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Anti-inflammatory effect of fucoxanthin derivatives isolated from *Sargassum siliquastrum* in lipopolysaccharide-stimulated RAW 264.7 macrophage

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ABSTRACT

In this study, the anti-inflammatory effect of fucoxanthin (FX) derivatives, which was isolated from *Sargassum siliquastrum* were evaluated by examining their inhibitory effects on pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. The FX derivatives were isolated from activity-guided chloroform fraction using inhibition of nitric oxide (NO) production and identified as 9′-*cis*-(6′*R*) fucoxnathin (FXA), and 13-*cis* and 13′-*cis*-(6′*R*) fucoxanthin complex (FXB) on the basis of a comparison of NMR spectroscopic data. Both FXA and FXB significantly inhibited the NO production and showed slightly reduce the PGE2 production. However, FXB exhibited cytotoxicity at the whole tested concentration, therefore, the results of FXA was only illustrate for further experiments. FXA induced dose-dependent reduction in the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) proteins as well as mRNA expression. In addition, FXA reduced the LPS-stimulated production and mRNA expressions of TNF-α and IL-6 in a dose-dependent manner whereas IL-1β production do not inhibit by addition of FXA. Taken together, these findings indicate that the anti-inflammatory properties of FXA may be due to the inhibition of iNOS/NO pathway which associated with the attenuation of TNF-α and IL-6 formation. Thus FXA may provide a potential therapeutic approach for inflammation related diseases.

1. Introduction

Inflammation is a physiological response of a body to stimuli, including infections and tissue injury, and protects a body from these inflammatory stimuli (Dung et al., 2009). Macrophage plays critical roles in immune reaction, allergy, and inflammation. These cells induce inflammatory reaction, and initiate and maintain specific immune responses by releasing different types of cytokines (Lee et al., 2011; Poltorak et al., 1998). Macrophage activation by lipopolysaccharides (LPS), which are derived from gram-negative bacteria cell walls, results in the release of several inflammatory mediators including nitric oxide (NO), cyclooxygenase (COX)-2, interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α (Kanno et al., 2006). Over-expression of the inflammatory mediators in macrophage is involved in many inflammation related diseases, such as atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease, and autoimmune diabetes (Coker and Laurent, 1998; Kern, 2007; Schroder et al., 2006). Thus, inhibition of inflammatory mediators produced by macrophages is believed to be crucial for managing inflammatory diseases. Many investigators have, therefore, focused either on identifying anti-inflammatory agent from natural resources or on developing synthetic anti-inflammatory compounds (Kazłowska et al., 2010; Michelini et al., 2008; Mueller et al., 2010; Paulino et al., 2009; Prawan et al., 2009; Van et al., 2009).

Carotenoids are natural pigments containing more than 600 members, which synthesized by many microorganisms and plants, so animals have to obtain them from food resources (Cardozo et al., 2007; Quirós and Costa, 2006). The carotenoids have recently attracted popular interest not only as a source of pigmentation but also for their beneficial effects on human health by functioning as antioxidant, which include a possible role in cancer preventation and enhancing immune responses (Kim et al., 2008). Among the carotenoids, fucoxanthin (FX) is one of the major carotenoid in brown algae, which has an unique structure featuring including an unusual allenic bond, conjugated carbonyl, epoxide, and acetyl group within its molecule. Many of the biological functions of FX have been previously characterized, including antioxidant, anti-obesity, antitumor, and UV-preventative activities (Heo and Jeon, 2009; Kim et al., 2010a; Maeda et al., 2005; Yan et al., 1999). More recently, in a previous study we isolated FX from brown algae and

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evaluated its potential anti-inflammatory activity (Heo et al., 2010; Kim et al., 2010b). However, the anti-inflammatory effects of FX derivatives have not yet been reported. Accordingly, the present study isolated the FX derivatives from *Sargassum siliquastrum* and their anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells were investigated.

2. Materials and methods

2.1. Materials

The brown alga, *Sargassum siliquastrum*, was collected along the coast of Jeju Island, Korea, between October 2009 and March 2010. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at −20 °C. Then, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

2.2. Extraction and isolation

The powdered *S. siliquastrum* was extracted three times with 80% aqueous methanol, and was evaporated under vacuum at 40 °C. The methanol extract was dissolved in distilled water and partitioned with hexane, chloroform ethyl acetate, and butanol. Since the chloroform fraction exhibited higher nitric oxide (NO) production inhibitory effects than that of other fractions, the chloroform fraction was fractionated by silica column chromatography with stepwise elution of chloroform–methanol mixture (100:1 → 1:1) to separate active fractions in chloroform fraction. A combined active fraction was further subjected to a Sephadex LH-20 column saturated with 100% methanol, and then purified by reversed-phase high performance liquid chromatography (HPLC) using a Waters HPLC system (Alliance 2690, NY, USA) equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 × 20 mm, 4 μm, YMC Co., Kyoto, Japan) by stepwise elution with methanol–water gradient (UV range: 440 nm, flow rate: 0.8 ml/min). Finally, the purified compounds were identified by comparing their ^{1}H and ^{13}C NMR data with literature (Haugan and Liaaen-Jensen, 1994; Heo et al., 2010). The purity of compounds were >97%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis. The compounds were dissolved in dimethylsulfoxide (DMSO) and employed in experiments in which the final concentration of DMSO in culture medium was adjusted to <0.01%.

2.3. Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Inc., NY, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (FBS; GIBCO Inc., NY, USA). The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and were sub-cultured every 3 days.

2.4. Determination of NO production

After pre-incubation of RAW 264.7 cells (1.5×10^5 cells/ml) with LPS (1 μg/ml) plus samples at 37 °C for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production (Lee et al., 2007). Briefly, a 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader (ThermoMax, CA, USA). Fresh culture medium was used as a blank in every experiment.

2.5. Lactic dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1.5×10^5 cells/ml) plated in 96 well plates were pre-incubated and then treated with LPS (1 μg/ml) plus samples at 37 °C for 24 h. The medium was carefully removed from each well, and the LDH activity in the medium was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, a 100 μl of reaction mixture was added to each well, and the reaction was incubated for 30 min at room temperature in the dark. The absorbance of each well was measured at 490 nm using a microplate reader.

2.6. Determination of prostaglandin E2 (PGE_2) production

Samples were diluted with DMEM before treatment. Cells were treated with LPS (1 μg/ml) to allow cytokine production for 24 h. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The production of PGE_2 was measured relative to that of control value.

2.7. Measurement of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) production

Samples solubilized with DMSO were diluted with DMEM before treatment. The inhibitory effect of samples on the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) production from LPS (1 μg/ml) treated RAW 264.7 cells was determined as described by Cho et al. (2000). Supernatants were used for pro-inflammatory cytokines assay using mouse ELISA kit (R & D Systems Inc., MN, USA).

2.8. RNA Isolation and RT-PCR analysis

Total RNA from LPS (1 μg/ml)-treated RAW 264.7 cells was prepared with Tri-Reagent (MRC, Cincinnati, OH, USA), according to the manufacturers protocol. RNA was stored at −70 °C until used. The reverse transcription of 1 μg RNA was carried out with M-MuLV reverse transcriptase (Promega, WI, USA), oligo dT-18 primer, deoxyribonucleotide triphosphates (dNTP, 0.5 μM) and 1 U RNase inhibitor. After this reaction cocktail was incubated at 70 °C for 5 min, 25 °C for 5 min, and 37 °C for 60 min in series, M-MuLV reverse transcriptase was inactivated by heating at 70 °C for 10 min. Polymerase chain reaction (PCR) was performed in reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3′- and 5′-primer (50 μM each) and 200 mM dNTP in 200 mM Tris–HCl buffer (pH 8.4) containing 500 mM KCl and 1–4 mM $MgCl_2$]. The PCR was performed in a DNA gene cycler (Bio-Rad, HC, USA) with amplification by 30 cycles of 94 °C for 45 s (denaturing), 60–65 °C for 45 s (annealing) and 72 °C for 1 min (primer extension). The PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

2.9. Immunoblotting

RAW 264.7 cells (1.0×10^6 cells/ml) were treated with LPS (1 μg/ml) plus samples for 24 h, and cellular proteins were extracted from the cells. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin (BSA) as a standard. Cell lysates (30–50 μg) were electrophoresed in SDS–polyacrylamide gels (8–12%), and the separated proteins were transferred to PVDF membranes (Bio-Rad) for 2 h. The membranes were pre- incubated with blocking solution (5% skim milk in Tris buffered saline containing Tween-20) at room temperature for 2 h and then incubated with anti-mouse iNOS (1:1,000; Calbiochem, La Jolla, CA, USA) and anti-mouse COX-2 (1:1,000; BD Biosciences Pharmingen, San Jose, CA, USA) for 2 h at room temperature. After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (1:5,000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 30 min. The bands were visualized on X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.10. Statistical Analysis

All the measurements were made in triplicate and all values were represented as means ± standard error. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Inhibitory effect of NO production of S. siliquastrum extracts and cytotoxicity

In an effort to express the potential anti-inflammatory effect of *S. siliquastrum* in LPS-induced RAW 264.7 cells, we investigated inhibitory effect of 80% methanol extracts of *S. siliquastrum* on NO production as well as its partitioned fraction with hexane, chloroform, ethyl acetate, and butanol, to detect bioactive compounds (Fig. 1). Among those tested samples, chloroform fraction showed the highest level of inhibitory effect (87.5%) on NO production than that of other tested samples. Hexane fraction also showed higher inhibitory effect (66.3%) on NO production, whereas the other samples evidenced less than 25% inhibitory activities. The cytotoxic effects of *S. siliquastrum* were assessed in the presence or absence of LPS via an LDH assay. As shown in the line graph of Fig. 1, only hexane fraction affect cell viability around 15.3%, however, other tested samples did not influence the cytotoxicity of RAW 264.7 cells. Thus, the chloroform fraction was selected for additional experiments, owing to its higher inhibitory effect of NO production and cell viability.