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RESEARCH ARTICLE

Protective Effect of Cinnamaldehyde on Streptozotocin-induced Damage in Rat Pancreatic β-Cells

Hai Dan Yuan, Bo Huang, and Sung Hyun Chung

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Abstract Cinnamaldehyde (CNA) is a primary constituent found in cinnamon (*Cortex cinnamomi*). Although anti-diabetic and anti-inflammatory activities of cinnamon extract have been investigated in recent years, whether CNA is responsible for these activities is yet to be explored. In the present study, we investigated the protective effect of CNA on streptozotocin (STZ)-induced β-cell dysfunction in RINm5F rat insulinoma cells. CNA markedly inhibited nitric oxide (NO) and prostaglandin E_2 (PGE_2) productions in concentration-dependent manners. Parallel with these observations, the protein and mRNA levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 enzymes were inhibited by CNA in concentration-dependent manners. CNA also inhibited STZ-induced nuclear factor (NF)-κB activation via the prevention of inhibitory κBα (IκBα) phosphorylation and degradation. Moreover, CNA significantly suppressed STZ-induced phosphorylations of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) in concentration-dependent manners. These results suggest that CNA is an active constituent of the cinnamon, and CNA protects against STZ-induced pancreatic β-cell damage by down-regulations of iNOS and COX-2 gene expression through blocking of NF-κB and MAPKs activities.

Keywords: cinnamaldehyde (CNA), streptozotocin (STZ), nuclear factor (NF)-κB, RINm5F cell

Hai Dan Yuan, Bo Huang, Sung Hyun Chung (✉)
Department of Pharmacology and Clinical Pharmacy, Kyung Hee University, Seoul 130-701, Korea
Tel: +82-2-961-0373; Fax: +82-2-957-0384
E-mail: suchung@khu.ac.kr

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease causing selective destruction of insulin producing β-cells of the Langerhans islets (1,2). It is generally thought that immune cells, cytokines, free radicals, and production of nitric oxide (NO) have an important role in the pathogenesis of T1DM (3,4). Streptozotocin (STZ) is a widely used diabetogenic agent that selectively destroys pancreatic β-cells, and thus induces experimental diabetes (5,6). Numerous studies indicate that oxygen free radicals or NO mediates deleterious effects of STZ on β-cell dysfunction and destruction (4).

Cinnamon is widely used as a spice and flavoring agent. Cinnamon is mentioned in the bible (Exodus and Proverb) and Chinese texts written 4000 years ago (7). Common and *Cassia cinnamon* contain volatile oils such as cinnamaldehyde, eugenol, and *trans*-cinnamic acid; phenolic compounds such as condensed tannin, catechins, and proanthocyanidins; monoterpenes, and sesquiterpenes; and trace coumarin (8,9). Among these components, cinnamaldehyde (CNA) is considered as the main active constituent in cinnamon. CNA has been shown to exert several pharmacological effects such as anti-inflammatory, anti-oxidant, anti-microbial, and anti-diabetic activities (10-13). Recently, CNA has been found to reduce lipopolysaccharide-induced nuclear factor (NF)-κB transcriptional activity in macrophages (14). In addition, CNA has been found to reduce interleukin (IL)-1β-induced cyclooxygenase (COX)-2 activity (15) and tumor necrosis factor (TNF)-α-induced NF-κB transcriptional activity in endothelial cells (16). However, there were no reports documenting protection activity of CNA against STZ-induced pancreatic β-cell damage. In the present study, we investigated the protective effect of CNA on STZ-induced β-cell dysfunction in rat insulinoma cell line (RINm5F), and observed that CNA prevented

STZ-induced pancreatic β-cell damage, and suppressed iNOS, COX-2 gene, and protein expressions via down-regulation of the mitrogen-activated protein kinases (MAPKs) and inactivation of NF-κB.

Materials and Methods

Materials Cinnamaldehyde (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.01% dimethyl sulfoxide (DMSO) and diluted with a cell culture medium. The chemical structure of CNA is depicted in Fig. 1A. The control cells were received the same amount of DMSO. Streptozotocin (Sigma-Aldrich) was dissolved in a cell culture medium. All other chemicals were of the highest grade commercially available.

Cell culture and viability assay RINm5F cells were purchased from American Type Culture Collection (Rockville, MD, USA) and grown at 37°C under a humidified, 5% CO_2 atmosphere in RPMI1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2.5 μg/mL of amphotericin B. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. In brief, RINm5F cells were seeded at 1×10^5 cells/well in 96-well plate and treated with the indicated concentrations of CNA for 3 h prior to the addition of STZ. After 24 h, 20 μL of MTS solution was added and incubated at 37°C for 1 h. At the end of incubation period, absorbance was recorded at 490 nm.

Nitrite measurement Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solution (17). Therefore, NO production can be determined by measuring the nitrite concentration in the cell-free culture supernatant using a colorimetric assay. RINm5F cells were seeded at 1×10^6 cells/well in 6-well plate and treated with the indicated concentrations of CNA for 3 h prior to the addition of STZ. After 24 h, 100 μL of culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1%(w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured using a microplate reader (Merck, Darmstadt, Germany).

PGE_2 analysis The RINm5F cells were seeded at 5×10^4 cells/well in 96-well plate and treated with the indicated concentrations of CNA for 3 h prior to the addition of STZ. After 24 h, the medium was collected and centrifuged at 2,300×*g* for 10 min. The supernatant was decanted into a new microcentrifuge tube, and the amount of PGE_2 was determined using a PGE_2 Enzyme-Immuno-Assay kit (R&D Systems, Inc., Minneapolis, MN, USA).

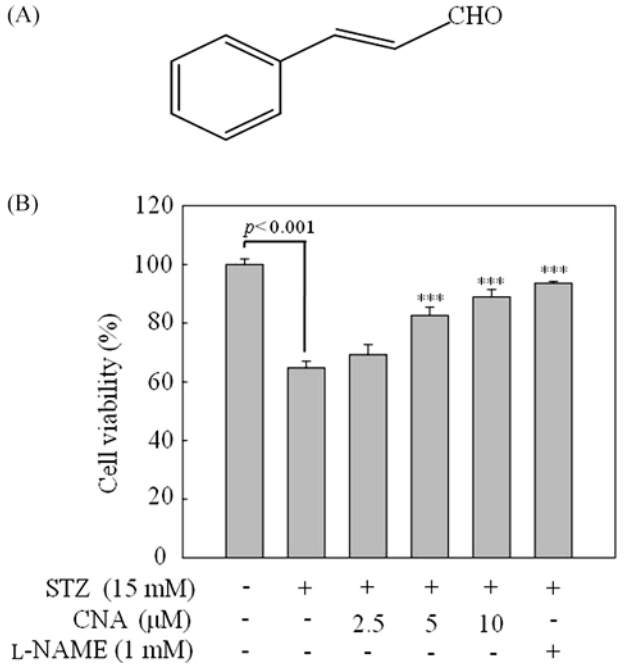

Fig. 1. Chemical structure of cinnamaldehyde (CNA) (A) and effect of CNA on STZ-induced cell damage in RINm5F cells (B). Percentage of viable cells after treatment were determined by MTS assay. Each value is expressed as the mean±SE of 3 independent experiments; ***p<0.001 vs. STZ group

Western blot analysis RINm5F cells (1×10^6 cells/well) were pretreated with various concentrations of CNA (2.5, 5, and 10 μM) for 3 h, and then treated with STZ (15 mM) for 24 h. After 24 h, cells were harvested, and total protein extracts were prepared using a protein extraction kit and insoluble protein was removed by centrifugation at 13,000 ×*g* for 15 min. The supernatant was collected from the lysates and protein concentrations were determined using a Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. For Western blotting, 40 μg protein was separated on a 8% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA, USA) in a transfer buffer containing 20 mM Tris-HCl, 154 mM glycine, and 20% methanol. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with specific antibodies and revealed with horseradish-peroxidase-conjugated secondary antibodies. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Uppsala, Sweden) and then exposed to X-ray film.

RNA isolation and reverse transcription (RT)-PCR Total RNA was isolated from RINm5F cells using an Easy Blue total RNA extraction kit (Intron Biotechnology Inc., Seoul, Korea) according to the manufacturer's instruction. Single-strand cDNA synthesis was performed as described previously (18), using 5 μg of RNA, oligo-dT primers and reverse transcriptase in total volume of 50 μL. PCR reactions were performed in a total volume of 20 μL comprising 2 μL of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer, and 1 units of Taq polymerase. Oligonucleotide primer sequences used in PCR amplification were as follows; iNOS sense 5'-ATGGCTTGCCCCTGGA AFT-3', anti-sense 5'-GTACTTGGGATGCTCCATGGTC A-3'; COX-2 sense 5'-ATGCTCTTCCGAGCTGTGCT-3', anti-sense 5'-TTACAGCTCAGTTGAACGCCTTTT-3'; CPN sense 5'-ATGGTCAACCCACCGTG-3', anti-sense 5'-TTAGAGTTGTCCACAGTTCGGAGA-3'. For iNOS, COX-2 and CPN, PCR was performed at 95°C for 5 min, 95°C for 30 s, followed by 57°C for 30 s and 72°C for 30 s, and amplified for 30 cycles. The RT-PCR products were electrophoresed on 1% agarose gels and visualized by 0.5 μg/mL ethidium bromide staining, and scanning densitometry was performed with the I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea). CPN was amplified as a control gene.

Electrophoretic mobility shift assay (EMSA) RINm5F cells (1×10^6 cells/well) were pretreated with various concentrations of CNA (2.5, 5, and 10 μM) for 3 h, and then 15 mM STZ was added to the culture media. After 1 h, the cells were harvested, and nuclear extract was prepared in nuclear extraction kit (Fermentas Inc., Glen Burnie, MD, USA). IκBa and p-IκBa in the cytoplasmic fractions were analyzed by Western blotting. Nuclear extract (2 μg) was mixed with the double-stranded NF-κB oligonucleotide 5-AGTTGAGGGGACT-TTCCCAG-GC3 end-labeled by [γ-^{32}P]dCTP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). The binding reactions were performed at 37°C for 30 min in 20 μL of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2.5 mg/mL BSA, 20 pg/μL poly (dI-dC), and 1 mM dithiothreitol. Electrophoresis was performed in a 0.5× Tris-borate buffer at room temperature at 180 V for 90 min on 5% polyacrylamide gels. The gels were then dried and protein-DNA complex formation was analyzed by autoradiography.

Statistical analysis Results were expressed as mean± standard error (SE), and differences between groups were analyzed by Student's *t*-test. Mean values were considered significantly different at $p<0.05$.

Results and Discussion

Cinnamon is one of the oldest spices used in naturopathic medicine, cited in Chinese books 4000 years ago (19), and traditionally used in Ayurvedic and Chinese medicine to treat diabetes (20). Interest in this spice has increased since the discovery of its insulin potentiating properties (21) and initial findings illustrating cinnamon's ability to reduce fasting blood glucose and plasma lipids (22). Recently, several groups reported anti-diabetic activity of cinnamon extract, and it's active ingredients are either A-type doubly linked procyanidin oligomers of the flavonoid catechins/ epicatechins (9,23), a naphthalenemethyl ester of 3,4-dihydroxyhydrocinnamic acid (24), or cinnamaldehyde (25). Kwon *et al.* (4) reported that cinnamon extract prevents STZ- and cytokine-induced β-cell damage by inhibiting NF-κB and Subash *et al.* (25) reported that CNA possesses hypoglycemic and hypolipidemic effects in STZ-induced diabetic Wister rats. Based upon these observations, it is suggested that cinnamon extract could ameliorate a hyperglycemia by overriding the inflammatory effects present in T1DM. Here, we tried to test the hypothesis that CNA exerts an anti-inflammatory activity and regulates genes encoding proinflammatory cytokines using RINm5F cells.

Effect of CNA on cell viability To investigate the effect of CNA on cell viability, RINm5F cells were treated with CNA (0, 2.5, 5, and 10 μM) for 3 h before exposure to STZ (15 mM). After 24 h, cell viability was determined by MTS assay. As shown in Fig. 1B, STZ significantly reduced cell viability to 35.3±2.3% when compared to control. Pretreatment of RINm5F cells with CNA and L-nitro-arginine methyl (L-NAME) (iNOS inhibitor) were both effective in preventing STZ-induced cytotoxicity in the RINm5F cells. This finding indicated that NO is an important mediator of STZ-induced toxicity of RINm5F cells, and the protective effect of CNA against STZ-induced RINm5F cell damage is due to the inhibition of NO generation.

Effects of CNA on nitrite, PGE_2 production, and iNOS, COX-2 expression NO is a free radical messenger molecule produced by iNOS in macrophages and in other cells that plays an important role in inflammatory responses (6). High levels of NO can provoke deleterious consequences in pancreatic islets such as decreases in glucose oxidation rate and insulin production (26). Similarly, COX-2 also plays an important role in pancreatic β-cell function and insulin secretion (27). COX-2, an inducible isoform of COX, is induced by pro-inflammatory stimuli, and is responsible for the production of pro-

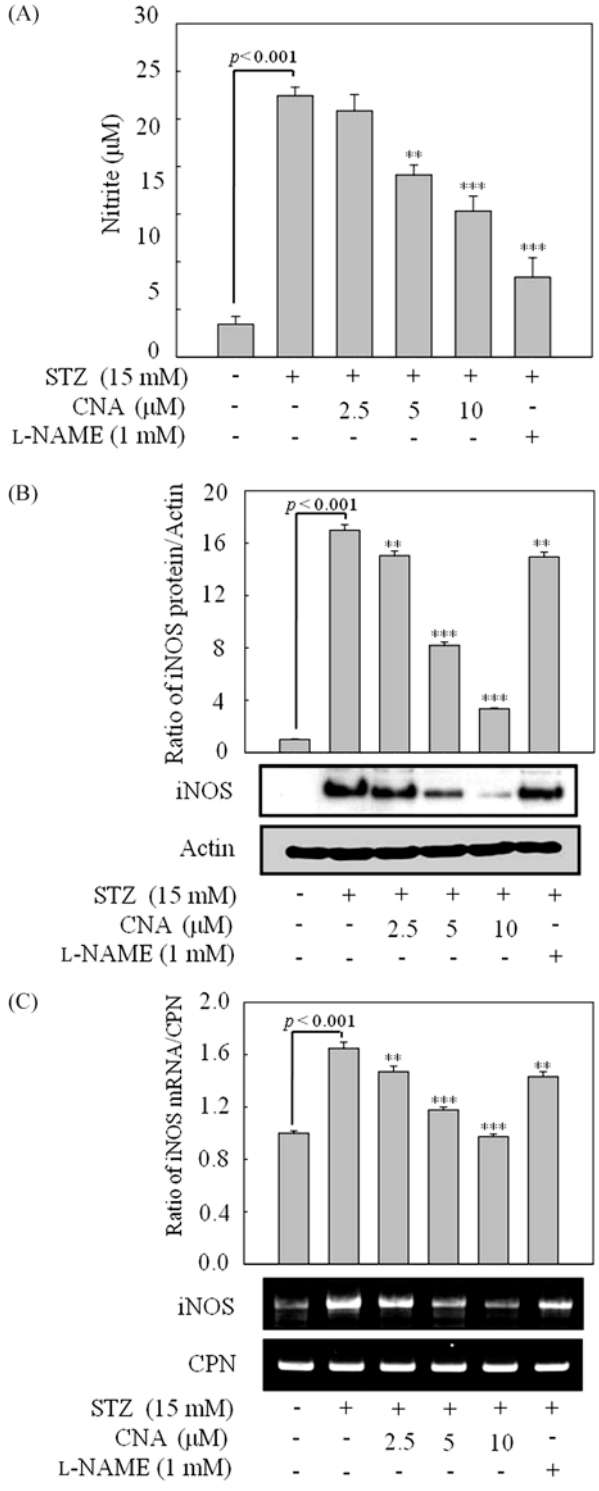

Fig. 2. Effect of CNA on STZ-induced NO production (A) and iNOS expression (B, C) in RINm5F cells. Results were expressed as mean±SE; **p<0.01, ***p<0.001 vs. STZ group

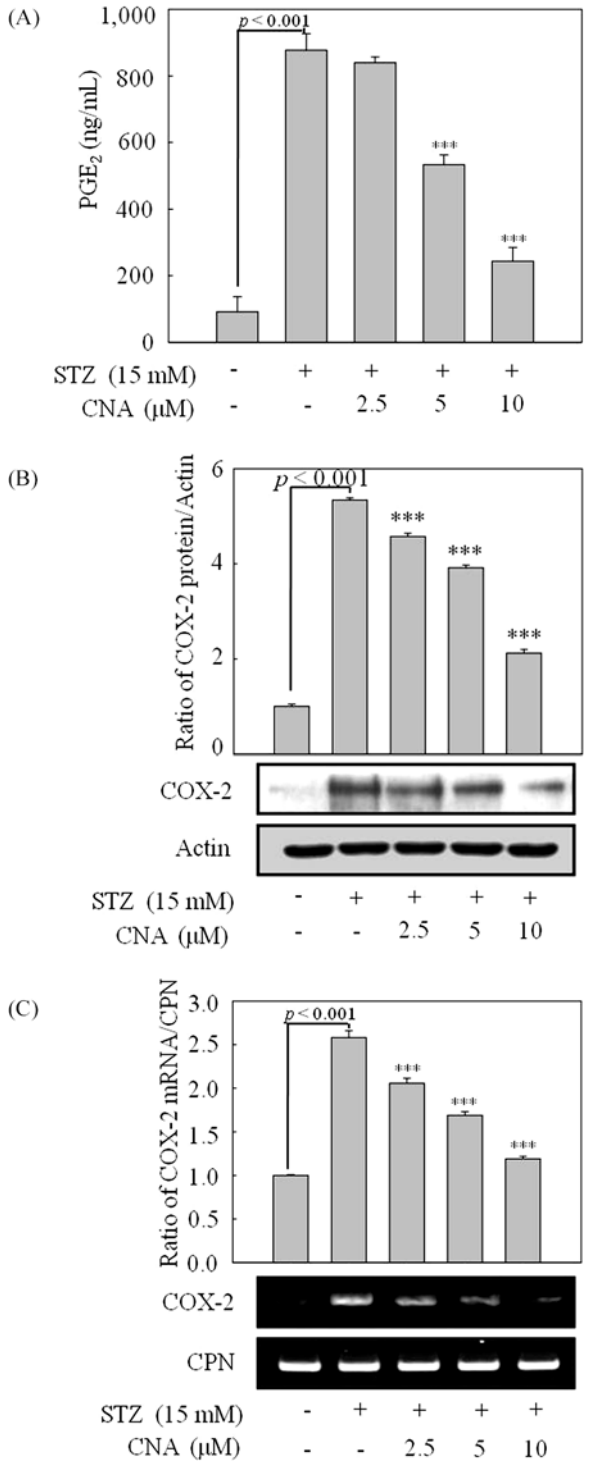

Fig. 3. Effect of CNA on STZ-induced PGE_2 production (A) and COX-2 expression (B, C) in RINm5F cells. Results were expressed as mean±SE; ***p<0.001 vs. STZ group

inflammatory prostaglandins (PGEs) at the inflammatory site (28). Thus, the inhibition of NO and PGE_2 production by suppressing iNOS and COX-2 expression is an important target in the development of anti-inflammatory agents. In the present study, CNA significantly inhibited nitrite (stable metabolism of NO) and PGE_2 levels in concentration-dependent manners (Fig. 2A, 3A). To further explore the mechanism underlying these inhibitions by CNA, we examined the expression levels of iNOS and COX-2 protein and mRNA. The inhibitions of nitrite and

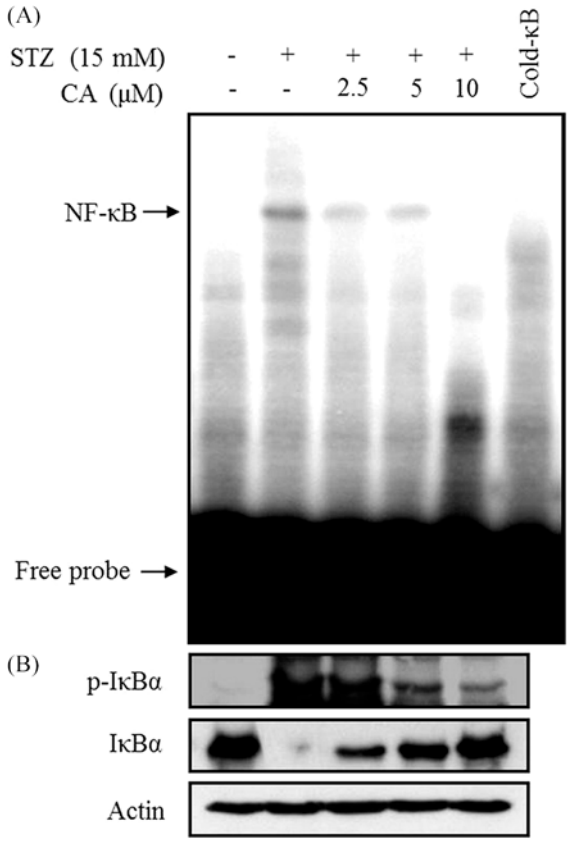

Fig. 4. Effect of CNA on STZ-induced translocation of NF-κB from cytosol to the nucleus (A) and IκBα degradation (B).

PGE_2 production were evidently shown by reductions in their protein (Fig. 2B, 3B) and mRNA levels (Fig. 2C, 3C). These observation suggests that inhibitions of the production of NO and PGE_2 may be attributed to the suppressions of the transcriptions of iNOS and COX-2 mRNA and subsequent reductions in their protein expressions.

Effect of CNA on STZ-induced NF-κB activation The transcriptional NF-κB has been implicated as a key mediator in the regulation of cells survival genes, and has been proposed to regulate transcription of both the COX-2 and iNOS gene (29). NF-κB, an inducible transcription factor, is active in response to various extracellular stimuli. In nonstimulated cells, NF-κB is present in the cytosol as either a homo- or a hetero-dimer and is linked to the IκB protein. The activation of NF-κB results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by the nuclear translocation and DNA binding of NF-κB (30,31). In the present study, our results showed that CNA concentration-dependently inhibited the nuclear translocation of the p65 protein and the STZ-stimulated DAN binding of NF-κB (Fig. 4A). In addition, the phosphorylation and degradation of IκBα, which is required for p65 activation, were inhibited when cells were treated with CNA (Fig. 4B). Therefore, these results suggest that CNA inhibits the expressions of iNOS and COX-2 via inactivation of NF-κB by suppressing IκBα phosphorylation and degradation.

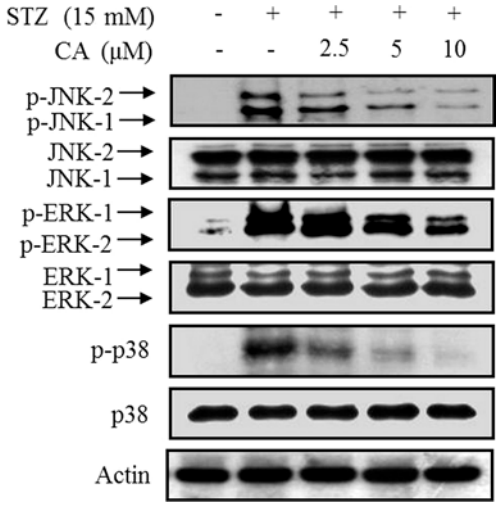

Fig. 5. Effect of CNA on STZ-induced phosphorylations of MAPKs in RINm5F cells.

Effect of CNA on MAPKs phosphorylation Activation of the NF-κB signaling pathway is closely related to the activations of MAPKs. Several studies have shown that MAPKs (ERK1/2, JNK1/2, and p38) play critical roles for the activation of NF-κB and subsequently, regulate COX-2 as well as iNOS-NO expression (32-34). To further clarify mechanism of action, effect of CNA on the STZ-induced phosphorylation of ERK1/2, JNK1/2, and p38 were examined. As expected, CNA concentration-dependently inhibited the phosphorylation of ERK1/2, JNK1/2, and p38 in STZ-induced RINm5F cells (Fig. 5). These results suggest that CNA's inhibition of NF-κB might be due to inhibition of MAPKs phosphorylation.

In summary, we provided the evidence that CNA prevents STZ-induced pancreas β-cell damage through suppression of iNOS and COX-2 expression via down-regulation of MAPK-NF-κB signaling pathway, and anti-diabetic activity of CNA may come from anti-inflammatory activity. CNA could be an adjunct to the treatment of diabetes caused by chonic inflammation, although further research is needed before definitive recommendations can be made.

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