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ORIGINAL CONTRIBUTION

Hexane fraction from *Laminaria japonica* exerts anti-inflammatory effects on lipopolysaccharide-stimulated RAW 264.7 macrophages via inhibiting NF-kappaB pathway

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Abstract
Purpose *Laminaria japonica* is a representative marine brown alga used as a culinary item in East Asia. *L. japonica* extract was shown to exert various biological activities; however, its anti-inflammatory activity has not been reported. The aim of this study is to investigate the molecular mechanisms underlying its anti-inflammatory action.
Methods Anti-inflammatory mechanisms of *L. japonica* *n*-hexane fraction (LHF) were assessed using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. An anti-inflammatory compound isolated from LHF by reverse-phase chromatography was identified using nuclear magnetic resonance (NMR) spectroscopy.
Results Our results indicate that LHF significantly inhibited LPS-stimulated nitric oxide (NO) and prostaglandin E_2 (PGE_2) secretion in a dose-dependent manner and suppressed the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) with no cytotoxicity. As results, levels of pro-inflammatory cytokines were significantly reduced by pretreatment of LHF in LPS-stimulated RAW 264.7 cells. Treatment of LHF strongly suppressed nuclear factor-κB (NF-κB) promoter-driven expression and nuclear translocation of NF-κB by preventing proteolytic degradation of inhibitor of κB (IκB)-α in LPS-stimulated RAW 264.7 cells. Moreover, LHF inhibited the phosphorylation of Akt and mitogen-activated protein kinase (MAPK) in LPS-stimulated RAW 264.7 cells. One of the anti-inflammatory compounds was isolated from LHF and identified as fucoxanthin.
Conclusions These results indicate that the LHF-mediated inhibition of NO and PGE_2 secretion in LPS-stimulated macrophages is regulated by NF-κB inactivation through inhibition of IκB-α, MAPKs, and Akt phosphorylation. LHF may be considered as a functional food candidate for the prevention or treatment of inflammatory diseases.

Keywords Anti-inflammation · Fucoxanthin · *Laminaria japonica* · MAP kinases · NF-kappaB · RAW 264.7 cells

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Introduction

Inflammation is a complex response of host defense against harmful stimuli, such as microbial infection, endotoxin exposure, or tissue injury, and ultimately leads to the restoration of normal cell structure and function. Macrophages are key regulators of the immune response to foreign invaders, such as infectious microorganisms, and are activated by exposure to interferon-γ, pro-inflammatory cytokines, and bacterial lipopolysaccharides (LPS) [1, 2]. Stimulated macrophages play a pivotal role in inflammatory diseases via excess production of cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, and other inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) [3, 4]. Excessive

production of inflammatory mediators and cytokines is involved in the pathogenesis of chronic diseases, such as atherosclerosis, inflammatory arthritis, obesity, and cancer [5–7]. Any substances that inhibit production of these molecules are considered as potential anti-inflammatory agents.

Marine macroalgae have been used as an important diet for centuries in Korea, Japan, and China. Due to richness of polysaccharides, minerals, and polyunsaturated fatty acids, macroalgae are known to be a good source of healthy food [8]. Recently, various studies have focused on their constituents as functional foods, including polysaccharides, phlorotannins, and other bioactive compounds [8–10]. Polysaccharides, such as alginate, fucoidan, and laminaran from *Laminaria japonica* classified as dietary fibers, are neither digested nor absorbed into the blood circulation; however, these soluble polysaccharides may also act as prebiotics, stimulating growth of beneficial bacteria in the colon [11]. Among them, fucoidan from *Laminaria* sp. exhibits anti-inflammatory effects by interaction with a membranous receptor of intestinal surface cells or cultured cells and stimulates a signal transduction system involved in immune response [12]. More recently, it was shown that sulfated fucans from *L. saccharina* have the anti-inflammatory, anticoagulant, anti-angiogenic, and antitumor activities [13].

L. japonica is used as a herbal medicine in China to treat goiter, scrofula, urinary disease, dropsy, stomach ailments, and hemorrhoids [14]. Fucoxanthin is one of the most abundant carotenoids found in brown macroalgae and the major functional pigment present in *L. japonica.* Fucoxanthin has been reported to have antioxidative [15–17], cytoprotective [18, 19], anti-inflammatory [20, 21], and anti-obese properties [22, 23].

As part of our ongoing effort to isolate lipophilic compounds with anti-inflammatory activities from marine brown algae, we found strong anti-inflammatory activity in *L. japonica n*-hexane fraction (LHF). To our knowledge, no previous study has been reported on the anti-inflammatory activity of *L. japonica* extracts. In this regard, we investigated the anti-inflammatory activity of LHF and its underlying mechanisms using cultured RAW 264.7 cells. Our data suggest that LHF may represent a source of functional food for prevention or treatment of inflammatory diseases.

Materials and methods

Microalgae material and reagents

L. japonica was collected from Gijang aquaculture farm, Busan, South Korea, in May 2010, and a voucher specimen was deposited in the laboratory (H.-R. Kim). Samples were rinsed in tap water to remove salt and dried in an air dryer at 60 °C for 40 h. A dried sample was ground with a hammer mill, and the powder was stored at −20 °C until used. CellTiter 96® AQueous One Solution cell proliferation assay kit, luciferase assay kit, murine NF-κB promoter/luciferase DNA, pRL-TK DNA, and reverse transcriptase were obtained from Promega (Madison, WI, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and GE Healthcare Bio-Science (Piscataway, NJ, USA), respectively. Lipofectamine/Plus, TRIzol, Dulbecco's modified Eagle's medium (DMEM), 4′,6-diamidino-2-phenylindole (DAPI), Alexa Fluor® 488-conjugated secondary antibody, luciferase assay kit, penicillin, and streptomycin sulfate were purchased from Invitrogen (Carlsbad, CA, USA). LPS (*Escherichia coli* O55:B5), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and the specific kinase inhibitors (LY294002, PD98059, SP600125, and SB203580) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Preparation of LHF and liquid chromatography

Dried powder (2.5 kg) of *L. japonica* was extracted three times with 96 % (v/v) ethanol (EtOH) for 3 h at 70 °C. The combined extracts were filtered and concentrated under reduced pressure to obtain the EtOH extract (446.6 g). For further fractionation of the EtOH extract, the extract was resuspended in water/EtOH (9:1, v/v) and partitioned successively with *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol, yielding the hexane (LHF, 36.2 g), dichloromethane (9.3 g), ethyl acetate (1.4 g), *n*-butanol (20.2 g), and water (370 g) fractions. Aliquots of LHF were separated by Shimadzu high-performance liquid chromatography (HPLC) system with Luna RP-18 [Luna C18(2), 5 μm, 250 × 10 mm, Phenomenex, Torrence, CA, USA]. The separation of LHF was conducted using 100 % methanol (solvent A) and 0.1 % formic acid in water (solvent B) as the mobile phase. The elution profile consisted of a linear gradient from A/B (78/22) to A/B (95/5) for 90 min and held for 10 min and then re-equilibration of the column with A/B (78/22) for 18 min. The flow rate was 0.34 mL/min at 35 °C oven temperature, and detection was performed at 410 nm. Fractions were collected and assessed for the ability to inhibit NO secretion using LPS-stimulated RAW 264.7 cells. The isolated compound (10 mg) was dissolved in 0.6 mL of CD_3OD and used for ^{1}H- and ^{13}C-NMR spectroscopy. NMR spectra were obtained by Fourier transform NMR JNM ECP-400 (JEOL, Tokyo, Japan). The chemical structure of the purified compound was identified by comparing its data with literature [16].

Cell culture and treatment

Murine macrophage RAW 264.7 cells (ATCC, Rockville, MD, USA) were cultured at 37 °C in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL). Cells placed in a 12-well plate at a density of 1×10^6 cells per well were incubated for 24 h. Cultured cells were treated with vehicle (control) and various concentrations of LHF for 1 h and then stimulated without or with 1 μg/mL of LPS for 0.5, 6, or 24 h.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter 96® AQueous One Solution cell proliferation assay kit according to the manufacturer's instructions. Briefly, cells were inoculated at a density of 1×10^5 cells/well into 96-well plate and cultured at 37 °C for 24 h. The culture medium was replaced with 200 μL of serial dilutions of LHF, and the cells were incubated for 24 h. The culture medium was removed and replaced with 95 μL fresh culture medium and 5 μL MTS solution. After 1 h, the absorbance at 490 nm was measured using a microplate reader (Glomax Multi Detection System, Promega).

Measurement of NO, PGE_2, TNF-α, IL-1β, and IL-6

RAW 264.7 cells were placed in a 12-well plate at a density of 1×10^6 cells per well and incubated for 24 h. Cultured cells were treated with vehicle or various concentrations of LHF for 1 h and then stimulated with 1 μg/mL LPS for 24 h. Cultured media were collected after centrifugation at $2{,}000 \times g$ for 10 min and stored at −70 °C until used. The nitrite concentration in the cultured media was measured as an indicator of NO secretion. Culture media (100 μL) were mixed with the same volume of Griess reagent (0.1 % naphthylethylene diamine dihydrochloride and 1 % sulfanilamide in 5 % phosphoric acid). Absorbance of the mixture at 540 nm was measured with a microplate reader. Levels of PGE_2, IL-1β, IL-6, and TNF-α in cultured media were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells placed in a 12-well plate were pretreated with LHF for 1 h and then stimulated with LPS for 6 h. Total RNA from each group was isolated with the TRIzol reagent. Five microgram of total RNA was used for reverse transcription using oligo-dT-adaptor primer and superscript reverse transcriptase. PCR was carried out with the gene-specific primers: COX-2 sense, 5′-CAGCAAATCCTTG CTGTTCC-3′; COX-2 antisense, 5′-TGGGCAAAGAAT GCAAACATC-3′; iNOS sense, 5′-TCTTCGAAATCCC ACCTGAC-3′; iNOS antisense, 5′-CCATGATGGTCA CATTCTGC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5′-TGGCACAGTCAAGGCTGAGA-3′; and GAPDH antisense, 5′-CTTCTGAGTGGCAGTGAT GG-3′. GAPDH was used as an internal standard to evaluate relative expression of COX-2 and iNOS. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II (ATTO & Rise Co., Tokyo, Japan) and CS analyzer ver. 3.00 software (ATTO).

Transient transfection and luciferase assay

Murine NF-κB promoter/luciferase DNA (1 μg) along with 20 ng control pRL-TK DNA was transiently transfected into 2×10^5 RAW 264.7 cells/well in a 24-well plate using Lipofectamine/Plus reagents for 40 h. Cells pretreated with 0–50 μg/mL LHF for 2 h were stimulated with LPS (1 μg/mL) for 6 h. Each well was washed twice with cold phosphate-buffered saline (PBS), harvested in 100 μL of lysis buffer (0.5 mM HEPES, pH 7.8, 1 % Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$), and used for the measurement of luciferase activity using a luciferase assay kit. Luminescence was measured on a top counter microplate scintillation and luminescence counter in single-photon counting mode for 0.1 min/well, following a 5-min adaptation in the dark. Luciferase activity was normalized to the expression of control pRL-TK.

Preparation of cytosolic and nuclear extracts

RAW 264.7 cells (5×10^6 cells/well) pretreated with LHF for 1 h were stimulated with LPS for 0.5 h. Cells were washed twice with cold PBS and harvested. Cell pellets were resuspended in 300 μL of hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9) and incubated on ice for 15 min. After vortexing for 10 s, homogenates were separated into supernatants (cytoplasmic compartments) and pellets (nuclear components) by centrifugation at $13{,}000 \times g$ for 10 min. The pellet was gently resuspended in 40 μL complete lysis buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10 % glycerol, and 0.5 mM PMSF, pH 7.9) and centrifuged at $13{,}000 \times g$ for 20 min at 4 °C. The supernatant was used as the nuclear extract.

Western immunoblot analysis

RAW 264.7 cells were incubated with various concentrations of LHF for 1 h and stimulated with LPS (1 μg/mL) for 30 min. RAW 264.7 cells were washed twice with cold PBS and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 % IGEPAL CA-630, 1 % Tween 20, 0.1 % SDS, 1 mM Na_3VO_4, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM PMSF) on ice for 1 h. After centrifugation at 18,000×*g* for 10 min, the protein concentrations in the supernatants were determined, and aliquots of the protein (40 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5 % nonfat dry milk in Tris-buffered saline with 0.1 % Tween 20 (TBST) buffer for 1 h, followed by the incubation for 2 h with primary antibody in TBST buffer containing 5 % nonfat dry milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer containing 5% nonfat dry milk for 1 h, and immune complexes were detected using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Little Chalfont, UK). Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software.

Immunofluorescence analysis

To analyze nuclear localization of NF-κB in RAW 264.7 cells, the cells were maintained on glass coverslips (SPL Lifesciences Co., Gyeonggi-do, Korea) in a 24-well plate for 24 h. Cells stimulated with LHF for 1 h were incubated with LPS (1 μg/mL) for 30 min. Cells were fixed in 4.0 % (w/v) paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.5 % (v/v) Triton

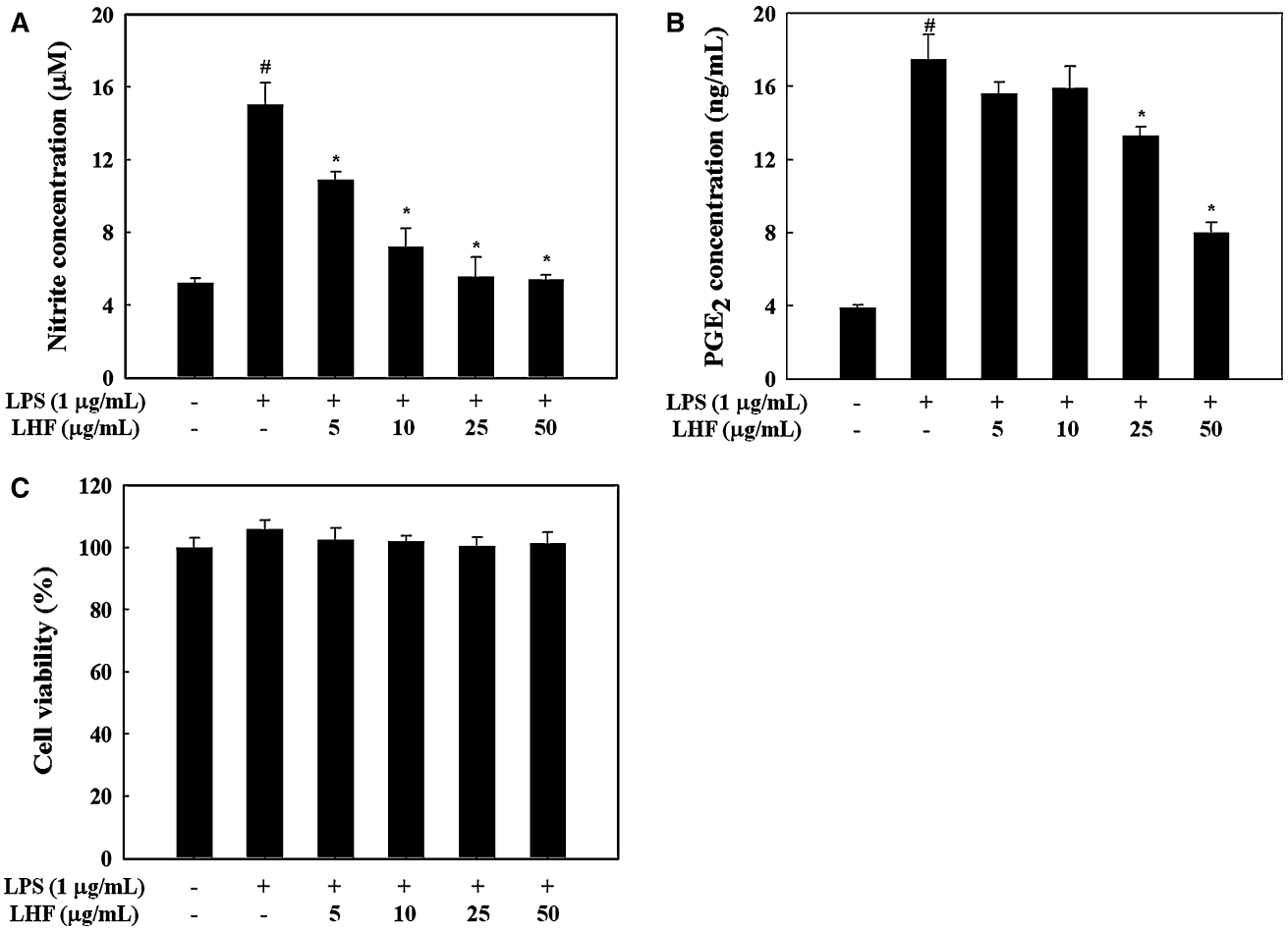

Fig. 1 Effect of LHF on LPS-stimulated NO and PGE_2 secretion in RAW 264.7 cells. Cells pretreated with different concentrations (5, 10, 25, 50 μg/mL) of LHF for 1 h were stimulated with LPS (1 μg/mL) for 24 h. **a**, **b** The treated culture media were used to measure the amount of NO and PGE_2 secretion. **c** Cytotoxic effect of LHF was measured by MTS assay. Values are the means ± SDs of three independent experiments. #$p < 0.05$ indicates significant differences compared to the control group. *$p < 0.05$ indicates significant differences compared to the LPS-only group

X-100 in PBS for 10 min. Permeabilized cells were washed with PBS and blocked with 3 % (w/v) BSA in PBS for 30 min. Thereafter, cells were incubated with an anti-NF-κB polyclonal antibody diluted in 3 % BSA/PBS for 2 h and incubated with Alexa Fluor® 488-conjugated secondary antibody diluted in 3 % BSA/PBS for 1 h. Cells treated with 2 μg/mL DAPI were mounted on glass slides using mounting solution, and the images were captured using an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Data were expressed as the means ± standard deviations (SDs) of at least three independent experiments unless otherwise indicated. Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Student's t tests for multiple comparisons. Differences were considered significant at values of $p < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS Inc., Chicago, IL).

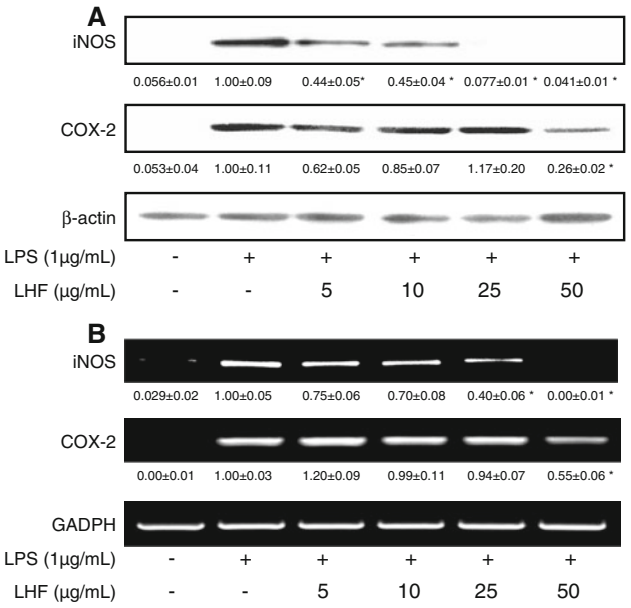

Fig. 2 Effect of LHF on LPS-stimulated iNOS and COX-2 expression in RAW 264.7 cells. **a** Cells were pretreated with indicated concentration of LHF for 1 h and stimulated with LPS (1 μg/mL) for 16 h. Thirty microgram of total proteins was subjected to 10 % SDS–PAGE. The expression of iNOS, COX-2, and β-actin protein was detected by Western blot using corresponding antibodies. **b** Cells were pretreated with LHF for 1 h and stimulated with LPS for 6 h, and then total RNA was prepared for RT-PCR. Relative densitometric ratios of each protein and mRNA over β-actin and GAPDH, respectively, are shown below the blots (means ± SDs of three independent experiments). *$p < 0.05$ indicates significant differences compared to the LPS-only group

Results

LHF inhibits NO and PGE_2 secretion

To evaluate the effect of LHF on NO secretion in LPS-stimulated RAW 264.7 cells, we measured nitrite concentrations in culture media using the Griess reagent. RAW 264.7 cells were pretreated with 0–50 μg/mL LHF for 1 h and stimulated with LPS for 24 h. NO secretion, measured as nitrite, was increased by treatment of LPS alone; however, LHF significantly reduced NO levels in LPS-stimulated cells in a dose-dependent manner ($p < 0.05$, Fig. 1a). To determine the effect of LHF on PGE_2 production in LPS-stimulated RAW 264.7 cells, PGE_2 concentrations in culture media were determined by ELISA. Increased PGE_2 secretion by LPS treatment was suppressed by LHF pretreatment (25 and 50 μg/ml) in RAW 264.7 cells (Fig. 1b). To exclude the possibility that the decreased NO and PGE_2 levels were due to cell death, we determined the effect of various LHF concentrations on cell viability. The MTS assay data demonstrated that LHF showed no cytotoxicity in RAW 264.7 cells up to 50 μg/mL (Fig. 1c). Thus, the inhibitory effects of LHF on NO and PGE_2 secretion were not due to cytotoxicity.

LHF inhibits the expression of iNOS and COX-2

Since iNOS and COX-2 are the key enzymes for the production of NO and PGE_2, respectively, we analyzed the expression of iNOS and COX-2 mRNA and proteins in LPS-stimulated RAW 264.7 cells by RT-PCR and Western blotting, respectively. As shown in Fig. 2a, LHF strongly inhibited the expression of iNOS protein in a dose-dependent manner; however, COX-2 protein expression was inhibited at 50 μg/mL LHF, which is similar to the suppression of PGE_2 secretion. In addition to iNOS protein, LHF also inhibited iNOS mRNA expression in a dose-dependent manner; however, iNOS gene expression was partially inhibited at 25 μg/mL LHF and completely inhibited at 50 μg/mL LHF (Fig. 2b). At the concentration of 25 μg/mL LHF, iNOS protein was not detected, but iNOS mRNA was partially inhibited. These results suggest that the LHF-mediated inhibition of NO and PGE_2 secretion in LPS-stimulated macrophages is associated with downregulation of iNOS and COX-2 expression at transcriptional level and that LHF-mediated inhibition of NO secretion is also contributed by the posttranscriptional or translational and posttranslational regulation of iNOS.

LHF inhibits production of pro-inflammatory cytokines

Since TNF-α, IL-1β, and IL-6 are early secreted pro-inflammatory cytokines and their elevated levels are

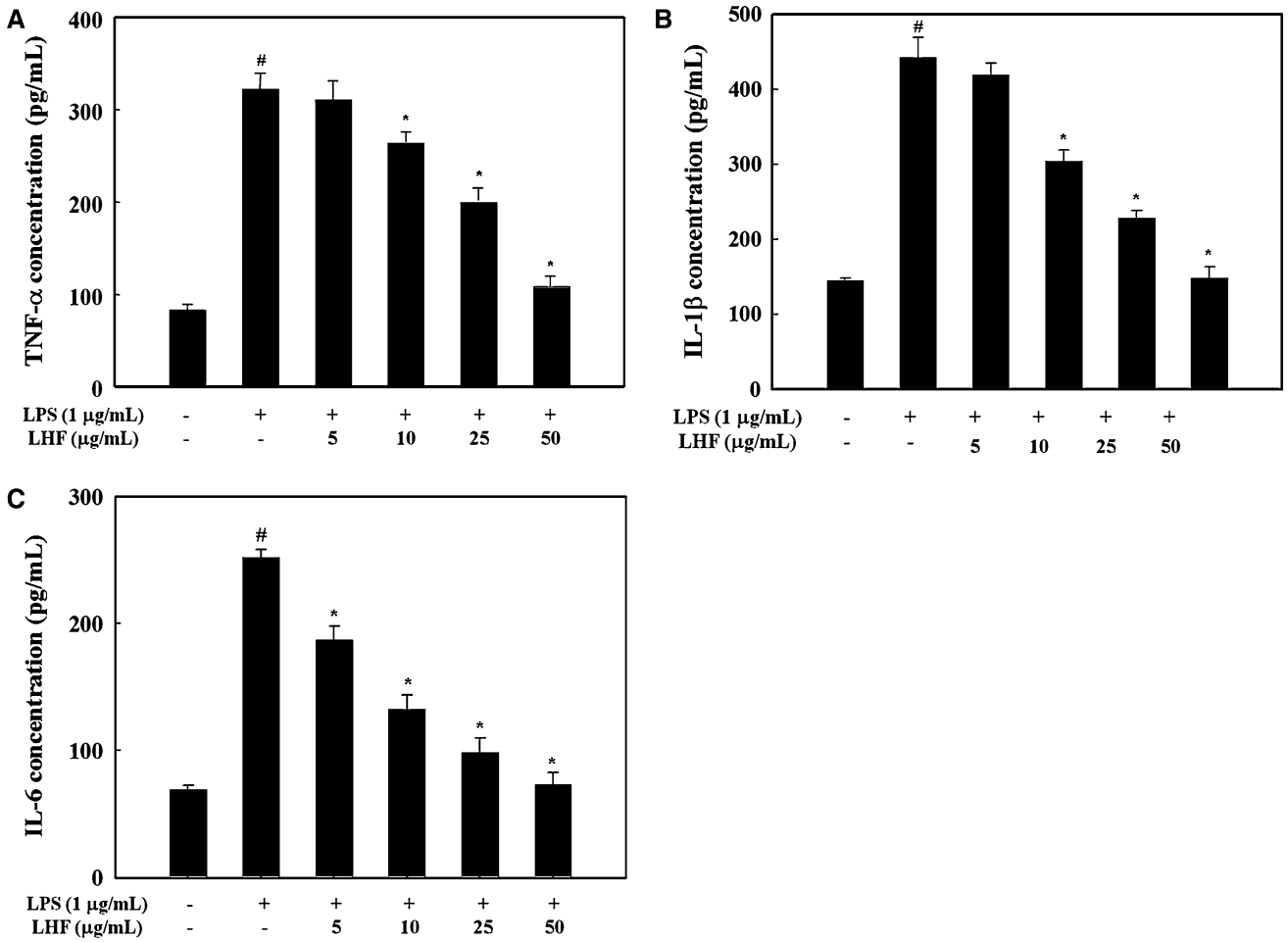

Fig. 3 Effects of LHF on pro-inflammatory cytokine secretions in LPS-stimulated RAW 264.7 cells. Cells were treated with various concentrations of LHF for 1 h and then stimulated with LPS for 24 h. TNF-α (**a**), IL-1β (**b**), and IL-6 (**c**) in the cultured media were measured by ELISA. Data are means ± SDs of three independent experiments. #$p < 0.05$ indicates significant differences compared to the control group. *$p < 0.05$ indicates significant differences compared to the LPS-only group

observed in a variety of acute and chronic inflammatory diseases, we determined the effects of LHF on the secretion of TNF-α, IL-1β, and IL-6 in LPS-stimulated cells. Stimulation of RAW 264.7 cells with LPS led to significantly increased levels of TNF-α (Fig. 3a), IL-1β (Fig. 3b), and IL-6 (Fig. 3c). However, pro-inflammatory cytokine secretion in LPS-stimulated cells was inhibited in a dose-dependent manner by the exposure to LHF ($p < 0.05$). This result indicates that LHF efficiently suppressed LPS-stimulated TNF-α, IL-1β, and IL-6 secretion, which supports the hypothesis that LHF inhibits the initial phase of the inflammatory response.

LHF inhibits NF-κB activation and translocation into the nucleus

Immunofluorescence data revealed that, in unstimulated cells, NF-κB/p65 was distributed mostly in the cytoplasm. After stimulation with LPS, most cytoplasmic NF-κB/p65 was translocated into the nucleus, as shown by strong NF-κB/p65 staining in the nucleus (Fig. 4a). The level of NF-κB/p65 in the nucleus was markedly reduced by pretreatment with LHF. To assess the molecular mechanisms underlying translocation of NF-κB from the cytosol to the nucleus in LPS-stimulated RAW 264.7 cells, we also investigated the inhibitory effect of LHF on LPS-stimulated degradation of IκB-α, which is responsible for the activation of NF-κB, by Western blotting. LPS treatment resulted in increased IκB-α degradation compared to controls, and LHF pretreatment recovered the level of cytosolic IκB-α in a dose-dependent manner (Fig. 4b). As a result of IκB-α degradation, the increased nuclear NF-κB level after LPS stimulation was reduced by LHF pretreatment in a dose-dependent manner (Fig. 4b). Considering the inhibitory effects of LHF on LPS-stimulated NF-κB activation, we next determined the effect of LHF on the

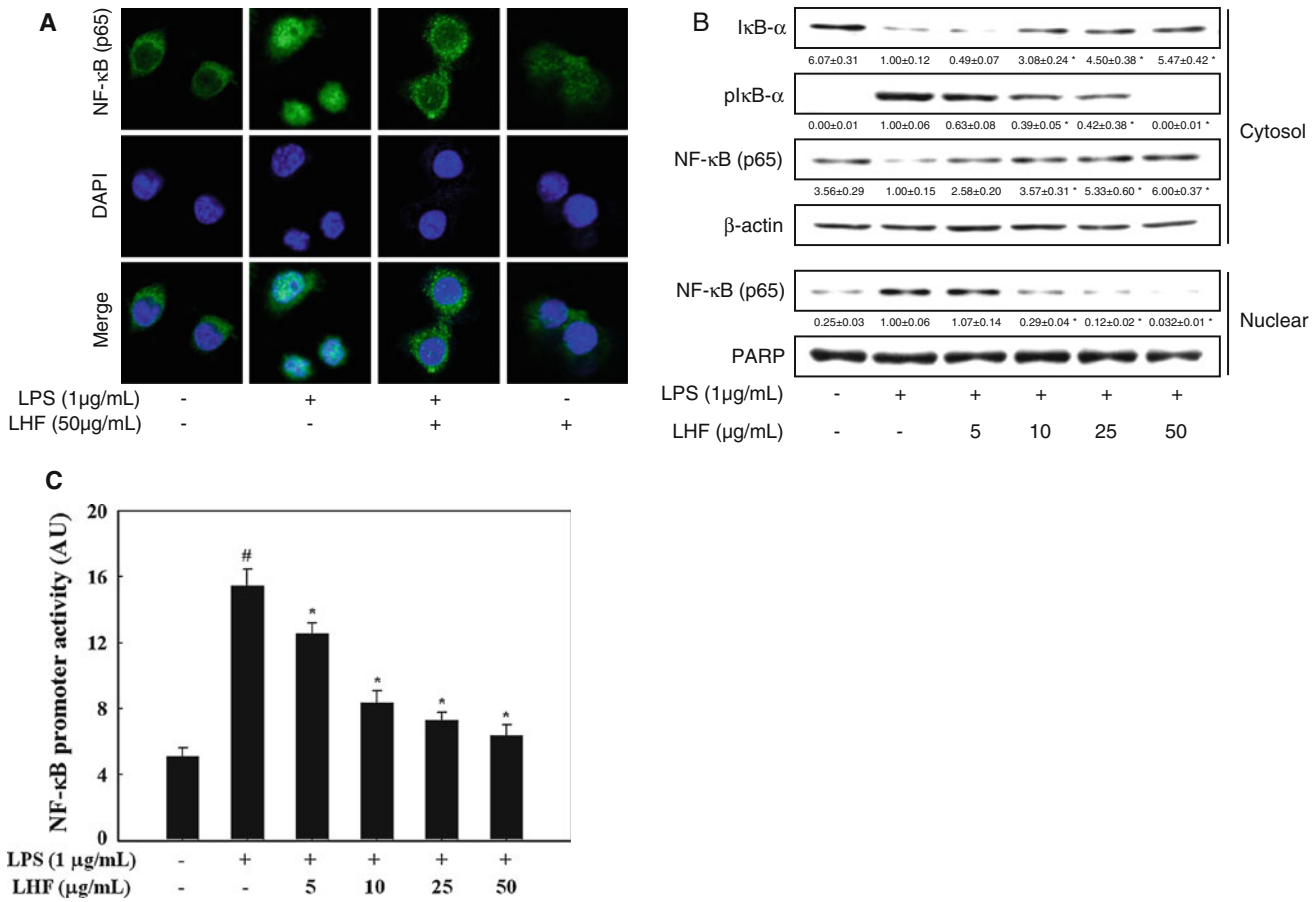

Fig. 4 Effect of LHF on the nuclear translocation and activation of NF-κB in LPS-stimulated RAW 264.7 cells. **a** Cells pretreated with LHF for 1 h were stimulated with 1 μg/mL LPS for 1 h. Cells and nucleus were stained by anti-NF-κB p65 antibody and DAPI, respectively, and then prepared for confocal microscopic analysis. The results shown (×100) are representative of those obtained from three independent experiments. **b** Cells were pretreated with indicated concentration of LHF and stimulated with LPS for 30 min. The cytosolic and nucleus extracts were separated by SDS–PAGE. The phosphorylations of IκB-α and the translocation of NF-κB were determined by Western blot analysis. Relative density ratios of proteins over β-actin and poly (ADP-ribose) polymerase (PARP) are shown below the blots (means ± SDs of three independent experiments). **c** Cells were co-transfected with 1 μg of NF-κB promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 40 h. Transfected cells were pretreated with LHF and then stimulated with LPS for 6 h. Data are means ± SDs of three independent experiments. #$p < 0.05$ indicates significant differences compared to the control group. *$p < 0.05$ indicates significant differences compared to the LPS-only group

promoter activity of NF-κB in LPS-stimulated macrophages. Data suggested that LHF pretreatment significantly inhibited LPS-induced NF-κB promoter-driven luciferase expression in macrophages ($p < 0.05$, Fig. 4c). These results indicate that the LHF-stimulated inhibition of iNOS, COX-2, and pro-inflammatory cytokine expression was regulated by the NF-κB pathway in LPS-stimulated macrophages.

LHF inhibits activation of MAPKs and Akt

To further investigate whether LHF regulates signaling proteins responsible for NF-κB activation, phosphorylation levels of MAPKs (JNK, p38, and ERK) and Akt were determined by Western blot. As shown in Fig. 5a, LHF inhibited phosphorylation of JNK, p38, ERK, and Akt induced by LPS treatment in RAW 264.7 cells. To further confirm the association of these signaling molecules with the LHF's anti-inflammatory effect, we compared NO secretion in LPS-stimulated RAW 264.7 cells in the presence of PI3K inhibitor (LY294002), ERK inhibitor (PD98059), JNK inhibitor (SP600125), or p38 inhibitor (SB203580). As shown in Fig. 5b, NO secretion was markedly suppressed by LHF as well as specific kinase inhibitors. These data suggest the additional characteristics of LHF to regulate NF-κB pathway via blocking the phosphorylation of MAPKs and Akt proteins in response to LPS.

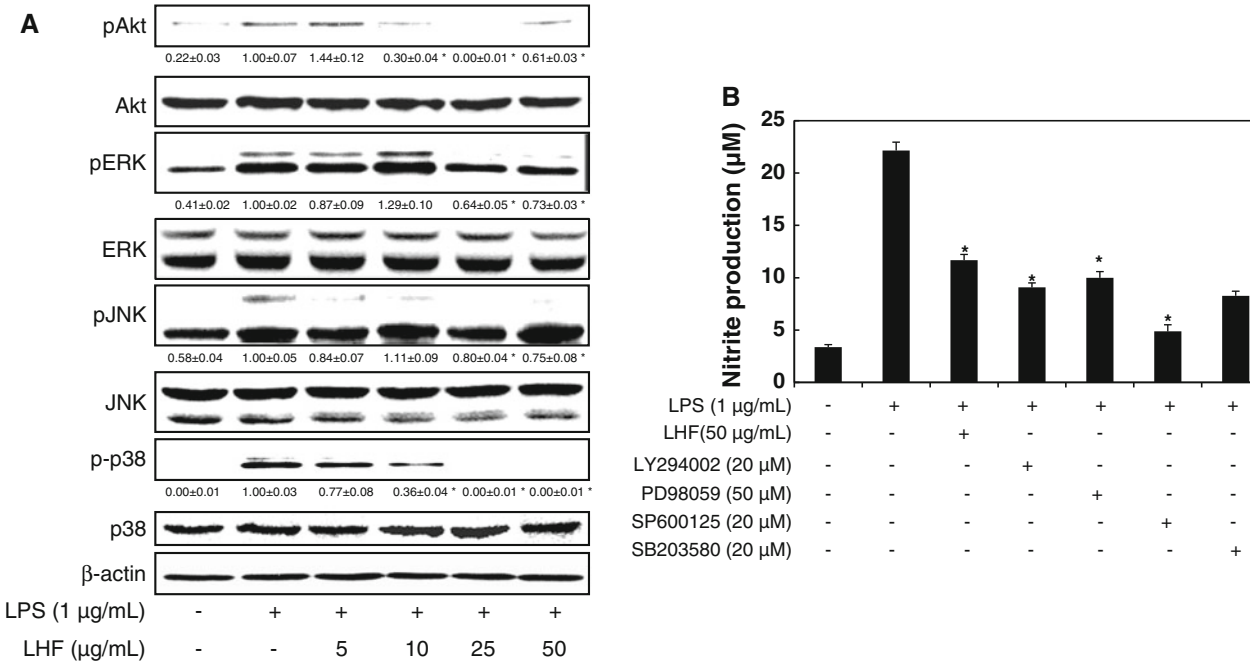

Fig. 5 Effects of LHF on phosphorylation of Akt and MAPKs in LPS-stimulated RAW 264.7 cells. Cells were incubated with various concentrations of LHF for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. **a** Whole cell lysates were prepared and analyzed by Western blot for measuring the phosphorylation (*p*) of Akt and MAPKs using corresponding antibodies. Relative density ratios of pAkt, pERK, pJNK, and pp38 over Akt, ERK, JNK, and p38, respectively, are shown below the blots (means ± SDs of three independent experiments). **b** Cells were pretreated with PI3K/Akt pathway inhibitor (LY294002), ERK inhibitor (PD98059), JNK inhibitor (SP600125), or p38 inhibitor (SB203580) for 30 min and then treated with LPS (1 μg/mL) for another 16 h. The culture media were used to measure the amount of nitrite to evaluate NO. Data are means ± SDs of three independent experiments. $*p < 0.05$ indicates significant differences compared to the LPS-only group

Fucoxanthin isolated from LHF inhibits NO and PGE_2 secretion

LHF was further separated into 11 fractions by reverse-phase column chromatography (Fig. 6a). Each fraction collected by the repeated chromatographies was dried *in vacuo*, redissolved in DMSO at 50 μg/mL concentration, and then used to assess for its anti-inflammatory activity in LPS-stimulated RAW 264.7 cells. The inhibitory activity on NO secretion was observed only in fraction 3 at concentration of 25 μg/mL (Fig. 6b). Fraction 3 was pooled by the repeated chromatographies and purified further by HPLC for NMR spectroscopy. The chemical structure of the compound in fraction 3 was identified as fucoxanthin from the comparison of its NMR spectra with the published spectral data (Table 1) [16]. We obtained 7.2 mg of fucoxanthin from 1 g of LHF. The purified fucoxanthin significantly inhibited LPS-stimulated NO and PGE_2 secretion in a dose-dependent manner ($p < 0.05$, Fig. 7a, b). Furthermore, the expression of iNOS and COX-2 protein was also markedly decreased by the fucoxanthin treatment in LPS-stimulated cells ($p < 0.05$, Fig. 7a, b). These data indicate that fucoxanthin is one of the anti-inflammatory compounds in LHF.

Discussion

We investigated the biological effects of LHF on production of inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. To further clarify the molecular mechanisms underlying the effect of LHF, we determined the effects of LHF on the secretion of NO, PGE_2, TNF-α, IL-1β, and IL-6; the expression of iNOS and COX-2; and the activation of NF-κB. Our data suggested that LHF effectively inhibited the secretion of NO, PGE_2, TNF-α, IL-1β, and IL-6 through downregulation of NF-κB activity via inactivation of MAPKs and Akt in LPS-stimulated macrophages. The inhibitory effect of LHF on the expression of inflammatory mediators suggests a mechanism responsible for its anti-inflammatory action and its potential to be a therapeutic or nutraceutical agent for inflammatory diseases.

Under pathological conditions, excess NO and PGE_2 participate in provoking inflammatory process and act synergistically with other inflammatory mediators [2, 3, 5]. Compounds able to reduce NO or PGE_2 secretion may be attractive as anti-inflammatory agents and, for this reason, the suppressive effects of natural compounds on NO or PGE_2 secretions have been intensively studied to develop

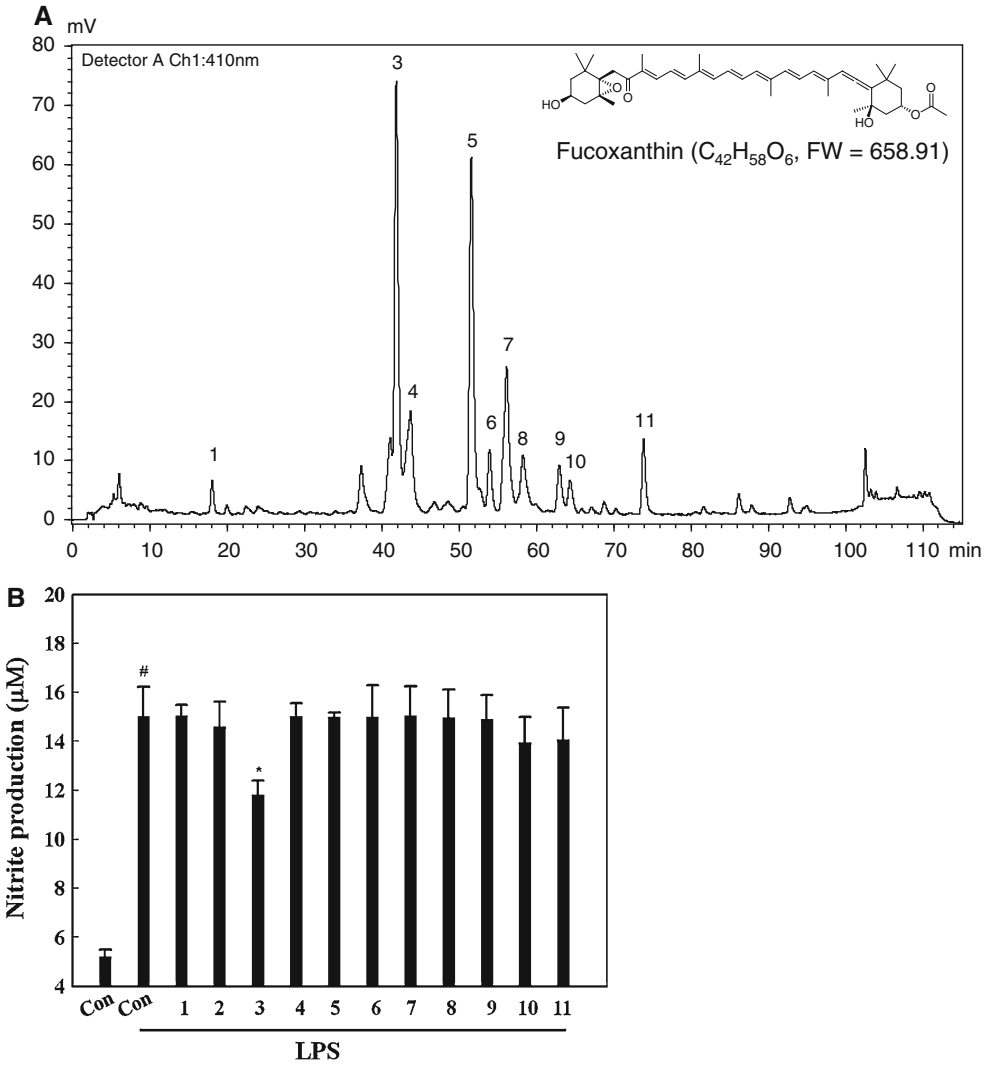

Fig. 6 Identification of fucoxanthin in LHF. **a** HPLC chromatogram of LHF and the chemical structure of fucoxanthin (insert). **b** Each fraction was repeatedly collected and dried under *vaccuo*. Quantified fractions were dissolved in DMSO, and cells pretreated with 25 μg/mL of each fraction of LHF were used to assay NO secretion

functional food for preventing inflammatory diseases [10, 20, 24, 25]. Recent studies have shown that in vivo or in vitro treatments of natural compounds are effective in reducing inflammation by the suppression of transcription factor responsible for the regulation of inflammatory proteins, which may ameliorate inflammation-related diseases, such as atherosclerosis, inflammatory bowel disease, and inflammatory arthritis [26–29]. Of interest, it has been previously reported that phlorofucofuroeckol A isolated from Laminariaceae has a strong ability to inhibit production of iNOS and COX-2 at the transcriptional level [30]. Thus, the regulation of iNOS and COX-2 is important in the inflammatory response. In this study, LHF-mediated inhibition of PGE_2 secretion in LPS-stimulated macrophages is associated with downregulation of COX-2 at the transcriptional level. However, while the expression of iNOS mRNA was progressively inhibited by LHF (Fig. 2b), that of iNOS protein was completely inhibited at lower concentration of LHF, suggesting that inhibition of NO by LHF is associated with downregulation of iNOS at translational or posttranslational level as well as transcriptional level in LPS-stimulated macrophage. These results seem to be the first study addressing the transcriptional inhibition of iNOS and COX-2 by LHF.

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, and the secreted TNF-α or LPS then induces release of IL-1β, IL-6, and IL-8 [4, 31]. TNF-α induces several physiological effects, including septic shock, inflammation, and cytotoxicity [32]. IL-1β is a major pro-inflammatory cytokine, which is produced mainly by macrophages and believed to play a significant role in the pathophysiology of endometriosis [33]. Moreover, IL-1β is important for the initiation and enhancement of the inflammatory response to microbial infection [4]. IL-6 is also pivotal pro-inflammatory cytokine synthesized mainly by macrophages; it plays a role in the acute-phase

Table 1 ^{13}C-NMR and ^{1}H-NMR spectral data of fucoxanthin isolated from LHF

Position	^{13}C (ppm)	^{1}H (ppm) (multiplicity, J in Hz)	Position	^{13}C (ppm)	^{1}H (ppm) (multiplicity, J in Hz)
1	35.33		1′	35.97	
2	45.65	1.35 (1H, *m*), 1.48 (1H, *d*, 12.11)	2′	45.61	1.4 (1H, *d*, 11.94), 1.97 (1H, *m*)
3	64.57	3.8 (1H, *d*, 10.00)	3′	68.19	5.35 (1H, *d*, 4.43)
4	41.82	1.76 (1H, *d*, 17.19), 2.3 (1H, *d*, 17.19)	4′	45.41	1.48 (1H, *m*), 2.26 (1H, *d*, 15.22)
5	66.33		5′	72.88	
6	67.25		6′	117.68	
7	40.98	2.58 (1H, *d*, 10.60), 3.6 (1H, *d*, 18.23)	7′	202.22	
8	198.29		8′	103.57	6.03 (1H, *s*)
9	134.7		9′	132.69	
10	139.32	7.13 (1H, *d*, 10.60)	10′	128.72	6.11 (1H, *d*, 11.24)
11	123.55	6.54 (1H, *d*, 13.14)	11′	125.89	6.58 (1H, *m*)
12	145.24	6.65 (1H, *d*, 14.91)	12′	137.29	6.33 (1H, *d*, 11.05)
13	135.62		13′	138.3	
14	136.83	6.39 (1H, *d*, 11.56)	14′	132.38	6.25 (1H, *d*, 11.98)
15	129.61	6.61 (3H, *s*)	15′	132.71	6.73 (1H, *m*)
16	25.21	1.01 (3H, *s*)	16′	31.49	1.05 (3H, *s*)
17	28.32	0.94 (3H, *s*)	17′	31.49	1.33 (3H, *s*)
18	20.76	1.2 (3H, *s*)	18′	29.39	1.36 (3H, *s*)
19	12.06	1.92 (3H, *s*)	19′	14.2	1.79 (3H, *s*)
20	12.99	1.97 (3H, *s*)	20′	13.12	1.97 (3H, *s*)
			3′OAc, CH_3	170.87	
			3′OAc, C=O	21.35	2.02 (3H, *s*)

Purified compound was dissolved in CD_3OD and analyzed using Fourier transform nuclear magnetic resonance spectrophotometer

immune response [32] and is regarded as an endogenous mediator of LPS-induced fever. Our data indicate that LHF significantly suppressed LPS-stimulated TNF-α, IL-1β, and IL-6 secretion, supporting the hypothesis that LHF inhibits the initial phase of LPS-stimulated inflammatory response.

NF-κB plays a pivotal role in the regulation of cell survival genes and coordinates the expression of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 [1, 4, 34]. NF-κB is associated with an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. Activation of NF-κB stimulated by LPS or pro-inflammatory cytokines leads to the phosphorylation of IκB-α kinase (IKK), which then phosphorylates IκB-α on serine residues 32 and 36, leading to subsequent degradation of IκB-α and inducing translocation of NF-κB into the nucleus [35]. In this study, we observed that downregulation of IκB-α by LPS was recovered by LHF treatment, suggesting that LHF protected the proteolytic degradation of IκB-α. Degradation of IκB-α involves its dissociation from the inactive complex, leading to activation of NF-κB in response to LPS, which is demonstrated by NF-κB promoter activity (Fig. 4c). Moreover, the immunofluorescence experiment revealed that nuclear translocation of NF-κB was significantly inhibited by LHF, supporting the inhibition of IκB-α degradation by LHF. From these data, the LHF-mediated downregulation of LPS-stimulated iNOS, COX-2, TNF-α, IL-1β, and IL-6 expression in RAW 264.7 cells is most likely largely associated with the ability of LHF to inhibit the NF-κB pathway. This is, to our knowledge, the first report addressing the negative regulation by LHF of the NF-κB pathway in response to LPS.

NF-κB activation is alternatively regulated by various cellular kinases including MAPKs and Akt, which are the groups of signaling molecules to play key roles in inflammatory reactions [30, 34]. MAPKs are involved in pro-inflammatory signaling cascades and regulation of iNOS and COX-2 through the activation of NF-κB in LPS-stimulated immune cells [27, 30, 34]. Therefore, anti-inflammatory mechanisms are closely related to inhibition of MAPKs in stimulated RAW 264.7 cells. In this study, we found that phosphorylation of MAPKs in response to LPS was inhibited by LHF pretreatment (Fig. 5a). Akt is also reported to act on upstream of IKK/NF-κB activation in LPS-stimulated cells [30, 36]. Results of this study showed that LHF significantly inhibited the phosphorylation of Akt (Fig. 5a), and these inhibitory effects lead to the inhibition of IκB-α degradation and NF-κB translocation in

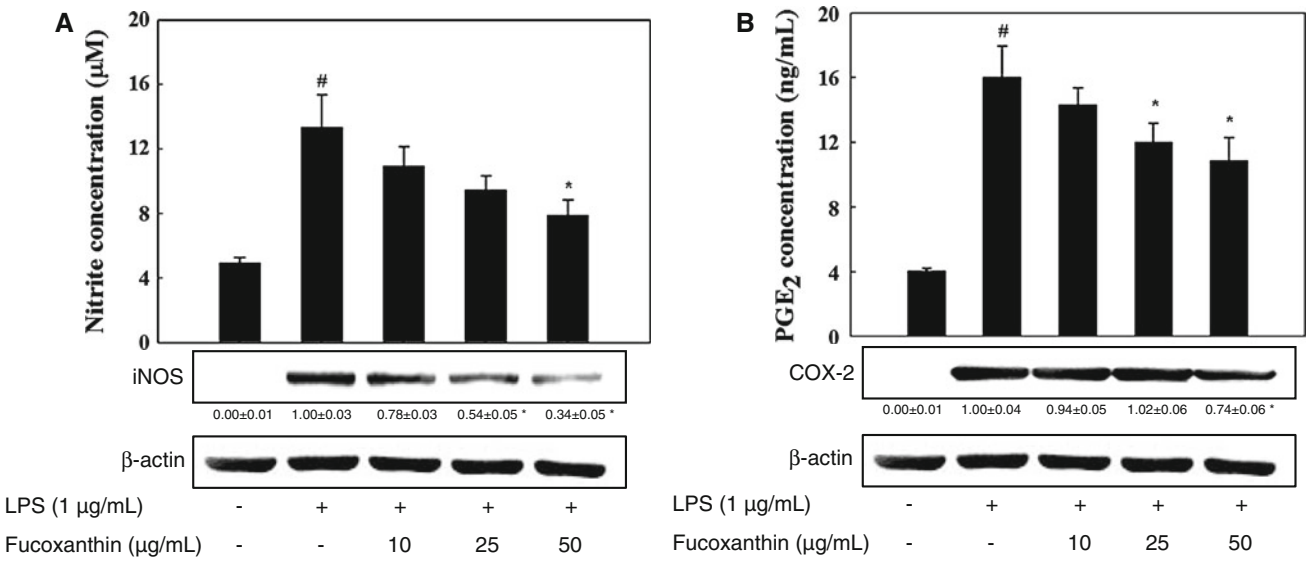

Fig. 7 Effect of fucoxanthin on LPS-stimulated NO and PGE_2 secretion in RAW 264.7 cells. Cells pretreated with different concentrations of fucoxanthin for 1 h were stimulated with LPS for 24 h. The treated culture media were used to measure the amount of NO (**a**) and PGE_2 (**b**) secretion. The expression levels of iNOS and COX-2 in cells were analyzed by Western blot using corresponding antibodies. Data are means ± SDs of three independent experiments. Relative density ratios of each protein over β-actin are shown below the blots. $^{\#}p < 0.05$ indicates significant differences compared to the control group. $^{*}p < 0.05$ indicates significant differences compared to the LPS-only group

LPS-stimulated RAW 264.7 cells (Fig. 4b), supporting that Akt signaling is, at least in part, involved in LHF-mediated anti-inflammatory activity of LHF. Thus, it is likely that inhibition of MAPKs and Akt phosphorylation by LHF may contribute to the LHF-mediated inhibition of iNOS and COX-2 expressions through the downregulation of NF-κB pathway in response to LPS.

Fucoxanthin is a major carotenoid present in the chloroplasts of brown macroalgae and is mostly isolated from *Sargassum* sp. [21, 22]. Recently, fucoxanthin isolated from *Sargassum siliquastrum* can effectively inhibit intracellular reactive oxygen species (ROS) formation induced by hydrogen peroxide treatment [16]. The ability of fucoxanthin isolated from *Myagropsis myagroides* and *Ishige okamurae* to reduce ROS formation may be involved in the inhibition of iNOS, COX-2, and pro-inflammatory cytokines expression and thus reduces inflammatory response [20, 21]. In this study, we isolated an anti-inflammatory compound from LHF using HPLC, and the compound was identified as fucoxanthin by NMR spectroscopy (Table 1). This is, to our knowledge, the first report addressing the isolation and identification of fucoxanthin from *L. japonica*. As shown earlier, fucoxanthin comprises less than 1 % of the LHF. Interestingly, fucoxanthin isolated from LHF showed much less suppressive activity in NO and PGE_2 secretion than LHF itself (Figs. 1 and 7). That is, in Fig. 1, a 50 μg/mL LHF (equivalent to less than 0.5 μg/mL fucoxanthin) reduced the nitrite concentration to near 99 % and the PGE_2 concentration to about 70 %, whereas in Fig. 7, a concentration of 50 μg/mL of fucoxanthin resulted in only 65 % inhibition of nitrite concentration and 50 % for PGE_2. These results suggest that very potent unidentified anti-inflammatory compound(s) may present in LHF or that fucoxanthin and other compounds may exert synergistic effect on the inhibition of inflammatory mediator production in LPS-stimulated RAW 264.7 cells.

In conclusion, we demonstrated that LHF inhibited the secretion of inflammatory mediators, such as NO and PGE_2, and pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in LPS-stimulated RAW 264.7 macrophages. Moreover, the inhibitory effect of LHF was associated with inactivation of the NF-κB pathway via blocking the phosphorylation of IκB, Akt, and MAPKs. We identified fucoxanthin, which effectively inhibits NO and PEG_2 production, from LHF. The anti-inflammatory activity of LHF and the mechanism underlying its effects will contribute to the further application of LHF in functional foods against inflammatory diseases. Future studies will be necessary in order to determine the bioavailability of this preparation and metabolites in the blood.

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Conflict of interest The authors declare that we have no conflict of interest.

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