Ethanolic Extract of *Chondria crassicaulis* Inhibits the Expression of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 in LPS-Stimulated RAW 264.7 Macrophages

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Abstract

Inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) have been implicated in various inflammatory diseases. In this study, we investigated the anti-inflammatory activities of *Chondria crassicaulis* ethanolic extract (CCE) by measuring its effects on the expression of iNOS and COX-2 proteins in lipopolysaccharide (LPS)-treated RAW 264.7 murine macrophages. CCE significantly and dose-dependently inhibited the LPS-induced release of nitric oxide and prostaglandin E_2, and suppressed the expression of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 cells, without causing any cytotoxicity. It also inhibited the production of the pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in LPS-stimulated RAW 264.7 cells. Moreover, treatment with CCE strongly suppressed nuclear factor-κB (NF-κB) promoter-driven expression in LPS-treated RAW 264.7 cells. CCE treatment blocked nuclear translocation of the p65 subunit of NF-κB by preventing proteolytic degradation of inhibitor of κB-α. These results indicate that CCE regulates iNOS and COX-2 expression through NF-κB-dependent transcriptional control, and identifies potential candidates for the treatment or prevention of inflammatory diseases.

Key words: *Chondria crassicaulis*, anti-inflammation, iNOS, COX-2, TNF-α, IL-1β, IL-6

Introduction

Macrophages are major inflammatory cells and immune effector cells. Activation of macrophages occurs in inflamed tissues and is induced by exposure to interferon-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and microbial lipopolysaccharide (LPS) (Xie et al., 1993). Activated macrophages produce excessive amounts of inflammatory mediators such as nitric oxide (NO) and prostaglandins (PGs), in addition to various pro-inflammatory cytokines (Nathan, 1992; Zhang and Ghosh, 2000). Excessive production of inflammatory mediators and cytokines contributes to the pathogenesis of chronic diseases such as atherosclerosis,

inflammatory arthritis, and cancer (Libby, 2006; Packard and Libby, 2008; Solinas et al., 2010). Substances that inhibit the production of these molecules are considered to be potential anti-inflammatory agents.

The expression of inflammatory proteins such as cyclooxyganase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines is primarily controlled at the transcription level (D'Acquisto et al., 1997; Kim and Moudgil, 2008). Transcriptional induction of COX-2 and iNOS is largely dependent on the cooperative activity of multiple transcription factors, including nuclear factor-κB

(NF-κB) and activator protein 1 (AP-1), which act on cognate *cis*-acting elements in the COX-2 and iNOS promoters (D'Acquisto et al., 1997; Marks-Konczalik et al., 1998). NF-κB plays pivotal roles in the immediate early stages of immune, acute-phase, and inflammatory responses, as well as in cell survival (Makarov, 2001). Under unstimulated conditions, NF-κB is located in the cytoplasm as an inactive complex with inhibitor of κB (IκB)-α. LPS treatment activates the IκB-α kinase complex, resulting in the phosphorylation, ubiquitination, and degradation of IκB-α and the subsequent translocation of NF-κB to the nucleus, where it promotes the transcription of target genes.

Marine algae have been identified as rich sources of structurally diverse bioactive compounds with great pharmaceutical potential (Abad et al., 2008; Blunt et al., 2010). A variety of biological compounds, including phlorotannins and fucoxanthin, were isolated from marine algae and characterized in terms of their biological activities (Kim et al., 2005, 2009, 2011; Woo et al., 2009). In Korea, *Chondria crassicaulis* is one of the most commonly consumed red seaweeds. As part of our ongoing investigation into the isolation of lipophilic compounds with anti-inflammatory activities from marine algae, we detected an anti-inflammatory activity in the ethanolic extract of *C. crassicaulis*. To the best of our knowledge, no previous study has reported on the anti-inflammatory activity, or other biological activities, of *C. crassicaulis*. This led us to investigate the anti-inflammatory action of *C. crassicaulis* ethanol extract (CCE) in cultured RAW 264.7 cells. The findings demonstrate that CCE may serve as a source of nutraceuticals for preventing or treating inflammation-related diseases.

Materials and Methods

Plant material and reagents

C. crassicaulis was collected along the coast of Busan, Korea, in January 2009, with voucher specimens being deposited in the laboratory of H.R. Kim. The samples were rinsed with tap water to remove salt and were dried in a cold-air drier (Shilla Ref. Co., Busan, Korea) at 60°C for 40 h. Dried samples were ground with a hammer mill, and the resulting powder was stored at -20°C until use. Antibodies against iNOS, COX-2, NF-κB, lamin, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IκB-α and p-IκB-α were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology.

Preparation of CCE

Dried powder from *C. crassicaulis* (100 g) was extracted

three times through incubation with 96% (v/v) ethanol for 3 h at 70°C. The extracts were combined, concentrated in a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 40°C, and lyophilized. Dried CCE (9.5 g) was dissolved in 100% dimethyl sulfoxide (DMSO) and further diluted with culture medium before treatment. The final concentration of DMSO in the cell culture medium was less than 0.1% (v/v).

Cell culture and viability assay

RAW 264.7 murine macrophages (ATCC, Rockville, MD, USA) were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere containing 5% CO_2. Cell viability was determined by a 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's manual. Briefly, cells were inoculated at a density of 1×10^5 cells/well into 96-well plates and cultured at 37°C for 24 h. The culture medium was replaced with 200 μL of serial dilutions of CCE (0-200 μg/mL), and the cells were incubated for 24 h. The culture medium was removed and replaced by 95 μL of fresh culture medium plus 5 μL of MTS solution. After 1 h, the absorbance at 490 nm was measured using an Ultraspec 2100 Pro microplate reader (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA).

Measurement of NO

RAW 264.7 cells (1×10^6 cells/mL) were transferred to 12-well plates, incubated with 0-200 μg/mL CCE for 1 h, and stimulated with 1 μg/mL LPS for 24 h. Cell culture medium was collected after centrifugation at 2,000 *g* for 10 min. The nitrite concentration in the culture medium was measured as an indicator of NO production. Then, 100 μL of the culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylene diamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the resulting mixture at 540 nm was measured with an Ultraspec 2100 Pro microplate reader. The concentration of nitrite was calculated using sodium nitrite as a standard.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to assess the inhibitory effect of CCE on the production of PGE_2 and the cytokines TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1×10^6 cells/mL) were transferred to 12-well plates, treated with 0-200 μg/mL CCE for 1 h, and stimulated with LPS (1 μg/mL) for 24 h. After incubation, the supernatants were collected and assayed with the appropriate ELISA kit (R&D Systems, Min-

neapolis, MN, USA) according to the manufacturer's protocol.

Western blot analysis

RAW 264.7 cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed through incubation in buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet-40, 1% Tween 20, 0.1% sodium dodecyl sulfate, 10 μg/mL leupeptin, 50 mM NaF, 1 mM PMSF) on ice for 30 min. After centrifugation at 18,000 g for 10 min, the protein content of the supernatant was measured. Protein aliquots (40 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween 20 (TBST) and then blocked with TBST containing 5% nonfat dried milk (w/v). The membranes were incubated at 4°C with antibodies specific for COX-2 (dilution 1:2,000; incubation period 10 h), iNOS (1:2,000; 10 h), p-IκB-α (1:2,000; 15 h), IκB-α (1:2,000; 15 h), NF-κB (1:2,000; 15 h), and actin (1:5,000; 10 h). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The membranes were washed three times with TBST at room temperature. Immunoreactivity was detected using ECL reagent (GE Healthcare Bio-Sciences Co.). Equal protein loading was assessed by measuring the actin protein levels.

Transient transfection and luciferase assay

One microgram of murine NF-κB promoter/luciferase DNA (Stratagene, Santa Clara, CA, USA) was transiently transfected with 200 ng of control pRL-TK DNA (Promega) into RAW 264.7 cells (2×10^6 cells/well) in a 24-well plate using Lipofectamine/Plus reagent (Invitrogen, Carlsbad, CA, USA) for 24 h. Cells pretreated with 0-200 μg/mL CCE were stimulated with LPS (μg/mL) for 6 h. Each well was then washed twice with cold PBS and harvested in 100 μL of lysis buffer (0.5 mM HEPES [pH 7.8], 1% Triton N-101, 1 mM CaCl$_2$ 1 mM MgCl$_2$) and analyzed for luciferase activity using a luciferase assay kit. Luminescence was measured using a TopCount microplate scintillation and luminescence counter in single-photon counting mode (0.1 min/well) following adaptation for 5 min in the dark. Luciferase activity was normalized to the expression of pRL-TK.

Preparation of cytosolic and nuclear extracts

RAW 264.7 cells were treated with various concentrations of CCE for 1 h and stimulated with LPS (1 μg/mL) for 30 min. Cells were washed twice with cold PBS, scraped into 200 μL of cold PBS, and pelleted through centrifugation of 300 g for 5 min. Cell pellets were resuspended in hypotonic buffer
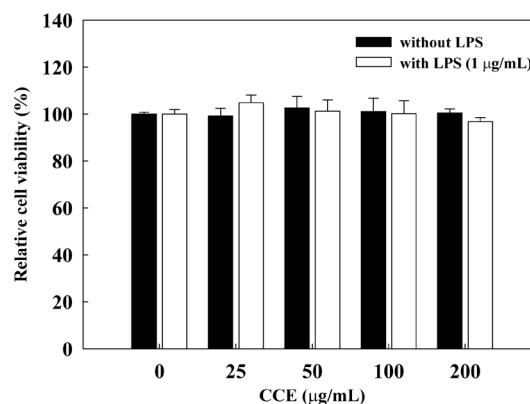

Fig. 1. Effect of *Chondria crassicaulis* ethanolic extract (CCE) on the viability of RAW 264.7 cells. Cells were treated with indicated concentration of CCE alone or CCE with lipopolysaccharide (LPS) (1 μg/mL) for 24 h. The cell viability was determined by MTS assay. Each column represents the means ± SDs from three independent experiments.

(10 mM HEPES/KOH, 10 mM KCl, 2 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9) and incubated on ice for 15 min. After vortexing for 10 s, homogenates were centrifuged at 13,000 g for 10 min. The resulting supernatant (cytosolic extract) was collected. The pellet was gently resuspended in 20 μL of complete lysis buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM PMSF, pH 7.9) and centrifuged at 13,000 g for 20 min at 4°C. The resulting supernatant was used as a nuclear extract.

Statistical analysis

Data were expressed as the mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA), and pairs of groups were then analyzed by Student's t-test. Differences were considered significant at $P < 0.05$. All analyses were performed using SPSS for Windows version 10.07 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effect of CCE on RAW 264.7 cell viability

To determine whether CCE causes cytotoxicity in RAW 264.7 cells, we assessed the effects of various concentrations of CCE on RAW 264.7 cell viability by MTS assay (Fig. 1). We found that CCE, alone or combined with LPS (1 μg/mL), did not affect the viability of RAW 264.7 cells at concentrations of up to 200 μg/mL. At a concentration of 400 μg/mL, CCE reduced the viability of RAW 264.7 cells to 78.8 ± 4.8% when applied alone and to 82.5 ± 5.1% when applied with LPS. Thus, we used CCE at concentrations of ≤200 μg/mL, which did not affect cell viability, in further studies of anti-

inflammatory activity and possible mechanisms of action in RAW 264.7 cells.

The results of several studies strongly suggest that phenolic compounds derived from botanical sources are able to suppress inflammation via removal of reactive oxygen species by antioxidants (Rahman et al., 2006; Kim and Kim, 2010). We previously described the anti-oxidative and anti-inflammatory activities of phloroglucinol derivatives of *Ecklonia stolonifera* (Kim et al., 2009; Kim et al., 2011). The effects of different solvents used to extract polyphenols from plant material have been tested (Pinelo et al., 2004). Although the extraction yield is dependent on the solvent used and the method of extraction, extracts from various red seaweeds show a relationship between antioxidant activity and polyphenolic content (Ganesan et al., 2008). Ethanol extraction gave a relatively high radical scavenging activity and a high yield of polyphenol compounds (López et al., 2011). Thus, we used ethanol to extract anti-inflammatory compounds from *C. crassicaulis*. We subsequently assessed the anti-inflammatory activity of the resulting ethanolic extract in LPS-stimulated RAW 264.7 cells.

Effect of CCE on the production of NO in LPS-stimulated cells

To determine the effect of CCE on NO production in LPS-stimulated RAW 264.7 cells, we measured culture medium nitrite concentrations using the Griess reagent. RAW 264.7 cells were pretreated with various concentration of CCE (50-200 µg/mL) for 1 h and stimulated with LPS for 24 h. Production of NO, measured as nitrite, was increased by LPS. CCE significantly and dose-dependently suppressed the production of NO by LPS-stimulated cells ($P < 0.05$) (Fig. 2A). Since iNOS is the enzyme responsible for the production of NO, we analyzed the effect of CCE on iNOS protein expression in LPS-stimulated RAW 264.7 cells by Western blotting. As shown in Fig. 2B, CCE strongly and dose-dependently suppressed the expression of iNOS protein. These results indicate that CCE-mediated inhibition of NO production in LPS-stimulated macrophages was associated with downregulation of iNOS. The MTS assay showed that the CCE caused no cytotoxicity at concentrations up to 200 µg/mL CCE in RAW 264.7 cells (Fig. 1). Thus, the inhibitory effect of CCE on NO production was not attributable to cytotoxic effects.

NO is synthesized from L-arginine and molecular oxygen in a reaction catalyzed by NOS. Under pathological conditions, a significant increase in iNOS-derived NO contributes to the induction of inflammation, acting synergistically with other inflammatory mediators (Nathan, 1992). iNOS is strongly induced following exposure to bacterial endotoxin and pro-inflammatory cytokines (Guha and Mackman, 2001). Compounds able to reduce NO production or iNOS activity may be attractive anti-inflammatory agents, and for this reason, the suppressive effects of natural marine compounds on NO production have been intensively studied with the aim of devel-

oping anti-inflammatory drugs (Abad et al., 2008; Jung et al., 2009; Heo et al., 2010; Jin et al., 2010; Kim and Kim, 2010). The present results suggest that CCE-mediated inhibition of NO production in LPS-stimulated macrophages was associated with downregulation of the iNOS protein.

Effect of CCE on PGE$_2$ production in LPS-stimulated cells

To evaluate the effect of CCE on PGE$_2$ production in LPS-stimulated RAW 264.7 cells, culture medium PGE$_2$ concentrations were determined by ELISA. The stimulation of PGE$_2$ production in RAW 264.7 cells by LPS was suppressed by CCE treatment ($P < 0.05$) (Fig. 3A). Since COX-2 is the enzyme responsible for the production of PGE$_2$, we analyzed the effect of CCE on COX-2 protein expression in LPS-stimulated RAW 264.7 cells by Western blotting. As shown in Fig. 3B, CCE strongly and dose-dependently suppressed the expression of COX-2 protein.

COXs regulate the conversion of arachidonic acid to PGE$_2$ and are rate-limiting enzymes in the biosynthesis of PGs (Du-
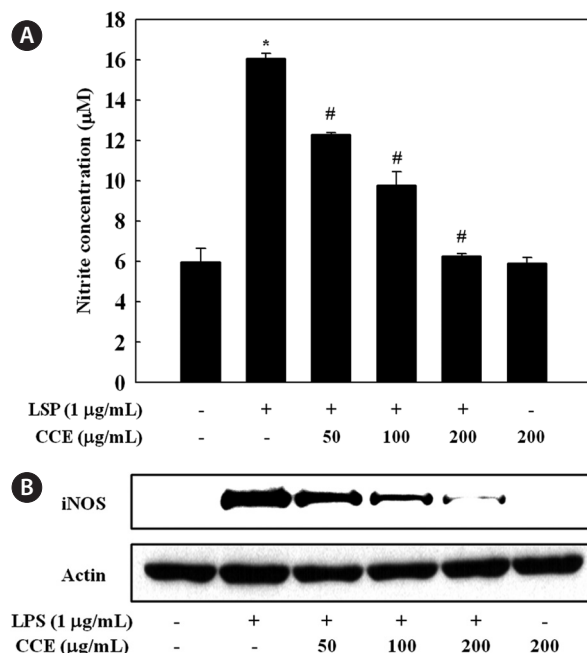
Fig. 2. Effect of *Chondria crassicaulis* ethanolic extract (CCE) on lipopolysaccharide (LPS)-induced nitrite production and inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells. (A) Cells pretreated with different concentrations (50, 100, 200 µg/mL) of CCE for 1 h were stimulated with LPS (1 µg/mL) for 24 h. The culture medium was used to measure the amount of nitrite to evaluate NO production by Griess reagents. (B) Cells pretreated with indicated concentration of CCE for 1 h were stimulated with LPS (1 µg/mL) for 16 h. Equal amounts of total proteins were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The expression of iNOS and actin protein was detected by Western blotting using responding antibodies. $^*P < 0.05$ indicates significant differences from the control group. $^#P < 0.05$ indicates significant differences from the LPS-treated group.

bois et al., 1998). COX-1 is constitutively expressed in many tissues, while COX-2 is known as an inducible enzyme that, in most cases, generates large amounts of PGs. COX-2 is highly expressed in inflammation-related cell types including macrophages and mast cells stimulated with pro-inflammatory cytokines and/or LPS (Nathan, 1992; Vane et al., 1994). Recent studies have shown that *in vivo* and *in vitro* treatments with natural compounds reduce inflammation by suppressing iNOS and COX-2 (Shin et al., 2010; Chung et al., 2011; Pan et al., 2011). The present results suggest that CCE-mediated inhibition of PGE_2 production in LPS-stimulated macrophages was associated with downregulation of COX-2. This is, to our knowledge, the first study to address the inhibition of iNOS and COX-2 expression by CCE.

Effect of CCE on the production of TNF-α, IL-1β, and IL-6 in LPS-stimulated cells

Since CCE was found to inhibit the inflammatory mediators NO and PGE_2 in a dose-dependent manner, we investigated the inhibitory effect of CCE on LPS-induced TNF-α, IL-1β,
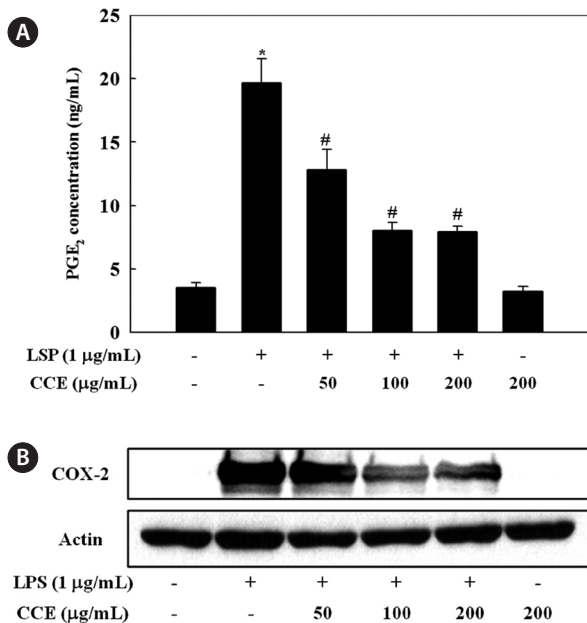
and IL-6 release by ELISA. Stimulation of RAW 264.7 cells with LPS significantly increased the levels of TNF-α, IL-1β, and IL-6. However, CCE, at concentrations ranging from 50 to 200 μg/mL, dose-dependently inhibited cytokine production in LPS-stimulated RAW 264.7 cells ($P < 0.05$) (Fig. 4). The
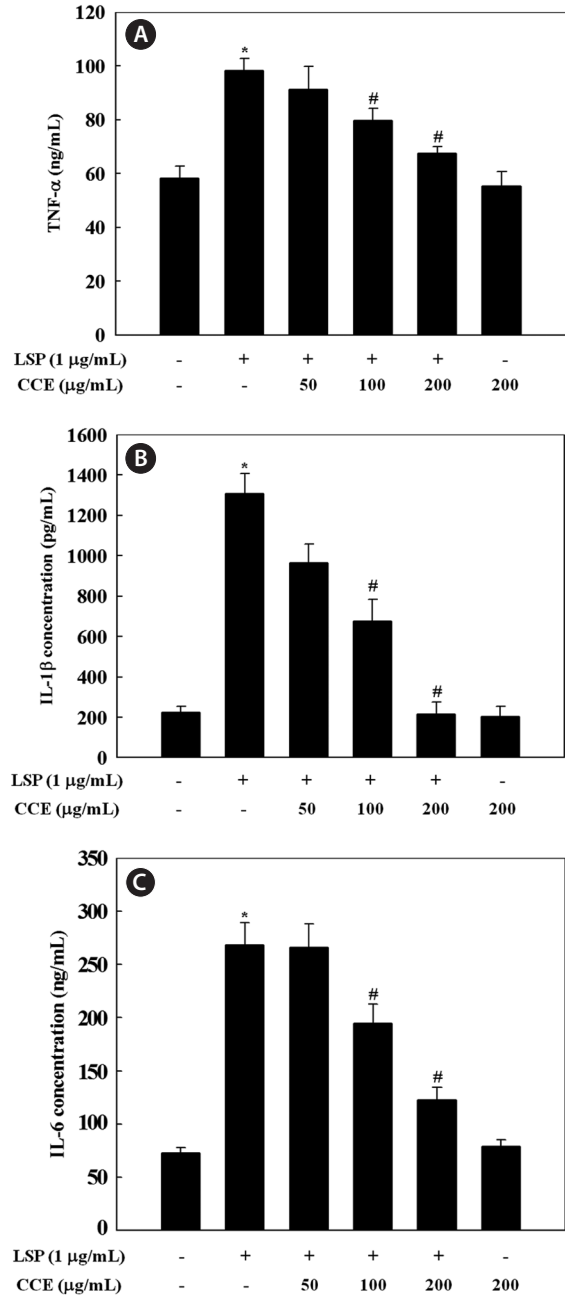
Fig. 3. Effect of *Chondria crassicaulis* ethanolic extract (CCE) on lipopolysaccharide (LPS)-stimulated prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) expression in RAW 264.7 cells. (A) Cells pretreated with different concentrations (50, 100, 200 μg/mL) of CCE for 1 h were stimulated with LPS (1 μg/mL) for 24 h. The culture medium was used to measure the amount of PGE_2 production by enzyme-linked immunosorbent assay. (B) Cells pretreated with indicated concentration of CCE for 1 h were stimulated with LPS (1 μg/mL) for 16 h. Equal amounts of total proteins of harvest cells were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The expression of iNOS and actin protein was detected by Western blotting using responding antibodies. *$P < 0.05$ indicates significant differences from the control group. #$P < 0.05$ indicates significant differences from the LPS-treated group.

Fig. 4. Effects of *Chondria crassicaulis* ethanolic extract (CCE) on pro-inflammatory cytokine productions in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW 264.7 cells pretreated with indicated concentration of CCE for 1 h were stimulated with LPS (1 μg/mL) for 24 h. Tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B) and IL-6 (C) in the cultured supernatant were measured by enzyme-linked immunosorbent assay. Data are mean ± SDs. of three independent experiments. *$P < 0.05$ indicates significant differences from the control group. #$P < 0.05$ indicates significant differences from the LPS-treated group.

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inhibitory effect of CCE on TNF-α, IL-1β, and IL-6 production was not attributable to cytotoxic effects, since cell viability was not altered by CCE at the concentrations used in this study (Fig. 1).

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are small secreted proteins that regulate immunity and inflammation. Bacterial LPS stimulates macrophages to release TNF-α. Secreted TNF-α or LPS then induces cells to release IL-1β and IL-6 (Beutler and Cerami, 1989). TNF-α induces several physiological processes, including septic shock, inflammation, and cytotoxicity (Dinarello, 1999). IL-1β is a major pro-inflammatory cytokine, which is mainly released by macrophages and is believed to play a key role in the pathophysiology of endometriosis (Lebovic et al., 2000). IL-1β is also important in the initiation and enhancement of inflammatory responses to microbial infection (Kim and Moudgil, 2008). IL-6 is a pivotal pro-inflammatory cytokine that is mainly synthesized by macrophages. It plays a role in the acute-phase immune response (Yoshimura, 2006) and is regarded as an endogenous mediator of LPS-induced fever. The results of the present study suggest that CCE significantly suppressed LPS-induced TNF-α, IL-1β, and IL-6 secretion, which supports the idea that CCE inhibits the initial phase of a LPS-stimulated inflammatory response.

Effect of CCE on NF-κB activation in LPS-stimulated cells

We further investigated whether CCE could inhibit the LPS-stimulated degradation of IκB-α in RAW 264.7 cells by Western blotting. LPS treatment resulted in degradation of IκB-α, a response that was dose-dependently reversed by CCE pretreatment (Fig. 5A). To investigate translocation of the p65 subunit of NF-κB from the cytosol to the nucleus after its release from IκBs, we examined NF-κB levels in cytosolic and nuclear extracts. CCE pretreatment dose-dependently increased cytosolic NF-κB levels (Fig. 5A). CCE dose-dependently reduced the nuclear p65 level, and in parallel increased the cytosolic p65 level. Considering the inhibitory effects of CCE on LPS-induced NF-κB activation, we next determined the effect of CCE on NF-κB promoter activity in LPS-stimulated macrophage cells. To achieve this, cells were transiently transfected with a luciferase construct containing the murine NF-κB promoter, pretreated for 2 h with various concentrations of CCE, and treated with LPS (1 μg/mL) for 6 h. Luciferase assay data show that CCE treatment significantly suppressed LPS-induced NF-κB promoter-driven luciferase expression in macrophages ($P < 0.05$) (Fig. 5B). These results suggest that CCE-mediated inhibition of iNOS, COX-2, and pro-inflammatory cytokine expression in LPS-stimulated macrophages was regulated by an NF-κB pathway.

The induction of inflammatory mediators such as NO and PGE$_2$, and of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, is dependent on NF-κB activation (Li and Ver-
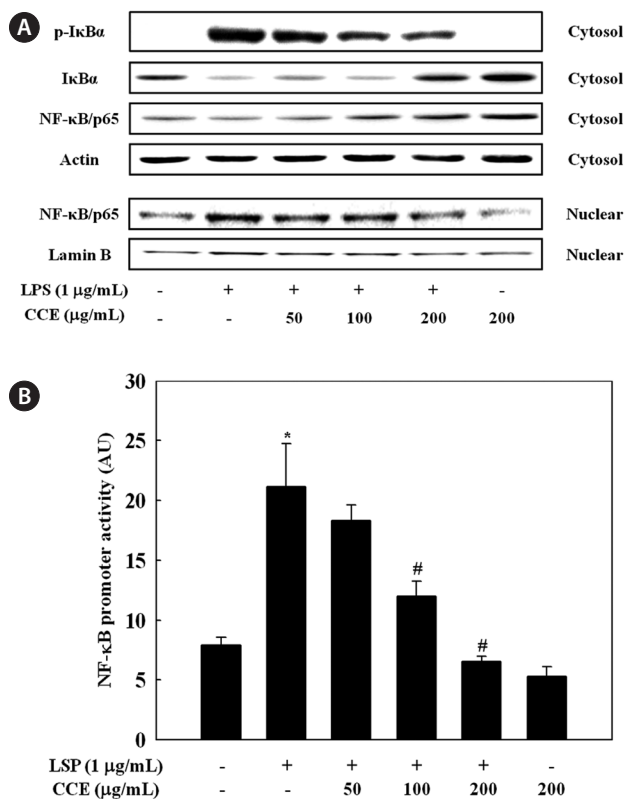

Fig. 5. Effect of *Chondria crassicaulis* ethanolic extract (CCE) on activation of nuclear factor-κB (NF-κB) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW 264.7 cells pretreated with indicated concentration of CCE for 1 h were stimulated with LPS (1 μg/mL) for 30 min. The expression of inhibitor of κB-α and NF-κB (A) were determined by a Western blot analysis. (B) RAW 264.7 cells were co-transfected with 1 μg of NF-κB promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Transfected cells pretreated with indicated concentration of CCE for 1 h were stimulated with LPS (1 μg/mL) for 6 h. Data are mean ± SDs of three independent experiments. *$P < 0.05$ indicates significant differences from the control group. #$P < 0.05$ indicates significant differences from the LPS-treated group.

ma, 2002). NF-κB is known to play a pivotal role in the regulation of cell survival genes and to coordinate the expression of pro-inflammatory enzymes and cytokines such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 (Xie et al., 1993; D'Acquisto et al., 1997; Marks-Konczalik et al., 1998; Makarov, 2001). NF-κB is associated with and tightly controlled by an inhibitory subunit, IκB, which retains NF-κB in the cytoplasm in an inactive form. Activation of NF-κB by LPS involves the phosphorylation of IκB-α kinase, which phosphorylates IκB-α at Ser32 and Ser36, leading to the subsequent degradation of IκB-α and inducing translocation of NF-κB into the nucleus (Chen et al., 1995). In the present study, we observed that degradation of IκB-α in response to LPS treatment was reversed by CCE treatment, suggesting that CCE protected IκB-α from proteolytic degradation. Moreover, we found that translocation of NF-κB into the nucleus was dose-dependently inhib-

ited by CCE. From these data, the CCE-mediated downregulation of LPS-induced iNOS, COX-2, TNF-α, IL-1β, and IL-6 expression in RAW 264.7 cells is likely largely dependent on the ability of CCE to inhibit NF-κB signaling. This is, to the best of knowledge, the first report addressing CCE's negative regulation of LPS-activated NF-κB signaling.

In conclusion, we demonstrated that CCE inhibited production of the inflammatory mediators NO and PGE$_2$ and the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 macrophages. Moreover, the inhibitory effects of CCE were found to be associated with the inactivation of NF-κB signaling through inhibition of IκB degradation. Verification of CCE's anti-inflammatory activity and mechanisms of action in cells will be beneficial to the further application of CCE in functional foods in the treatment of inflammatory diseases. These results suggest the need to perform additional studies to identify the compounds in CCE that contribute to its anti-inflammatory activity.

Acknowledgments

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