were measured by Northern blot analysis as described in Section 2. Equal loading of sample (10 μg) was verified by the ribosomal 18S and 28S bands. Results are representative of three independent experiments. CON: control without any treatment; IL, IL-1β.

3.5. Epicatechin inhibits IL-1β-induced IκBα protein degradation and NF-κB activation

Since the activation of transcription factor NF-κB is an essential step for the IL-1β-induced iNOS expression, it is possible that epicatechin might have inhibited NF-κB activation to inhibit iNOS expression. First, the degradation of IκBα protein should be requisite for the binding of NF-κB to κB sites in the promoter region. As shown in Fig. 5A, epicatechin inhibited the IL-1β-induced IκBα protein degradation. Using immunfluorescent staining, we localized the p65 NF-κB subunit in RINm5F cells (Fig. 5B). In the cells of control and epicatechin treatment alone, a diffuse cytoplasmic staining was observed (Figs. 5B1 and B2), while cells treated with IL-1β had a clear nuclear staining, indicating nuclear translocation of p65 (Fig. 5B3). Pretreatment with epicatechin prevented the nuclear translocation of p65 in some cells (Fig. 5B4). This inhibitory effect of epicatechin on the nuclear translocation of NF-κB was verified by an EMSA study. The activity of specific NF-κB binding with κB sites by IL-1β was markedly suppressed by the pretreatment with epicatechin (Fig. 5C). DNA binding of NF-κB by IL-1β was super shifted by the addition of antibodies against NF-κB subunits p65 and p50, but not by the anti-c-Rel antiboby (Fig. 5D). The specificity of this binding to the κB site was confirmed by the competition analysis with a 50-fold excess of the unlabeled probe (data not shown). These findings indicate that one mechanism by which epicatechin prevents the IL-1β-induced iNOS expression in RINm5F cells is through the inhibition of the IL-1β-induced NF-κB nuclear localization.

3.6. Epicatechin inhibits IL-1β-induced iNOS promoter activity

We performed a luciferase reporter gene assay to examine whether IL-1β-induced NF-κB binding to iNOS promoter leads to NF-κB-dependent iNOS gene transcription. Following transient transfection of the luciferase reporter gene containing two κB binding sites of iNOS promoter, RINm5F cells were stimulated with IL-1β for an additional 8 h following 30-min pretreatment of epicatechin. As shown in Fig. 6, IL-1β alone induced NF-κB-dependent luciferase expression about 2.9-fold compared to control, and pretreatment of epicatechin significantly inhibited the iNOS promoter activity.

3.7. Epicatechin inhibits IL-1β-induced inhibition of insulin release

Insulin release is drastically inhibited at a high concentration of and a long exposure to cytokines such as IL-1β. We examined the protective effect of epicatechin on the IL-1β-induced inhibition of insulin release in RINm5F cells and islets. Since RINm5F cells are insensitive to D-glucose due to lack of type 2 glucose transporter [20], ATP was applied to stimulate insulin release via P_{2Y} receptor. As shown in Fig. 7A, epicatechin significantly inhibited the IL-1β-induced inhibition of insulin release in RINm5F cells. To examine the effect of epicatechin on the basal insulin release in islets, 5.6 mM D-glucose was applied. As shown in Fig. 7B1, epicatechin alone did not affect insulin release compared to control, whereas IL-1β alone inhibited insulin release. Also, epicatechin tended to attenuate, though not significantly, the inhibitory effect of IL-1β on insulin release. Meanwhile, to examine the effect of epicatechin on the glucose-induced insulin release, 20 mM D-glucose was applied to islets (Fig. 7B2). Except for the group of IL-1β alone treatment, all the other groups showed the increase of insulin release about two-fold than that of 5.6 mM D-glucose stimulation (Fig. 7B1). Epicatechin significantly restored, though not completely, the IL-1β-induced inhibition of insulin release.

4. Discussion

NO synthesized by iNOS has been implicated in IL-1β-induced β-cell damage. Since NO is very unstable, it is then readily converted to highly toxic peroxynitrite [21]. The clinical relevance of NO in the incidence of type 1 diabetes mellitus has been demonstrated in the epidemiologic studies. High intakes of nitrite and *N*-nitroso compounds were observed in those groups with a high incidence of type 1 diabetes mellitus [22,23]. These facts have evoked a lot of interest in the protective effects of free radical scavengers, particularly with the natural and synthetic antioxidants on the incidence of type 1 diabetes mellitus. It is well known that pancreatic β-cells show remarkably low antioxidant enzyme activities. The expression and activity of the main antioxidant enzymes, such as superoxide dismutase, peroxidase and catalase are significantly lower in the islet than are those in the liver or in the kidney [24,25]. These low antioxidant defense systems of β-cells are considered to be a target of free radicals, leading to the β-cell damage and type 1 diabetes mellitus [26].

It has been well known that IL-1β induces iNOS expression and the overproduction of NO, and this leads to the dysfunction and destruction of β-cells [3]. The RINm5F cell is comparable to the primary β-cells in the aspects of IL-1β-mediated NO production, iNOS expression, and cytotoxicity [27,28]. We observed that the responses of RINm5F cells to IL-1β were similar to those of islets in nitrite formation and iNOS protein expression, although they lack type 2 glucose transporter. This result strongly suggests that RINm5F cells may be an appropriate substitute for the primary β-cells.

The 0.8 mM concentration of epicatechin used in this study was determined with references to the IC_{50} values of nitrite release and to the proliferation assays in which cell viability was not affected. This concentration of epicate-

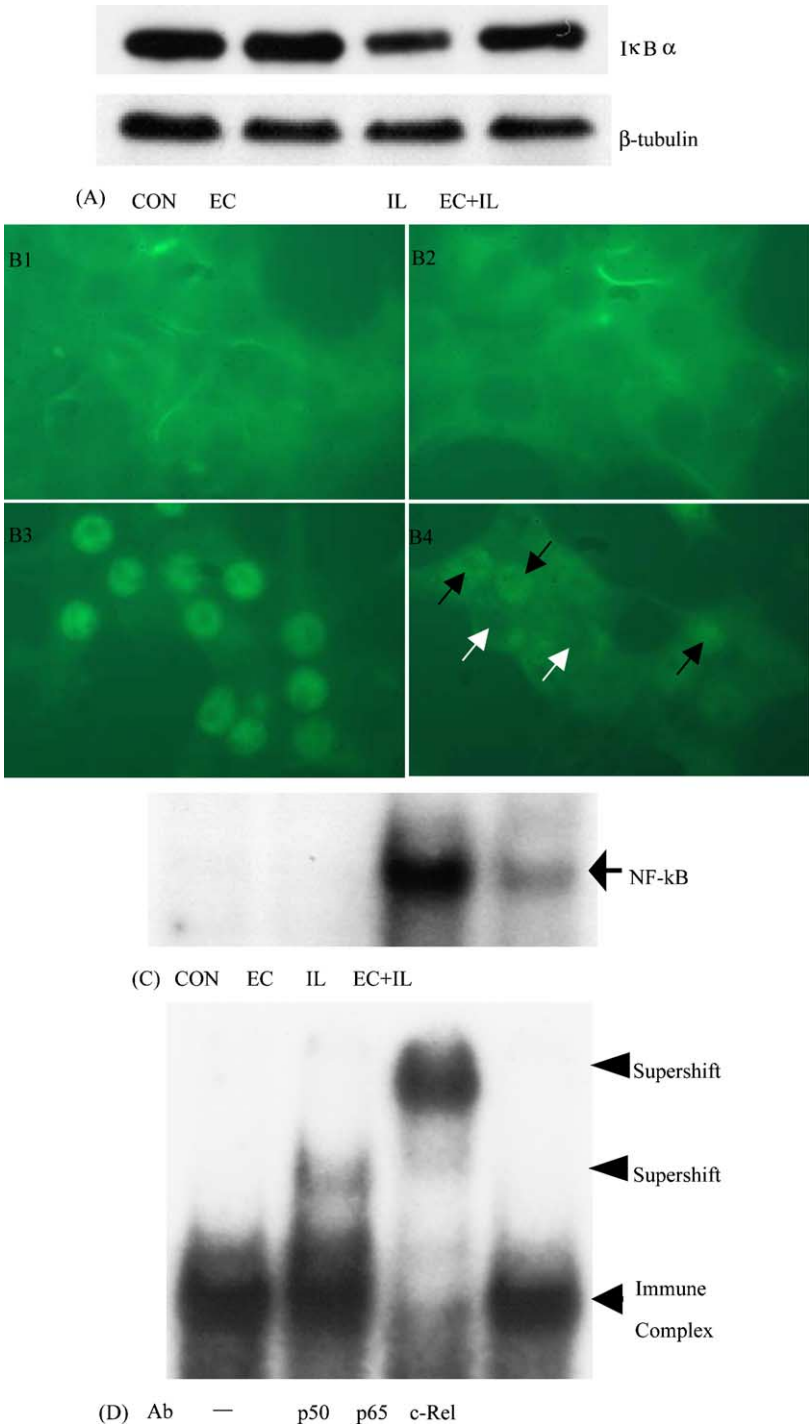

Fig. 5. The effect of epicatechin (EC) on IL-1β-induced NF-κB activation in RINm5F cells. RINm5F cells were pretreated with EC (0.8 mM) for 10 min, then cultured for 30 min with IL-1β (100 pg/mL). (A) Total cellular proteins (30 μg) were separated on a 8% SDS–PAGE and the levels of IκBα protein were measured by Western blot analysis as described in Section 2. Equal loading of protein was verified by probing the same blot for β-tubulin. (B) Immunofluorescent staining for p65 NF-κB subunit. In the cells of control (B1) and epicatechin treatment alone (B2), immunostaining was exclusively observed in the cytoplasm. When treated with IL-1β, an intense nuclear staining was detected in all the cells (B3). Meanwhile, epicatechin pretreatment inhibited IL-1β-induced nuclear translocation in some cells (white arrows), but not in all cells (black arrows) (B4). (C): Nuclear extracts from RINm5F cells were analyzed on electrophoretic mobility shift assay (EMSA) with a NF-κB consensus probes (5′-AGTTGAGGGGACTTTCCCAGGC-3′). (D) For supershift EMSA experiments, specific antibodies (0.2 μg) against NF-κB subunits (p50, p65, and c-Rel) were incubated with nuclear extracts for 30 min before the addition of probes. The arrows indicate bands super shifted by anti-p50 (middle) and anti-p65 (upper) NF-κB subunits. Results are representative of three independent experiments. CON: control without any treatment; IL, IL-1β.

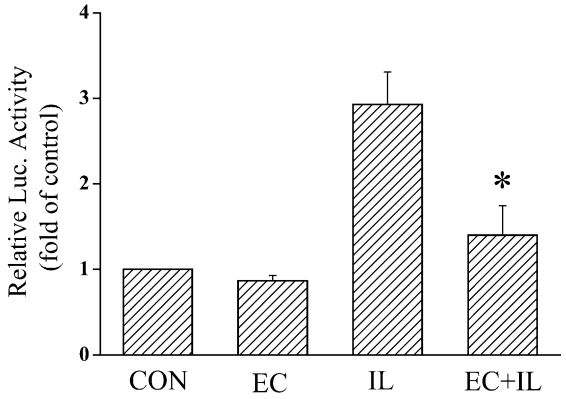

Fig. 6. The effect of epicatechin (EC) on IL-1β-induced iNOS promoter activity in RINm5F cells. RINm5F cells were transiently cotransfected with piNOS-Luc (about 1.2 kb fragments of the 5′ flanking region of the rat iNOS gene) and pCMV-β-gal expression vectors. Following overnight incubation, cells were pretreated with EC (0.8 mM) for 30 min, then cultured for an additional 8 h with IL-1β (100 pg/mL). Cells were then solubilized in lysis buffer and luciferase and β-galactosidase activities were measured as described in Section 2. Transfection efficiencies were normalized by a ratio of luciferase activity to β-galactosidase activity. Data are expressed as mean ± S.D. and each value is fold increase of control value. $^{*}P < 0.05$ versus treatment with IL-1β alone (ANOVA/Bonferroni's test). CON: control without any treatment; IL, IL-1β.

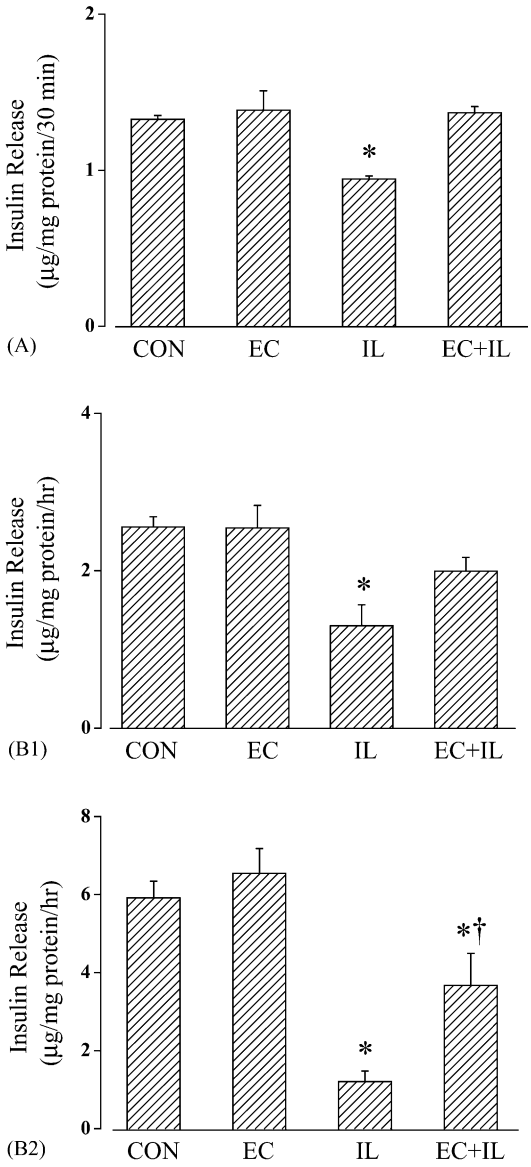

Fig. 7. The effect of epicatechin (EC) on IL-1β-induced inhibition of insulin release in RINm5F cells and rat islets. RINm5F cells (A) and islets (B) were pretreated with EC (0.8 mM) for 1 h, and then incubated with IL-1β (100 pg/mL) for an additional 24 h. The cells and the islets were washed with KRB buffer, and then incubated with ATP (100 μM, A) for 30 min or D-glucose [either 5.6 mM (B1) or 20 mM (B2)] for 1 h. Insulin released into the medium was measured radioimmunochemically using rat anti-insulin antibody. For correction of differences in the cell mass of each group, six identical procedures were performed, and insulin content is expressed per protein content. Data are expressed as mean ± S.D. $^{*}P < 0.05$ versus CON, EC, and EC+IL (in A); CON and EC (in B and C). $^{\dagger}P < 0.05$ versus IL. CON: control without any treatment; IL, IL-1β.

chin was also within the ranges of other pancreas studies [29,30], and this specific concentration had no effect on both the insulin release and the islet integrity in our previous study [14].

Epicatechin inhibited both the IL-1β-induced nitrite production and the iNOS protein while L-NMMA, an NOS inhibitor, inhibited the IL-1β-induced nitrite production only. These results suggest that epicatechin might have inhibited the action of IL-1β at the level of protein or above. Interestingly, L-NMMA tended to slightly increase the density of the iNOS band, and this implies that L-NMMA possibly inhibited the enzyme activity of iNOS, which resulted from the binding of L-NMMA to iNOS to protect from degradation. Similar findings were also reported elsewhere [31].

The peak time of iNOS mRNA expression in response to IL-1β was consistent with others' results [7,32]. A pretreatment with epicatechin inhibited the iNOS mRNA expression induced by IL-1β, implying that epicatechin might regulate the expression of iNOS at the transcriptional level. NF-κB is the primary transcription factor in the regulation of iNOS mRNA expression [28,33]. NF-κB activation by cytokines such as IL-1β promotes the expression of pro- or anti-apoptotic genes [34]. In a resting state, NF-κB is sequestered in the cytosol, where it is bound to the inhibitory protein IκB. Upon its activation, IκB becomes phosphorylated and it then triggers a proteolytic degradation of IκB, which leads to the release and translocation of the NF-κB dimer to the nucleus, where binding to the κB binding sites in the promoter region occurs, and this leads to the increased gene transcription [4,35]. In our study, the pretreatment with epicatechin inhibited IL-1β-induced IκBα protein degradation. This finding is consistent with the observation that EGCG blocks LPS-stimulated NF-κB activation by blocking IκBα protein degradation in mice peritoneal macrophages [36]. Then, we observed that epicatechin inhibited IL-1β-induced nuclear translocation of the p65 NF-κB subunit through a morphological approach, and that epicatechin inhibited

IL-1β-induced NF-κB DNA binding activity through EMSA (Fig. 5). The NF-κB complex is composed of hetero or homodimerization of proteins in the Rel family including p50 and p52. The activated form of the NF-κB complex varies depending on cell types and stimulants [37,38]. Through an antibody super shift experiment, we observed that the IL-1β-induced NF-κB complex was composed of p50–p65 subunits [39]. In transient transfection experiment IL-1β-induced iNOS promoter activity was significantly inhibited by the pretreatment of epicatechin. Thus, the actions of epicatechin appear to be associated with the inhibition of NF-κB activation and its nuclear localization, and this might be done by inhibiting IκBα protein degradation.

IL-1β has been shown to inhibit mitochondrial aconitase activity and the oxidation of glucose to CO_2, leading to the reduction of the cellular ATP levels. NO mediates the inhibitory effects of IL-1β on insulin release through this mitochondrial dysfunction [40,41]. In our study, epicatechin restored, although not completely, IL-1β-induced inhibition of insulin secretion in RINm5F cells. Also, epicatechin attenuated, though it did not restore to control level, the IL-1β-induced inhibition of glucose-stimulated insulin release in islets. This effect of epicatechin might be partly due to the inhibition of NO production. While the inhibitory effects of epicatechin on the IL-1β-mediated iNOS pathway were significant, its action on the insulin secretory function was modest. Accordingly, to understand the inhibitory mechanism of epicatechin on the β-cell damage, a study verifying the action of epicatechin on other inflammatory agents such as cyclooxygenase-2 is needed.

In conclusion, this study suggests that epicatechin efficiently inhibits IL-1β-induced NO production and iNOS gene expression via the inhibition of IκBα protein degradation and of NF-κB activation in pancreatic β-cells.

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