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Codium fragile Ethanol Extraction Inhibited Inflammatory Response through the Inhibition of JNK Phosphorylation

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Abstract

***Codium fragile* (CF) is an edible green alga consumed as a traditional food source in Korea. In this study, the ethanol extract of CF was evaluated to determine if it has anti-inflammatory activity. Lipopolysaccharide (LPS), a toxin from bacteria, is a potent inducer of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Therefore, we studied whether CF extracts have an anti-inflammatory effect in LPS-induced murine macrophage cell lines (RAW 264.7). In the present study, IL-6 production was measured using an enzyme-linked immunosorbent assay (ELISA), prostaglandin E_2 (PGE_2) production was measured using the EIA kit, and cyclooxygenase (COX)-2 and mitogen-activated protein kinase (MAPK) activation were determined by Western blot analysis. IL-6 mRNA, COX-2 mRNA and iNOS mRNA expression were measured using reverse transcription-polymerase chain reaction (RT-PCR). The results indicated that CF extracts inhibit LPS-induced IL-6, NO and PGE_2 production in a dose-dependent manner, as well as expression of iNOS and COX-2. CF extracts significantly inhibited LPS-induced c-Jun N-terminal kinase (JNK) 1/2 phosphorylation. Taken together, these findings may help elucidate the mechanism by which CF modulates RAW 264.7 cell activation under inflammatory conditions.**

Key words: *Codium fragile*, lipopolysaccharide, anti-inflammatory effect

INTRODUCTION

Bacteria stimulate the innate immune system of a host, resulting in the release of inflammatory molecules, such as cytokines and chemokines, in response to infections (1,2). Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, is a well-known activator of the innate immune system during Gram-negative infections (3). LPS induces most of the clinical manifestations of bacterial infections, including inflammation, fever, and septic shock (4). Most of these effects are due to the activation of macrophages and generation of proinflammatory cytokines, such as tumor necrosis factor, interleukin (IL)-6, and IL-8 (5-8).

Macrophages play an important role in host defenses against noxious substances, and are involved in a variety of disease processes, including autoimmune diseases, infections, and inflammatory disorders (9). In addition to stimulating the production of IL-6, an inflammatory stimulus such as LPS can activate macrophages to produce a variety of other inflammatory mediators, including prostaglandin (PG) E_2 and nitric oxide (NO), which are synthesized by cyclooxygenase (COX)-2 and inducible NO synthase (iNOS), respectively. These inflammatory mediators are involved in the pathogenesis of many inflammation-associated human diseases (10-12). The following three well-defined mitogen-activated protein kinases (MAPKs)－extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK)－have been implicated in the transcriptional regulation of the iNOS gene. Also, specific MAPK inhibitors suppress the expression of the iNOS gene (13-15).

Marine bioresources are of interest to scientists and nutritionists because some marine plants contain compounds that have bioactivities not found in land plants. Screening of algal extracts for biologically active compounds began in the 1950s with simple antibiotic assays and expanded to include testing for products with antiviral, antibacterial, antifungal, anti-mitotic, and antitumorigenic activities (16-19). Studies on the antitumor effects of algal species have been reported by a number of researchers (20-23). Sea staghorn, *Codium fragile*

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(Suringar) Hariot is a siphonous marine green alga belonging to the family Codiaceae (order Codiales, class Chlorophyceae). In China, it is eaten as food by some of the people along the coastlines and is used as anticancer, antiviral, antipyretic, and helminthic agents in Chinese traditional medicine (24). However, there is yet no report regarding sea staghorn inducing anti-inflammatory effect in macrophage cells. Understanding the underlying mechanism related to the inhibition of inflammation by *Codium fragile* will benefit the potential new source of drugs for the treatment of inflammatory diseases.

MATERIALS AND METHODS

Plant material and extract

Codium fragile was presented from Dr. H.G. Choi (Faculty of Biological Sciences, Wonkwang University). The *C. fragile* was air-dried in the shade at room temperature (RT) and then ground into a powder using a mechanical grinder. Approximately 500 g of the powdered material was then dissolved in 1500 mL of ethanol (EtOH) for 7 days (room temperature). The EtOH extract yield was 36.49 g. The extract was filtered (pore size, 0.45 μm), lyophilized, and kept at 4°C. The dried extract was then dissolved in phosphate buffered saline (PBS) in preparation for use. A sample of the *C. fragile* has been deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Iksan.

Materials

RPMI 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). Bovine serum albumin, LPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). COX-2, iNOS, p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK, β-actin, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-mouse IL-6 antibody and biotinylated anti-mouse IL-6 antibodies were purchased from BD Biosciences (San Jose, CA, USA). COX-2, iNOS, IL-6, and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejeong, Korea).

Cell culture

The murine macrophage cell line, RAW 264.7, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Seoul, Korea) and grown in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL of penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. To stimulate the cells, the medium was replaced with fresh RPMI 1640 medium, LPS was added in the presence or absence of CF for the indicated periods.

Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded at 5×10^5/mL per well in 24-well tissue culture plates and pretreated with various concentrations of CF (100 g/mL or 50 g/mL) for 30 minutes before LPS (200 ng/mL) stimulation for 24 hours. ELISA plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) were coated overnight at 4°C with anti-mouse IL-6 antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed four times with PBS containing 0.05% Tween 20. The nonspecific protein binding sites were blocked with assay diluent (PBS containing 10% fetal bovine serum, pH 7.0) for at least 1 hour. Immediately, each sample and IL-6 standard were added to the wells. After incubation for 2 hours, a working detector (biotinylated anti-mouse IL-6 monoclonal antibody and streptavidin-horseradish peroxidase reagent) was added and incubated for 1 hour. Accordingly, substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 minutes in the dark before the reaction was stopped with stop solution (2 N H_3PO_4). The absorbance was read at 450 nm. All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Measurement of NO production

NO production was assayed by measuring the nitrite in the supernatants of cultured RAW 264.7 cells. The cells were seeded at 1×10^5/mL in 96-well culture plates. After preincubation of the RAW 264.7 cells for 18 hours, the cells were pretreated with CF (100 g/mL or 50 g/mL) and stimulated with LPS (200 ng/mL) for 24 hours. The supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 minutes. The concentrations of nitrite were measured by reading at 570 nm. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

Measurement of PGE_2 production

The RAW 264.7 cells were cultured in 24-well culture plates (5×10^5/mL). CF (100 μg/mL or 50 μg/mL), LPS (200 ng/mL) was added to the culture medium and incubated at 37°C for 24 hours. The medium was collected in a microcentrifuge tube and centrifuged. The supernatant was decanted into a new microcentrifuge tube, and the amount of PGE_2 was determined by a PGE_2 Enzyme Immuno-Assay Kit (Amersham Biosciences, Little Chalfont, UK) according to the procedure de-

scribed by the manufacturer.

Western blot analysis

Protein expression was assessed by Western blot analysis according to standard procedures. The RAW 264.7 cells were cultured in 60-mm-diameter culture dishes (3×10^6/mL) and pretreated with various concentrations of CF (100 μg/mL or 50 μg/mL). After 30 minutes or 1 hour, LPS (200 ng/mL) was added to the culture medium, and the cells were incubated at 37°C for the times indicated in figure legends. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). The cell pellets were resuspended in lysis buffer on ice for 15 minutes, and cell debris was removed by centrifugation. The protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene membrane (Millipore, Bedford, MA, USA).

The membrane was blocked with 5 % nonfat milk in Tris-buffered saline with Tween 20 buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.05% Tween 20, pH 7.4). After blocking, the membrane was incubated with primary antibodies for 18 hours. The membrane was then washed with Tris-buffered saline with Tween 20 and incubated with antimouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Milan, Italy).

RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR)

The RAW 264.7 cells were cultured in 30-mm-diameter culture dishes (3×10^6/mL) and pretreated with various concentrations of CF (100 μg/mL, or 50 μg/mL). After 30 minutes, LPS (200 ng/mL) was added to the culture medium, and the cells were incubated at 37°C for 24 hours. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Total cellular RNA was isolated using RNeasy Mini kit (QIAGEN) and 1 μg of total RNA was reverse-transribed using QuantiTect Reverse Transcription Kit (QIAGEN), according to the manufacturer's instructions. The total RNA (2 μg) was converted to cDNA by treating it with 200 units of reverse transcriptase and 500 ng of oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates at 42°C for 1 hour. The reaction was stopped by heating at 70°C for 15 minutes, and the cDNA mixture (3 μL) was used for enzymatic amplification. PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 units of Taq DNA polymerase, and 0.1 M each iNOS, COX-2, IL-6, and β-actin primers, respectively.

The conditions for amplification were as follows: denaturation at 94°C for 3 minutes for the first cycle and for 30 seconds starting from the second cycle, of iNOS at 56°C for 30 seconds, of COX-2 at 53°C for 30 seconds, and of IL-6 at 57°C for 45 seconds, and extension at 72°C for 90 seconds for 35 cycles. Final extension was performed at 72°C for 7 minutes. The PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

The primers used were as follows: 5-CATGTTCTCTGGGAAATCGTGG-3 (sense) and 5-AACGCACTAGGTTTGCCGAGTA-3 (antisense) for IL-6; 5-AGCCCAACAATACAAATGACCCTA-3 (sense) and 5-TTCCTGTTGTTTCTATTTCCTTTGT-3 (antisense) for iNOS; 5-CACTCAGTTTGTTGAGTCATTC-3 (sense) and 5-GATTAGTACTGTAGGGTTAATG-3 (antisense) for COX-2 and 5-ATGAAGATCCTGACCGAGCGT-3 (sense) and 5-AACGCAGCTCAGTAACAGTCCG-3 (antisense) for β-actin.

Statistical analysis

The results are expressed as mean ± SE values for the number of experiments. Statistical significance was compared between each treated group and the control and was determined by Student's *t*-tests. Each experiment was repeated at least three times and yielded comparable results. Values with $p<0.05$ and $p<0.005$ were considered significant.

RESULTS

Effect of CF extracts on LPS-induced IL-6 and mRNA expression

Since CF extracts were found to inhibit pro-inflammatory mediators, we used ELISA and RT-PCR to investigate the effect of CF on LPS-induced IL-6 production. Pretreatment with CF extracts reduced IL-6 production and mRNA expression in a dose-dependent manner (Fig. 1).

Effect of CF extracts on LPS-induced PGE_2 production

To assess the effect of CF extracts on the LPS-induced PGE_2 production in RAW 264.7 cells, cell culture media was harvested and PGE_2 levels were measured. To examine whether CF inhibits PGE_2 production, cells were pre-incubated with CF extracts for 1 hour and then activated with LPS (200 ng/mL) for 24 hours. As shown in Fig. 2, CF extracts inhibited the production of PGE_2 in dose-dependent manner (Fig. 2).

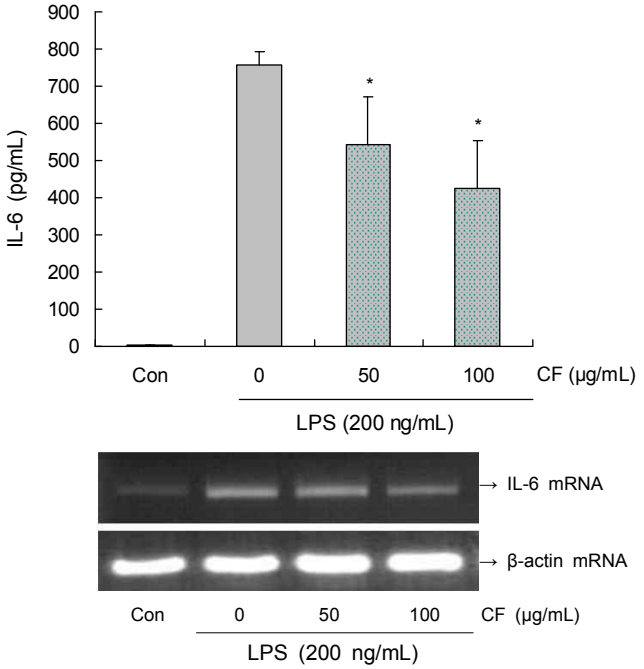

Fig. 1. Effects of CF extracts on LPS-induced IL-6 production and mRNA expression. RAW 264.7 cells were pretreated with the indicated concentrations of CF for 1 hour before being incubated with LPS (200 ng/mL) for 24 hours. Production of IL-6 was measured by ELISA. IL-6 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of CF for 1 hour before being incubated with LPS (200 ng/ml) for 24 hours. The β-actin mRNA was carried out in parallel to confirm equivalency of cDNA preparation. The experiment was repeated three times and similar results were obtained. Results are mean±SE. Statistical significance: $p<0.05$ vs the LPS treated group; significances between treated groups were determined using the Student's *t*-test.

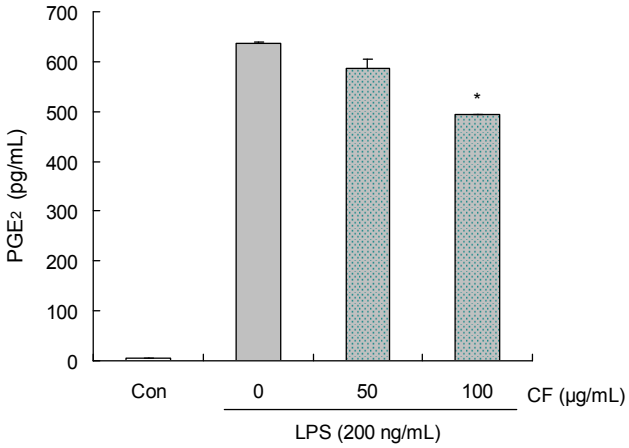

Fig. 2. Effects of CF extracts on LPS-induced PGE_2 production in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentration of CF for 1 hour before being incubated with LPS (200 ng/mL) for 24 hours. Control cells were incubated with the vehicle alone. $^{*}p<0.05$ vs the LPS treated group; significances between treated groups were determined using the Student's *t*-test.

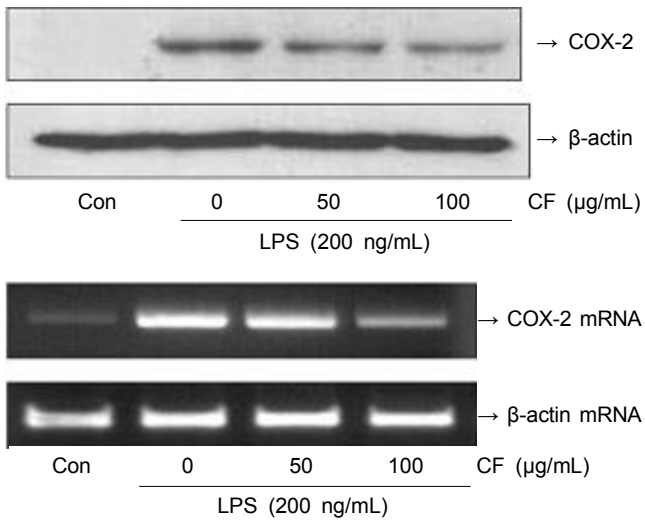

Fig. 3. Effect of CF extracts on LPS-induced COX-2 protein and mRNA expressions in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of CF for 1 hour before being incubated with LPS (200 ng/mL) for 24 hours. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with COX-2 antibody. Equal loading of protein was verified by β-actin. COX-2 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of CF for 1 hour before being incubated with LPS (200 ng/mL) for 24 hours. The β-actin mRNA was carried out in parallel to confirm equivalency of cDNA preparation. The experiment was repeated three times and similar results were obtained.

Effect of CF extracts on LPS-induced COX-2 protein and mRNA expression

Western blot and RT-PCR analysis were performed to determine whether the inhibitory effect of CF extracts on PGE_2 is related to the modulation of the expression of COX-2. In unstimulated RAW 264.7 cells, COX-2 protein and mRNA were undetectable. However, in response to LPS, COX-2 is strongly expressed. CF extracts significantly inhibited COX-2 expression in a dose-dependent manner. RT-PCR analysis showed that COX-2 mRNA expressions related with their protein levels (Fig. 3).

Effect of CF extracts on the phosphorylation of MAPKs in LPS-stimulated RAW 264.7 cells

MAPKs are essential for LPS-induced iNOS expression to occur in RAW 264.7 macrophages. Therefore, the effect of CF extracts on the activation of MAPKs in LPS-stimulated RAW 264.7 cells was evaluated. As shown in Fig. 4, CF extracts markedly inhibited the activating phosphorylation of JNK 1/2, whereas phosphorylation of ERK 1/2 and p38 MAPK phosphorylation were unaffected by treatment with CF extracts (Fig. 4).

Effect of CF extracts on LPS-induced NO production and iNOS mRNA expression

To assess the effect of CF extracts on the LPS-induced NO production in RAW 264.7 cells, cell culture media

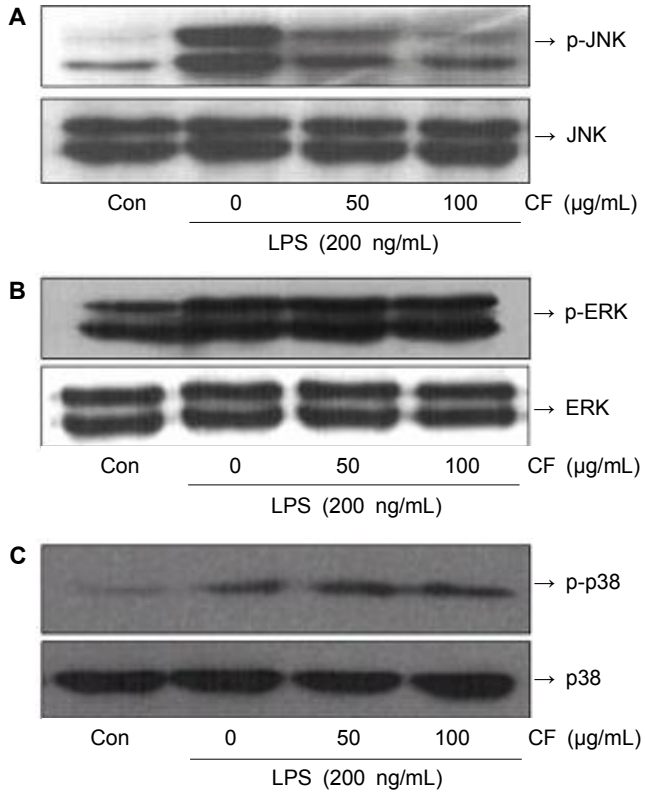

Fig. 4. Effect of CF extracts on phosphorylation of MAPKs in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of CF for 30 min before being incubated with LPS (200 ng/mL) for 30 min. Whole cell lysates were analyzed by Western blot analysis. The experiment was repeated three times and similar results were obtained.

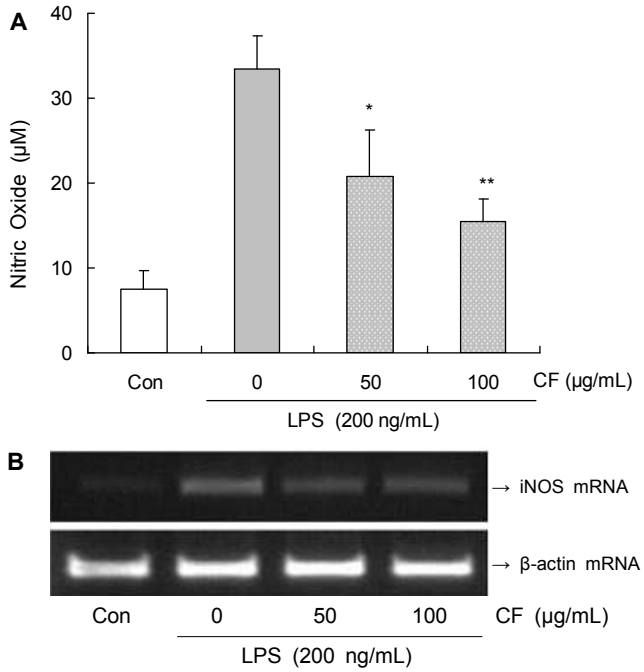

Fig. 5. The effect of CF extracts on LPS-induced NO production in RAW 264.7 macrophages. (A) RAW 264.7 cells were pretreated with the indicated concentrations of CF for 1 hour prior to being incubated with LPS (200 ng/mL) for 24 hours. The culture supernatants were subsequently isolated and analyzed for nitrite levels. The effect of MEAD on LPS-induced iNOS mRNA expression in RAW 264.7 macrophages. (B) iNOS mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of CF for 1 hour prior to being incubated with LPS (200 ng/mL) for 24 hours. Analysis of the β-actin mRNA was conducted in parallel to confirm equivalency of the cDNA preparation. The experiment was repeated three times and similar results were obtained. The values were expressed as a percentage of the maximal band intensity in the culture treated with LPS alone. Data are the mean±SEM of iNOS/ β-actin based on at least three separate experiments. $^{*}p<0.05$, $^{**}p<0.005$ vs the LPS treated group; significances between treated groups were determined using the Student's *t*-test.

was harvested and nitrite levels were measured. CF treatment inhibited NO production dose-dependently in LPS stimulated RAW 264.7 cells. CF extracts inhibited NO production by 53.8% at high concentration, but only by a little at lower concentrations (Fig. 5A).

The effect of CF extracts on the LPS-induced expression of iNOS by RAW 264.7 cells was evaluated to identify the anti-inflammatory mechanism. iNOS was strongly expressed in cells that were treated by LPS, and RT-PCR analysis showed that iNOS mRNA expression was related to the protein levels (Fig. 5B). The results indicate that pretreatment with CF extracts results in a reduced expression of iNOS mRNA and protein in response to LPS stimulation.

DISCUSSION

In this study, we evaluated the pharmacological basis for using extracts from *Codium fragile* (CF) as a treatment for various inflammatory diseases. The effects of CF extracts on macrophage functions related to inflammation were investigated to verify possible mechanisms underlying the alga's beneficial effects. We demonstrated that CF inhibits LPS-induced pro-inflammatory mediators, including NO and PGE_2. To explore the mechanism of inhibition of NO and PGE_2 production in RAW 264.7 cells, the effect of CF on the iNOS and COX-2 gene was examined.

NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels of NO have been reported in a variety of pathological processes, including various forms of inflammation, circulatory shock, and carcinogenesis (25-27). Therefore, an inhibitor of iNOS might be effective as a therapeutic agent for inflammatory diseases (28). The results of this study showed that CF extracts inhibited LPS-induced NO production in RAW 264.7 macrophages. To further investigate the mechanism underlying

these inhibitions by CF extracts, the expression of iNOS mRNA levels was examined by RT-PCR, respectively, which revealed that CF extracts reduced iNOS mRNA expression (Fig. 5). Taken together, these results indicate that CF has a potent anti-inflammatory effect that occurs through inhibition of the expression of iNOS and NO production.

CF extracts inhibited the expression of COX-2 protein, COX-2 mRNA and iNOS mRNA in dose-dependent manner, as assessed by Western blot analysis and RT-PCR, respectively. These results imply that CF exerts its effects through the inhibition of the iNOS and COX-2 transcription.

The MAPKs play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokines and stress (29). Several studies have demonstrated that MAPKs are involved in LPS-induced iNOS expression (30-32). Therefore, the effects of CF extracts on the LPS-induced phosphorylation of extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK were evaluated in this study. Interestingly, pretreatment of macrophages with CF extracts inhibited JNK 1/2 phosphorylation, but not ERK 1/2 and p38 MAPK phosphorylation (Fig. 4), which indicates that JNK 1/2 is likely responsible for the suppressive effect of CF on iNOS induction. These findings suggest that JNK 1/2 is involved in the inhibition of pro-inflammatory mediators by CF extracts in RAW 264.7 cells.

In conclusion, we have shown that extracts from CF inhibited LPS-induced NO, PGE_2, and IL-6 productions in macrophages, as well as iNOS and COX-2 expression and the phosphorylation of, JNK 1/2. These results suggest that CF may represent a new functional food source for the treatment of inflammatory diseases. Therefore, further research is clearly required to identify the active principle(s) within CF.

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