Anti−inflammatory Activity of the Methanol Extract from the Stem of *Coriandrum Sativum* in RAW 264.7 Cells

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ABSTRACT

Objectives : *Coriandrum sativum* is a medicinal herb that is used to enhance organoleptic quality and food flavor and as source of natural antioxidants. This research investigated the anti−inflammatory activity of *Coriandrum sativum* stem methanol extract (CSSE) using RAW 264.7 cells.
Methods : Production of tumor necrosis factor−α (TNF−α), interleukin (IL)−1β, IL−6, and nitric oxide (NO) in the culture supernatant, protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase−2 (COX−2), and nuclear factor−kappa B (NF−κB) in the extract were assayed.
Results : Treatment with CSSE (100 μg/mℓ) resulted in inhibited levels of protein expression of lipopolysaccharide− (LPS−) induced iNOS, COX−2, and NF−κB as well as production of TNF−α, IL−1β, IL−6, and NO induced by LPS.
Conclusions : These results demonstrate that CSSE exhibits anti−inflammatory activities via decreasing production of pro−inflammatory mediators through suppression of the pathways of NF−κB in LPS−induced RAW 264.7 cells. Thus, CSSE may have therapeutic potential for a variety of inflammation−mediated diseases.

Key words : *Coriandrum sativum* stem, Anti−inflammation, Nuclear factor−kappa B, pro−inflammatory mediators

Ⅰ. Introduction

Coriandrum sativum is a medicinal herb of the Apiaceae family that is used to enhance organoleptic quality and aromatic flavor and as source of natural antioxidants. In addition, this plant is extensively cultivated in Central Europe, Russia, India, and Morocco to be used in food industry[1−3]. Different parts of the plant are traditionally used to alleviate giddiness, bronchitis, gout, gastric complaints, and spasms[4]. Previous reports on the *C. sativum* indicate their diverse medicinal activities, such as antioxidant, anticancer, antidiabetic, hypocholesterolemic, antibacterial, hepatoprotective, and anxiolytic properties[5,6]. The phenolic compounds, catechin, apigenin and p−coumaric acid, and alkanals and aliphatic alkenals were found in *C. sativum* aerial parts (stem and leaf)[7,8].

Inflammation, indicated by the important signs (heat, redness, pain, and swelling) is a host response to invading pathogens. This response may cause progressive damage, contributing to the pathogenesis of various disorders (arthritis and asthma) in the long term[9]. Several pro−inflammatory mediators are involved in inflammatory response. Among them, COX−2, iNOS and cytokines (TNF−α, IL−1β and IL−6) play important roles in the inflammatory processes[9,10]. COX−2 and iNOS which contribute to production of PGE$_2$ and NO are proteins whose expression is regulated by activation of NF−κB[11]. NF−κB acts as a transcription factor involved in transactivation of a variety of genes associated with regulation of immune and inflammatory responses, cellular proliferation, and tumorigenesis[12]. Moreover, involvement of inflammatory cytokines (TNF−α, IL−1β and IL−6) in promotion of inflammatory responses has been recognized[13,14].

According to several studies there is a dispute about *C. sativum* activities between antioxidant and anti−inflammatory effects[15,16]. Furthermore, the molecular

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mechanisms underlying anti−inflammatory activities of *Coriandrum sativum* stem methanol extract (CSSE) has not been elucidated. Therefore, we investigated the effects of CSSE on NF−κB−signaling pathway and NF−κB−regulated induction of inflammatory cytokines, COX−2 and iNOS in RAW 264.7 cells. These data provide a basis for the molecular mechanism for understanding the effects of CSSE on inflammation.

Ⅱ. Materials and methods

1. Chemicals and reagents

Anti−COX−2, anti−NF−κB p65, anti−p−IκBα, and anti−lamin A/C antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and peroxidase−conjugated secondary antibody was purchased from Santa Cruz Biotechnology Inc. Anti−iNOS and Anti−β−actin antibodies were purchased from Calbiochem (San Diego, Calif.). In addition, the TNF−α, IL−1β and IL−6 ELISA kits were obtained from Pierce Endogen (Rockford, IL, USA). 3−(4,5−dimethylthiazol−2−yl)−2,5−diphenyl tetrazolium bromide (MTT), sulfanilamide, dimethyl sulfoxide, lipopolysaccharide (LPS) and all other chemicals were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2. Preparation of CSSE

The dried stems of *Coriandrum sativum* were purchased from Seomun market (Daegu, Republic of Korea). The stems of *Coriandrum sativum* (100 g) were extracted with 2 L of methanol for 3 h, and filtered through a filter paper (Hyundai Micro No. 20). The supernatant was filtered through a 0.2 μm filter (Nalgene, New York, USA), and the filtrate was then lyophilized. The yield of lyophilized CSSE was 19.8%. The lyophilized powder of CSSE was dissolved in dimethyl sulfoxide prior to use.

3. Cell culture

RAW 264.7 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Thermo Scientific Inc., Bremen, Germany) supplemented with 10% heat−inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 U/mℓ of penicillin, and 100 μg/mℓ of streptomycin (Gibco/BRL, Grand Island, NY) at 37 ℃ in a 5% CO_2 incubator.

4. MTT assay for cell viability

For determination of cytotoxic concentrations of CSSE, RAW 264.7 cells were plated in a 96−well plate at a density of 5 × 10^4 cells per well. Cells were serum−starved for 16 h, followed by pretreatment with 30 and 100 μg/mℓ of CSSE for 1 h, followed by stimulation with 1 μg/mℓ of LPS. Cells were then incubated for the next 18 h at 37 ℃, in a 5% CO_2 incubator. Following incubation of the cells, viable cells were stained with MTT (0.5 ㎎/mℓ, 4 h). Media were then removed and formazan crystals produced in the wells were dissolved by addition of 200 μℓ dimethyl sulfoxide. Absorbance was measured at 570 ㎚ using an ELISA microplate reader (Tecan). Cell viability was defined relative to untreated control cells (that is, viability (% of control) = 100 × (absorbance of treated sample)/(absorbance of control)).

5. Measurement of NO production

Following pre−incubation of RAW 264.7 cells (5 × 10^5 cells/mℓ) for 24 h, cells were pretreated with 30 and 100 μg/mℓ of CSSE for 1 h, followed by stimulation with 1 μg/mℓ of LPS. Cells were then incubated for 18 h at 37 ℃, in a 5% CO_2 incubator, followed by collection of culture supernatants. Nitric oxide was measured by reaction with 100 μℓ of Griess reagent (1% sulfanilamide and 0.1% N−[1−naphthy]−ethylenediamine dihydrochloride in 5% phosphoric acid; Roche) to 100 μℓ of culture supernatant for 15 min at room temperature in the dark. Absorbance was determined at 540 ㎚ using an ELISA microplate reader (Tecan, USA). A standard curve was generated in the same fashion using $NaNO_2$.

6. TNF−α, IL−1β, and IL−6 assays

RAW 264.7 cells (5 × 10^5 cells/ml) were pre−incubated for 24 h. Cells were then pretreated with 30 and 100 μg/mℓ of CSSE for 1 h, followed by stimulation with 1 μg/mℓ of LPS. Following collection of culture supernatants at 18 h after LPS stimulation, enzyme−linked immunosorbent assay (ELISA) was performed according to the manufacturer's protocol for quantification of levels of TNF−α, IL−1β and IL−6.

7. Western blot analysis

Control and CSSE−treated RAW 264.7 cells were collected by centrifugation and washed once with phosphate−buffered saline (PBS). Washed cell pellets were resuspended in extraction lysis buffer [50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P−40, 1 mM PMSF, 0.5 mM dithiothreitol, 5 mM NaF, and 0.5 mM sodium orthovanadate] containing 5 μg/mℓ

each of leupeptin and aprotinin and incubated for 20 min at 4 ℃. Microcentrifugation was performed for removal of cell debris, followed by rapid freezing of supernatants. Bio-Rad protein assay reagent was used for determination of protein concentrations, according to the manufacturer's instructions. The cellular proteins from treated or untreated cell extracts were separated on 8% SDS-polyacrylamide gels, followed by electroblotting onto nitrocellulose membranes, followed by incubation overnight with blocking solution (5% skim milk) at 4 ℃, and then with primary antibody for 2 h. Blots were then washed three times with Tween 20/Tris-buffered saline (TTBS), incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and rewashed three times with TTBS. ECL Western detection reagents (Amersham Bioscience, Piscataway, NJ) were used for development of blots.

8. Preparation of nuclear extracts

Dishes were washed with ice-cold PBS, scraped, and transferred to microtubes. Cells were allowed to swell by addition of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2% Nonidet P-40, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA)]. Samples were incubated for 10 min on ice and centrifuged for 5 min at 4 ℃. Pellets containing crude nuclei were resuspended in 50 $\mu\ell$ of extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 420 mM NaCl, 20% glycerol, and protease inhibitor cocktail, followed by incubation for 30 min on a shaker at 4 ℃. Samples were centrifuged at 16,000 × g for 10 min in order to obtain supernatant containing nuclear extracts.

9. Statistical analysis

Data were expressed as mean ± S.D. Multiple comparison tests were performed for different dose groups. The Levene test was used for examination of variance homogeneity. If results of the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed using a one way ANOVA test followed by least-significant differences (LSD) multicomparison test, for determination of which pairs of the group comparison was significantly different, and independent t-test. SPSS for Windows (Release 14.0K, SPSS Inc., Chicago, IL, USA) was used in performance of statistical analyses. Differences were considered significant at $p < 0.05$.

Ⅲ. Results

1. Effects of CSSE on LPS-induced production of NO and cell viability

We examined the effects of CSSE on LPS-inducible production of NO. Different dosages of CSSE (30 and 100 $\mu g/m\ell$) were used for evaluation of the inhibitory effects of CSSE on LPS-induced production of NO in RAW 264.7 cells. Compared to control, treatment with LPS resulted in significantly increased production ($p < 0.01$) of NO. However, treatment with CSSE (100 $\mu g/m\ell$) resulted in significantly reduced production ($p < 0.01$) of LPS-induced NO (Fig. 1A). Therefore, CSSE exhibited an inhibitory activity for induction of NO in macrophages. In addition, the MTT assay was used for examination of possible cytotoxic effects of CSSE in RAW 264.7 cells. As shown in Fig. 1B, no significant change in cell viability was observed according to the different concentrations of CSSE treatment.
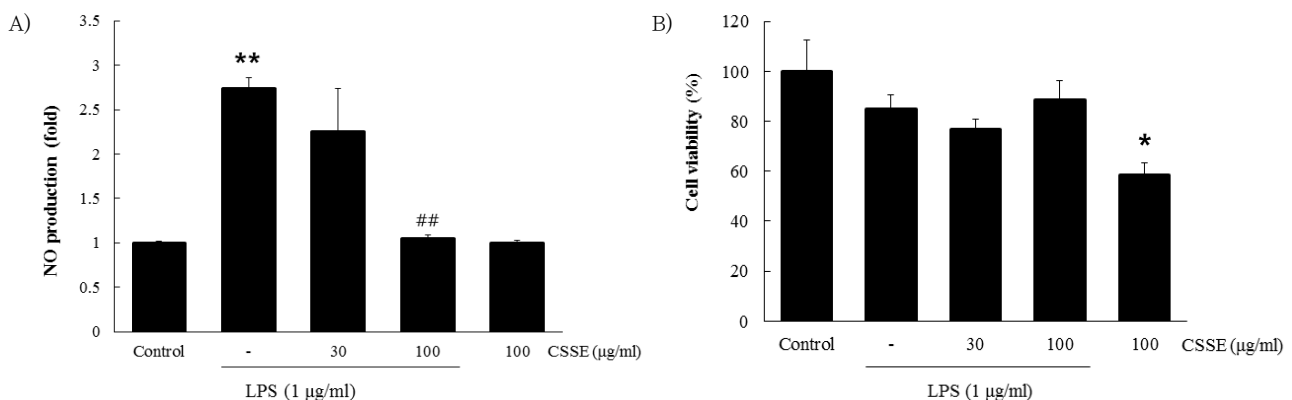

Fig. 1. Inhibitory effects of CSSE on LPS-induced production of NO (A) in RAW 264.7 cells. The cells (5 × 10^5 cells/mℓ) were treated with various concentrations (30 and 100 $\mu g/m\ell$) of CSSE for 1 h, followed by continuous incubation with LPS (1 $\mu g/m\ell$) for the next 18 h. Concentrations of NO in culture medium were monitored as described in the methods section. The MTT assay was used for measurement of viability of cells exposed to CSSE (B). Data represent the mean ± S.D. from three separate experiments. $^*p < 0.05$, $^{**}p < 0.01$ significant compared with vehicle-treated control; $^{##}p < 0.01$ significant compared with LPS alone.

2. Effects of CSSE on LPS−induced production of pro−inflammatory cytokines (TNF−α, IL−1β, and IL−6)

We evaluated the effects of CSSE on LPS−inducible production of TNF−α, IL−1β, and IL−6 by enzyme immunoassay. Compared to control, treatment with LPS resulted in significantly increased production ($p < 0.01$) of TNF−α, IL−1β, and IL−6 in culture supernatants of RAW 264.7 cells. However, at the concentrations of 30 and 100 $\mu g/m\ell$, treatment with CSSE resulted in significantly ($p < 0.01$) inhibited LPS−induced production of TNF−α, IL−1β, and IL−6 (Fig. 2 A, B, and C). Therefore, these results indicate that CSSE is a potent inhibitor of induction of TNF−α, IL−1β, and IL−6.
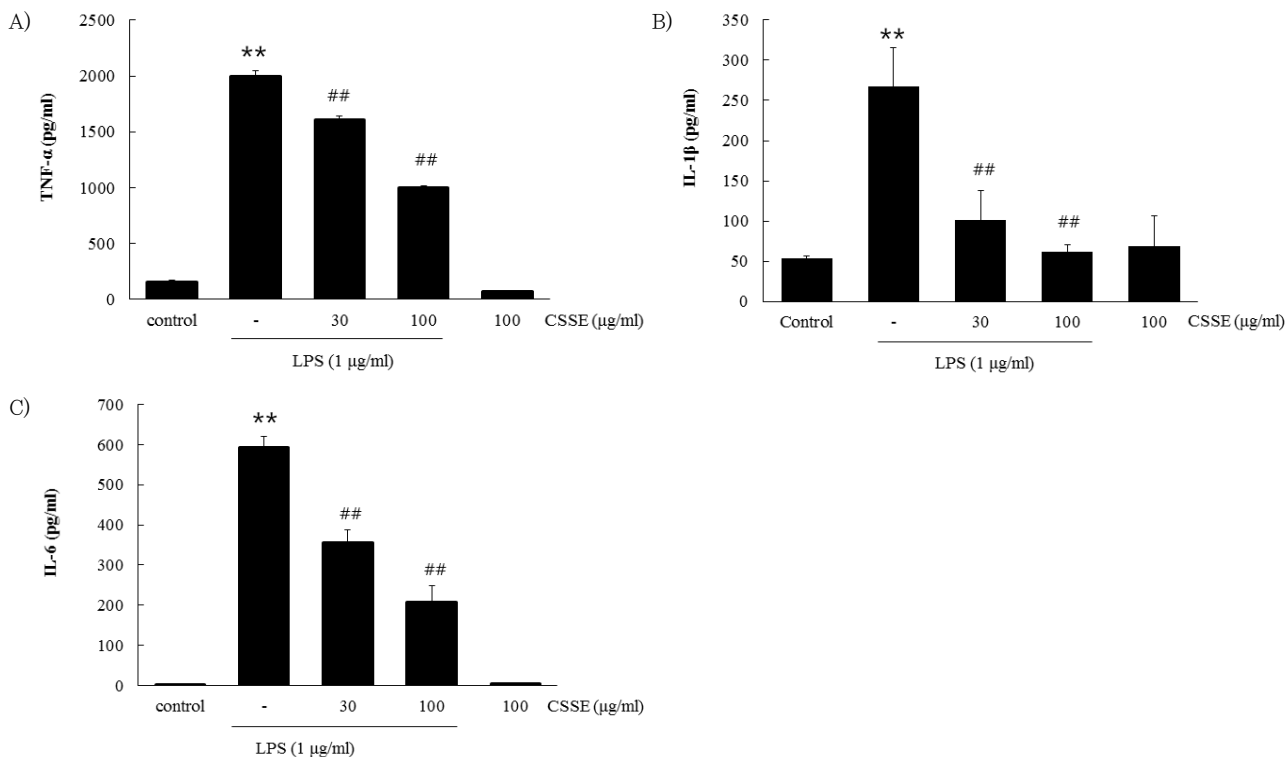

Fig. 2. Inhibitory effects of CSSE on LPS−induced production of pro−inflammatory cytokines (TNF−α (A), IL−1β (B), and IL−6 (C)) in RAW 264.7 cells. The cells (5 × 10^5cells/mℓ) were treated with various concentrations (30 and 100 $\mu g/m\ell$) of CSSE for 1 h, followed by continuous incubation with LPS (1 $\mu g/m\ell$) for the next 18 h. Concentrations of pro−inflammatory cytokines in culture medium were monitored as described in the methods section. Data represent the mean ± S.D. from three separate experiments. **$p < 0.01$ significant compared with vehicle−treated control; ##$p < 0.01$ significant compared with LPS alone.

3. Effects of CSSE on LPS−induced protein expression of iNOS and COX−2

As shown in Fig. 3, outstandingly up−regulated levels of iNOS and COX−2 protein were observed in response to LPS. Treatment with CSSE (100 $\mu\ell/m\ell$) resulted in significant inhibition of levels of LPS−stimulated iNOS and COX−2 protein (Fig. 3A) ($p < 0.01$ for iNOS (B); $p < 0.05$ for COX−2 (C), Fig. 3). Accordingly, these data show that CSSE is an inhibitor of induction of iNOS and COX−2 protein.

4. Effects of CSSE on LPS−induced activation of NF−κB

To examine if the reduction of nuclear translocation of NF−κB (p65) by CSSE was due to the inhibition of p−IκBα, Western blot analysis was conducted to evaluate levels of p−IκBα and nuclear NF−κB (p65). Treatment with LPS significantly increased the level of p−IκBα ($p < 0.01$) and CSSE (100 $\mu g/m\ell$) significantly blocked the LPS−stimulated increase ($p < 0.01$, Fig. 4A and C). In addition, NF−κB (p65) was accumulated in the nucleus after treatment with LPS and the LPS−stimulated NF−κB accumulation was significantly inhibited by CSSE (100 $\mu g/m\ell$) ($p < 0.01$, Fig. 4B and D).
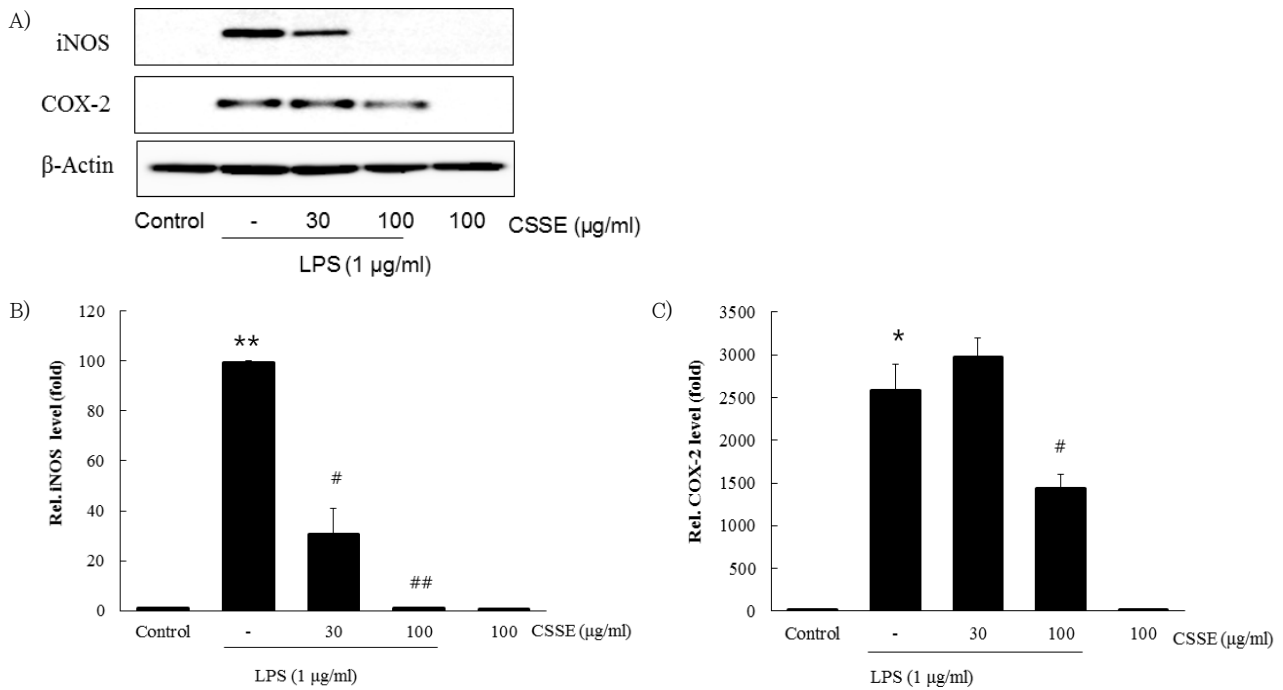
Fig. 3. Inhibitory effects of CSSE on LPS−induced expressions of iNOS and COX−2 (A). RAW 264.7 cells (5×10^5cells/ml) were treated with various concentrations (30 and 100 μg/ml) of CSSE for 1 h, followed by continuous incubation with LPS (1 μg/ml) for the next 18 h. Control cells were incubated with vehicle alone. Western blot analysis was performed for determination of protein levels of iNOS and COX−2. β−Actin was used as a loading control. The blots shown are representative of three blots yielding similar results. The densities of iNOS (B) and COX−2 (C) were measured via densitometry. Data represent the mean ± S.D. from three separate experiments. *$p < 0.05$, **$p < 0.01$ significant compared with vehicle−treated control; #$p < 0.05$, ##$p < 0.01$ indicate significant differences from the LPS−induced group.
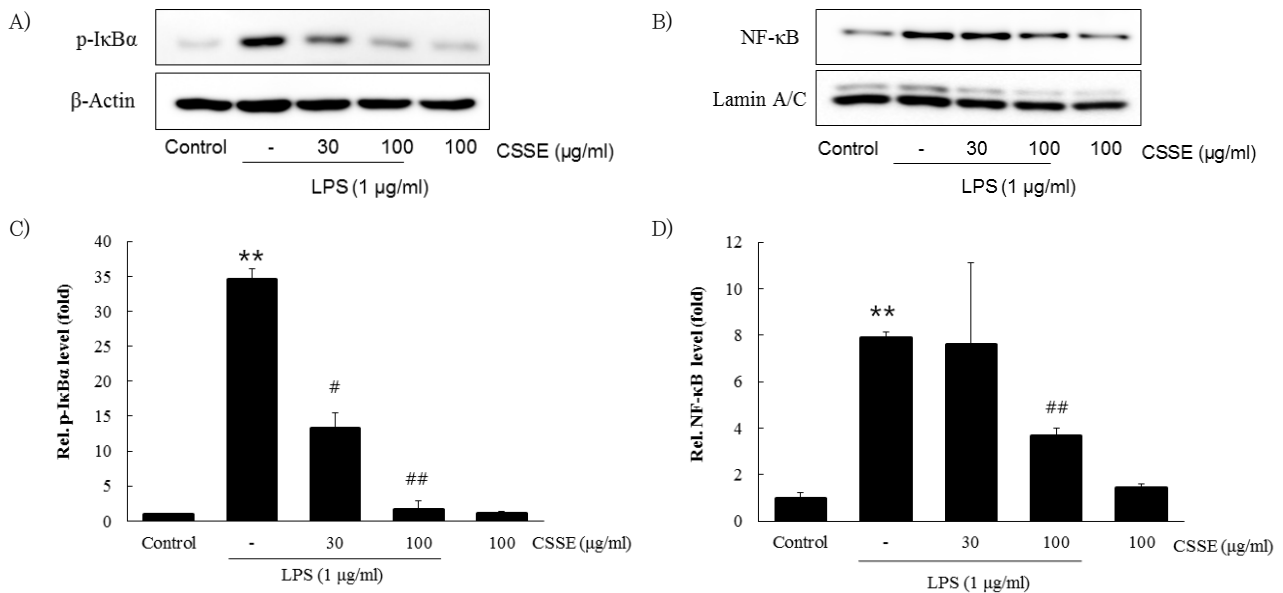

Fig. 4. Inhibitory effects of CSSE on the LPS−induced activation of p−IκBα (A and C) and nuclear NF−κB (p65) (B and D). RAW 264.7 cells at a concentration of 5×10^5cells/ml were treated with CSSE (30 and 100 μg/ml) for 1 h, then with LPS (1 μg/ml) for 15 min. Control cells were treated with vehicle only. Western blot analysis was performed to determine the protein levels of p−IκBα and NF−κB (p65). The densities of p−IκBα and NF−κB (p65) were measured by densitometry. Data are given as the mean ± S.D. of three replicates for each sample. **$p < 0.01$ significant compared with vehicle−treated control. #$p < 0.05$ and ##$p < 0.01$ significant compared with LPS only group.

Ⅳ. Discussion

C. sativum is traditionally used to alleviate giddiness, bronchitis, gout, gastric complaints, and spasms[4].

However, there has been little scientific evidence to prove the benefits of CSSE. Accordingly, we investigated the effects of CSSE on inflammatory response *in vitro*. In cell viability test, CSSE alone 100 μg/ml has cytotoxicity

compared with control, but CSSE 100 $\mu g/m\ell$ + LPS do not have any cytotoxicity compared with LPS alone treatment.

Inflammation is a complex biological response to harmful stimuli during the host defense mechanism[9,17]. NO produced by iNOS is involved in maintenance of the inflammatory response[18]. In addition, increases of iNOS activity related to the pro-inflammatory agents (IL-1β, TNF-α and interferon-γ) may cause shock and inflammatory responses[19,20]. According to our results, treatment with CSSE resulted in significantly inhibited induction of LPS-stimulated production of NO and expression of iNOS protein. These findings show that the therapeutic effect of CSSE on some inflammatory diseases may be due in part to its inhibition of NO production and expression of iNOS protein. COX-2 is also involved in angiogenesis, pathological processes, and inflammatory disease[21,22]. Results of COX-2 (western blotting) show that treatment with CSSE resulted in significantly inhibited induction of expression of COX-2 protein in cells, suggesting that CSSE might have a therapeutic effect in treatment of pathogenic pain. TNF-α is a well-known pro-inflammatory cytokine[23], which is also involved in the pathogenesis of Carrageenan (CA)-induced inflammation[24]. In addition, marked increases of IL-1β expression after CA treatment were previously reported[25,26]. IL-6 is also involved in a variety of immunological reactions and interactions with a variety of target cells[14]. In this study, our results showed that treatment with CSSE resulted in significantly inhibited secretion of LPS-stimulated pro-inflammatory cytokines. These results suggest that the inhibitory effects of CSSE on pro-inflammatory cytokines may involve an important mechanism for cure of inflammatory disorders.

NF-κB is a typical transcription factor that controls the expression of genes related to apoptosis, immune responses, and cell cycle. Unsuitable NF-κB activation may mediate tumorigenesis and inflammation. However, suppression of NF-κB activity should be useful in the treatment and prevention of cancer[27]. Our study demonstrated that CSSE significantly prevented LPS-stimulated NF-κB activation. It has been suggested that NF-κB is associated with regulation of COX-2 and iNOS protein expression, and that several chemopreventive phytochemicals inhibited COX-2 and iNOS protein expression by suppressing NF-κB activation[28,29]. Our results revealed that LPS increased levels of p-IκBα and NF-κB (p65), whereas treatment with CSSE resulted in reduced p-IκBα levels and inhibited nuclear translocation of NF-κB. Taken together, these data indicate that suppression of NF-κB activation by CSSE was correlated with inhibition of induction of iNOS, COX-2 and pro-inflammatory cytokines by CSSE.

V. Conclusions

In conclusion, above results demonstrate that CSSE exhibits anti-inflammatory activities via decreasing production of pro-inflammatory mediators (such as NO, iNOS, COX-2, TNF-α, IL-1β, and IL-6) through suppression of the NF-κB pathways in LPS-induced RAW 264.7 cells. Thus, CSSE may have therapeutic potential for a variety of inflammation-mediated diseases.

Conflict of Interest

The authors declare that they have no conflict of interest.

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