Celastrol ameliorates cytokine toxicity and pro-inflammatory immune responses by suppressing NF-κB activation in RINm5F beta cells

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The hallmark of type 1 diabetes mellitus (T1DM) is selective destruction of the insulin-producing pancreatic β-cells via the inflammatory response (reviewed in [1]). Various cytokines, such as interleukin-1β (IL-1β), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α), are produced from activated immune cells that infiltrate the islets during development of T1DM. These pro-inflammatory cytokines are important mediators that induce loss of pancreatic β-cells (2, 3).

INTRODUCTION

The molecular mechanisms underlying β-cell destruction include upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), leading to enhanced production of nitric oxide (NO) and prostaglandin E2, respectively (2, 4). In addition, various chemokines including CC chemokine ligand 2 (CCL2; monocyte chemoattractant protein-1), CX3 chemokine ligand 8 (CXCL8; IL-8), and CXCL10 (IFN-γ-induced protein-10) may contribute to β-cell destruction by recruiting various immune cells into the pancreatic islets (5, 6).

RESULTS

Celastrol reverses the cytotoxic effect of cytokines in RINm5F cells. We used the RINm5F rat pancreatic β-cell line, which is a pancreatic β-cell line stimulated with a combination of interleukin-1β (IL-1β), interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNFα), and interleukin-6 (IL-6) to induce cytokine toxicity and pro-inflammatory responses in RINm5F rat pancreatic β-cells (2, 3).

Celastrol ameliorates cytokine toxicity and pro-inflammatory immune responses by suppressing NF-κB activation in RINm5F beta cells. The mechanisms of action of celastrol against cytotoxicity and pro-inflammatory immune responses in the RINm5F rat pancreatic β-cell line stimulated with a combination of interleukin-1 beta, tumor necrosis factor-alpha, and interferon-γ. Celastrol significantly restored cytokine-induced cell death and significantly inhibited cytokine-induced nitric oxide production. In addition, the protective effect of celastrol was correlated with a reduction in pro-inflammatory mediators, such as inducible nitric oxide synthase, cyclooxygenase-2, and CC chemokine ligand 2. Furthermore, celastrol significantly suppressed cytokine-induced signaling cascades leading to nuclear factor kappa B (NF-κB) activation, including IκB-kinase (IκK) activation, IκB degradation, p65 phosphorylation, and p65 DNA binding activity. These results suggest that celastrol may exert its cytoprotective activity by suppressing cytokine-induced expression of pro-inflammatory mediators by inhibiting activation of NF-κB in RINm5F cells.

The molecular mechanisms underlying β-cell destruction include upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), leading to enhanced production of nitric oxide (NO) and prostaglandin E2, respectively (2, 4). In addition, various chemokines including CC chemokine ligand 2 (CCL2; monocyte chemoattractant protein-1), CX3 chemokine ligand 8 (CXCL8; IL-8), and CXCL10 (IFN-γ-induced protein-10) may contribute to β-cell destruction by recruiting various immune cells into the pancreatic islets (5, 6).

Nuclear factor kappa B (NF-κB) signaling pathways predominantly regulate cell death and expression of various inflammatory mediators, such as iNOS, COX-2, and CCL2 in pancreatic β-cells (7). Cytokine stimulation initially activates the IκB-kinase (IκK) complex, which consists of two kinase subunits (IκKα and IκKγ) and the IκKα/NEMO regulatory subunit. In turn, IκK rapidly phosphorylates IκBα on Ser32 and Ser36, resulting in its ubiquitination and subsequent proteasomal degradation (8). NF-κB dissociates from IκBα and translocates to the nucleus, where it activates gene transcription of inflammatory mediators.

Celastrol, a quinone methide triterpenoid, is a major constituent from Tripterygium wilfordii Hook with biological activities and has been used widely as a traditional medicine to control various inflammatory diseases (9). Celastrol has anti-inflammatory activities in various inflammatory disease models (reviewed in [10]). Although celastrol does not prevent diabetes in NOD mice, it transiently lowers blood glucose (11). In addition, celastrol inhibits insulin resistance and diabetic nephropathy, possibly by inhibiting NF-κB activity in a type 2 diabetic animal model (12). Despite its beneficial effects on several diabetic conditions, the protective effect of celastrol on pancreatic β-cells has not been determined. In this study, we investigated the regulatory effect of celastrol on cytokine-induced cell death, expression of pro-inflammatory mediators, and NF-κB signaling cascades in RINm5F rat pancreatic β-cells.

Keywords: β-cell, Celastrol, Cytokine, Inflammation, NF-κB

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were evaluated using the Griess reaction. Stimulating RINm5F cells with cytokines for 24 h, and then nitrite levels in the medium with various concentrations of celastrol for 1 h, stimulated cytokine-induced NO production, RINm5F cells were pretreated (13). To examine the regulatory effect of celastrol on cytoplasmic metabolism and modifying proteins in pancreatic \( \beta \)-cells is a major mediator inducing cell death by altering mitochondrial metabolism and modifying proteins in pancreatic \( \beta \)-cells (13). To examine the regulatory effect of celastrol on cytokine-induced NO production, RINm5F cells were pretreated with various concentrations of celastrol for 1 h, stimulated with cytokines for 24 h, and then nitrite levels in the medium were evaluated using the Griess reaction. Stimulating RINm5F cells with cytokines markedly increased NO production, whereas a 1 h pretreatment with celastrol resulted in a significant reduction in NO levels in a dose-dependent manner in cytokine-stimulated RINm5F cells. These results contribute to the protective effect of celastrol against cytokine-induced cell death.

Celastrol inhibits iNOS and subsequent production of NO in cytokine-stimulated RINm5F cells

Inflammatory cytokines, such as IL-1\( \beta \), TNF-\( \alpha \), and IFN-\( \gamma \), exert toxic effects on pancreatic \( \beta \)-cells by inducing iNOS expression and subsequent NO production (reviewed in [7]). NO is a major mediator inducing cell death by altering mitochondrial metabolism and modifying proteins in pancreatic \( \beta \)-cells (13). To examine the regulatory effect of celastrol on cytokine-induced NO production, RINm5F cells were pretreated with various concentrations of celastrol for 1 h, stimulated with cytokines for 24 h, and then nitrite levels in the medium were evaluated using the Griess reaction. Stimulating RINm5F cells with cytokines markedly increased NO production, whereas a 1 h pretreatment with celastrol resulted in a significant reduction in NO levels in a dose-dependent manner in cytokine-stimulated RINm5F cells. These results contribute to the protective effect of celastrol against cytokine-induced cell death.

Celastrol inhibits cytokine-induced expression of COX-2 and CCL2 in RINm5F cells

Stimulating pancreatic \( \beta \)-cells with cytokines, such as IL-1\( \beta \), TNF-\( \alpha \), and IFN-\( \gamma \), induces the expression of pro-inflammatory mediators, such as COX-2 and chemokines including CCL2, CCL8, and CXCL10 (5, 6, 14). These chemokines are implicated in the recruitment and activation of immune cells, such as monocytes and T cells, into pancreatic islets during development of T1DM (6, 14). We further examined the effect of celastrol on the expression of pro-inflammatory mediators, such as COX-2, CCL2, CCL8 and CXCL10, in cytokine-stimulated RINm5F cells. The cells were pretreated with celastrol for...
Celastrol inhibits cytokine-induced signaling cascades leading to NF-κB activation in RINm5F cells

NF-κB is a principal transcription factor for the expression of various proinflammatory mediators, such as iNOS, COX-2, and cytokines/chemokines (7, 15). Signaling cascades leading to NF-κB activation include IKK activation, IkBα degradation, and p65 phosphorylation (8). We first investigated the effect of celastrol on cytokine-induced IkKαβ activation. Total and phosphorylated forms of IkK in cell lysates were determined to evaluate the degree of IkKαβ activation. As shown in Fig. 4A, celastrol significantly inhibited cytokine-induced IkKαβ phosphorylation. Next, we evaluated the suppressive effect of celastrol on cytokine-induced downstream signaling cascades leading to NF-κB activation after IkK activation. As shown in Fig. 4B, celastrol significantly suppressed IkBα degradation and p65 phosphorylation in cytokine-stimulated RINm5F cells. In addition, celastrol inhibited cytokine-induced p65 DNA-binding activity, as measured by electrophoretic mobility shift assay (EMSA) (Fig. 4C). However, celastrol did not affect cytokine-induced signal transducer and activator of transcription 1 (STAT1) activation (Fig. 4D). These findings suggest that suppressing signaling cascades leading to NF-κB activation is a mechanism by which celastrol inhibits expression of proinflammatory mediators, such as iNOS, COX-2 and CCL2, in cytokine-stimulated RINm5F cells.

DISCUSSION

The most common feature of T1DM is loss of pancreatic β-cells via autoimmune responses (1). When stimulated by inflammatory cytokines (e.g., IL-1β, TNF-α, and IFN-γ), insulin-producing pancreatic β-cells express increased levels of iNOS and COX-2, as well as chemokines such as CCL2, which mediate destructive events in the pancreatic islets (3, 6). Therefore, any interventions in the steps involved in cell death and...
pro-inflammatory responses may provide a rationale for the development of therapeutic agents against T1DM.

During pathogenic processes of inflammatory diseases including type 1 diabetes, macrophages and T cells infiltrate the pancreas, and produce various cytokines, such as IL-1β, TNF-α, and IFN-γ, which mediate selective β-cell dysfunction and death (reviewed in [7]). Previous reports have suggested that these cytokines induce cell death in synergistic fashion (reviewed in [16]). The combination of IL-1β, TNF-α, and IFN-γ under our experimental conditions induced cell death in about 60% of RINm5F cells. However, cytokine-induced cell death was significantly inhibited by pretreatment with celastrol (Fig. 1C), indicating its protective effect in cytokine-stimulated RINm5F cells. It has been suggested that cytokine-induced iNOS expression and subsequent NO production have functional correlations with the dysfunction and destruction of β cells (4). We observed that celastrol suppressed iNOS expression and subsequent NO production in cytokine-stimulated RINm5F cells (Fig. 2). In addition, celastrol significantly inhibited cytokine-induced COX-2 expression (Fig. 3A and B). The inhibited iNOS and COX-2 expression may have contributed to the protective effect of celastrol against cytokine-induced cell death.

Chemokines, such as CCL2, CXCL8, CXCL10, are also mediators of β-cell death by recruiting various immune cells into the pancreatic islets (5, 6). Previous studies have demonstrated that increased CCL2, CXCL8, and CXCL10 expression levels are associated with disease progression in T1DM models (5, 6, 17). Although resident macrophages and endothelial cells primarily secrete these chemokines, β cells release various chemokines including CCL2 upon stimulation by cytokines (reviewed in [16]). We found that celastrol significantly decreased CCL2 production in cytokine-stimulated cells, whereas CXCL8 and CXCL10 levels were not lowered (Fig. 3). The differential effect of celastrol on chemokine expression may be due to the distinct pathways leading to chemokine expression.

NF-κB activation is involved in pro-apoptotic and inflammatory responses. Therefore, modulating NF-κB activation is a promising strategy to develop therapeutic targets against T1DM (18). Various stimuli including cytokines activate the IKK complex, consisting of IKKα, IKKβ, and IKKγ/NEMO, which in turn activates downstream signaling cascades leading to NF-κB activation (19). As shown in Fig. 4A, celastrol significantly inhibited cytokine-induced IKK phosphorylation. A previous study revealed that celastrol suppresses IKK activity, possibly by directly targeting cysteine 179 in IKK (20). Celastrol also suppressed cytokine-induced IkBα degradation, p65 phosphorylation, and DNA-binding activity of p65 NF-κB (Fig. 4B and C). Cytokines utilize diverse transcriptional factors other than NF-κB, such as STAT1 and activator protein 1, leading to up-regulation of gene transcription related to β-cell death (reviewed in [7]). However, celastrol did not affect cytokine-induced STAT1 (Fig. 4D) or c-Jun activation (data not shown) under our experimental conditions. Taken together, these data suggest that celastrol exerts protective effects against cytokine-induced cell death and pro-inflammatory responses by suppressing NF-κB signaling pathways.

In conclusion, we provide evidence that celastrol regulated cytokine-induced cell death and pro-inflammatory responses by downregulating iNOS and COX-2, as well as chemokines such as CCL2 in RINm5F cells. Celastrol exerted its protective effects against cytokine-stimulated RINm5F cells by intervening in NF-κB signaling pathways. Elucidating the mechanisms of action by which celastrol modulates dysfunction of pancreatic β-cells may provide the molecular basis for developing therapeutic agents against T1DM.

MATERIALS AND METHODS

Cell culture and reagents
The RINm5F rat pancreatic β-cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO2 and 95% air. IL-1β, TNF-α, and IFN-γ were obtained from R&D Systems (Minneapolis, MN, USA). Primary antibodies against iNOS, COX-2, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Celastrol, HRP-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis, MO, USA). All other reagents were from Sigma-Aldrich.

Cell viability assay
Cell viability was monitored by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay with a slight modification (21). Briefly, RINm5F cells were seeded at 1 × 104 cells/well in a 96-well plate. The cells were incubated with different concentrations of celastrol with or without a combination of cytokines (5 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ) for 24 h in serum-free media. MTT solution (1 mg/ml) was added to each well and incubated at 37°C for 30 min. After the culture supernatants were removed, 0.5 ml isopropanol was added to dissolve the blue formazan crystals. Absorbance was measured at 570 nm with a microplate reader.

Nitrite determination
RINm5F cells seeded at 1 × 104 cells/well in a 6-well plate were cultured for 24 h in complete media. The cells were treated with celastrol for 1 h and then stimulated with cytokines (5 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ) for 24 h in serum-free media. The amount of nitrite in the culture medium was measured using the Griess reagent system (Promega, Madison, WI, USA) (21).

Western blot analysis
Cellular or nuclear extracts were prepared in a lysis buffer (50 mM Tris-HCl, 150 ml NaCl, 1 mM EDTA, 1 mM PMSF, 1
µg/ml leupeptin, 1 mM Na3VO4, 1 mM NaF, 1% NP-40, and 0.25% sodium deoxycholate). The protein concentrations in the samples were measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins in samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% nonfat dry milk in TBST buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.6, and 0.1% Tween 20) for 1 h, and incubated with primary antibodies at 4°C overnight. After washing with TBST three times, the membranes were incubated with HRP-conjugated primary antibodies for 2 h at room temperature. Immunoreactive proteins were detected by a chemiluminescence system (Amersham Life Sciences, Parsippany, NJ, USA) (22).

RT-PCR analysis
Total cellular RNA was extracted from RINm5F cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA), according to the manufacturer’s instructions. cDNA was generated from total RNA (2 µg) using 10,000 U of reverse transcriptase and 0.5 µg/µl oligo-(dT)15 primer (Promega, Madison, WI, USA) (23). PCR amplification of cDNA was performed using the following sense and antisense primers (5'-3'): iNOS sense, TTG GGT CTT CTT AGC CTA GTC; iNOS antisense, TGT GCA GTC GTC CCA GTG AGG AAC; COX-2 sense, CTG TAT CCC GCC CCG GTG CTG GT; COX-2 antisense, ACT TGC GYY GAT GGC CAC TCT CAC ATT TC; CXCL10 sense, CGC CAC CTA TCG CTC TCA CAC ATT TC; CXCL10 antisense, CTT GGG GAC ACC TTT TAG CAT CTT TGG G; beta-actin sense, GAC CGA GCC GGG TGG GTA TCC CAG C; beta-actin antisense, TGT GCA GCC GGG TGG GTA TCC CAG C; beta-actin antisense, TGT CAG CGA TGC CTG GTG AC. PCR products were resolved on a 1% agarose gel and visualized with UV light after staining with ethidium bromide.

ELISA
RINm5F cells were pretreated with celastrol for 1 h and then stimulated with cytokines (5 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ) for 24 h. Culture supernatants were collected and analyzed for CCL2 production with an ELISA kit (R&D Systems), according to the manufacturer’s instructions.

EMSA
RINm5F cells were pretreated with varying doses of celastrol for 1 h, and then stimulated with cytokines (5 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ) for 30 min. Nuclear extracts were prepared, and the EMSA was performed as described previously (24). Nuclear extracts (10 µg) were incubated with 32P-end labeled double-stranded NF-κB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3'); Promega) in binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 10% glycerol, 0.25 mM DTT, and 1 µg of poly d(dC) for 30 min. The DNA-protein complexes were analyzed by electrophoresis on a 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA). Then, the gels were dried and examined by autoradiography.

Statistical analysis
Results are expressed as mean ± standard deviation from at least three independent experiments. The values were evaluated by one-way analysis of variance, followed by Duncan’s multiple range test using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). Null hypotheses of no difference were rejected if P values were less than .05.

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REFERENCES
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