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Full Length Article

Anti-inflammatory Effect of *Petalonia binghamiae* in LPS-Induced Macrophages is Mediated by Suppression of iNOS and COX-2

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ABSTRACT

A number of seaweed species are used as traditional medicine in different parts of the world. However, very few data on the anti-inflammatory effect of seaweed have been published. *Petalonia binghamiae* (Miyeoksoi), a brown alga, is a traditional food in the southern regions of the Korea peninsula. In this study, the anti-inflammatory effects of the ethyl acetate extracts of *P. binghamiae* were evaluated on lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Our results supports that *P. binghamiae* potently inhibited LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production, exhibiting IC_{50} values of 38.8 and 9.3 μg/mL. Consistent with these findings, *P. binghamiae* reduced the LPS-induced expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein level in a concentration-dependent manner, as determined by Western blotting. In addition, the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) released into the medium were reduced by *P. binghamiae* in a dose-dependent manner, exhibiting IC_{50} values of 19.4 μg/mL for IL-6. Based on these results, the anti-inflammatory effects of *P. binghamiae* extracts, together with their low cell toxicity, suggest potential therapeutic applications in the regulation of the inflammatory response.

Key Words: COX-2; Inflammation; iNOS; *Petalonia binghamiae*

INTRODUCTION

For centuries, people in coastal regions of Far East countries such as Korea and Japan, have known of and collected edible algae from seashores. It has been reported that 988 different species of algae can be found in the coast of the Korea Peninsula facing the middle of the East Sea and Jeju Island. Approximately 40 of these algae species are edible. However, most edible algae are only available for harvesting and eating during specific seasons. Recently, various types of marine algae, including inedible seaweed, have gained consideration as resources for bio-active compounds, bio-ethanol production and the extruded pellet of aquacultured fish. Some are reported to protect against gastrointestinal injury, peptic ulcers and liver injury, as well as possess anti-inflamatory, antioxidant and anticoagulant activities (Hwang *et al*., 2008; Choi *et al*., 2009 & 2010)

Petalonia binghamiae (J. Agardh), called "Miyeoksoi" in Korea, is a brown alga and widely distributed along the coasts of Korea, Japan, and western USA (Kuda *et al*., 2006; Kang *et al*., 2008; Pedroche *et al*., 2008). The shape of *P. binghamiae* is an aggregate of several leaves that are 20-30 mm in width and approximately 250 mm in length. Although *P. binghamiae* grows well along many coasts of Korea and other countries, it is consumed as an edible alga and traditional food only in fishing towns. Usually, the alga is eaten after drying and addition to a Korean Rockfish (*Sebastes schlegeli*) soup. There are reports that *P. binghamiae* extracts possess anti-diabetic (Kang *et al*., 2008), anti-allergic (Kimiya *et al*., 2008) and antioxidant (Kuda *et al*., 2006) activities. However, the anti-inflammatory effect of *P. binghamiae* extract has not been reported until now. Therefore, we conducted a detailed study to investigate the anti-inflammatory effects of *P. binghamiae* extract in RAW 264.7 cells.

Seaweed-derived anti-inflammatory compounds have been investigated for their potential inhibitory effects *in vitro* using lipopolysaccharide (LPS)-stimulated macrophages. In this system, bacterial LPS is one of the best-characterized stimuli used to induce upregulation of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).

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Inducible COX-2 is responsible for the high prostaglandin levels observed in much inflammatory pathology. Similarly, iNOS produces large amounts of nitric oxide (NO) and is thought to play a central role in inflammatory disease. Therefore, the present study focused on whether the ethyl acetate fraction from *P. binghamiae* inhibited NO and PGE_2 production and iNOS and COX-2 expression in LPS-stimulated macrophages.

MATERIALS AND METHODS

Plant material: *P. binghamiae* thalli were collected in March 2006 at Jeju Island, Korea and then identified by Wook Jae Lee. The voucher specimen with the number JBR-163, is deposited at the herbarium of Jeju Biodiversity Research Institute. The plant materials were cleaned, dried at room temperature for 2 weeks and ground into a suitable powder for extraction. The powdered alga (30 g) was extracted by using 80% ethanol (EtOH; 2 L) at room temperature for 24 h and the extract concentrated in a rotary evaporator and then evaporated to dryness under a vacuum. The dried EtOH extract (8 g) was suspended in water (1 L) and partitioned with ethyl acetate (EtOAc; 1 L) and the EtOAc fraction separated and collected. The partitioning was repeated three more times. Yields and ratios of these four solvent partitions are represented in parentheses for EtOAc (0.2734 g, 3.4%)

Cell culture: Murine RAW 264.7 (1×10^6 cells per well) macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin, all from GIBCO (Grand Island, NY, USA), incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell viability: Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells (1×10^5 cells mL^{-1}) were cultured in 96-well plates for 18 h, followed by treatment with various concentrations (12.5, 25, 50 µg mL^{-1}) of PBE. After 24 h of incubation, MTT was added to the medium for 4 h. Finally, the supernatant was removed and the formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm. Percent of cell viability/toxicity was determined relative to the control group.

Determination of NO concentration: Nitric oxide (NO) production was assayed by measuring the nitrite in the supernatants of cultured RAW 264.7 cells as described previously (Yang *et al*., 2009). RAW 264.7 cells (2.5×10^5 cells mL^{-1}) were incubated on 24-well dishes for 18 h with 1 µg mL^{-1} of LPS and a variety of concentrations of PBE. The presence of nitrite was determined as a stable oxidized product of NO in cell culture media by modifying the Griess method. Briefly, the culture supernatant (100 µL) was mixed with the same volume of Griess reagent (1% sulfanilamide & 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) for 10 min and absorbance was measured at 540 nm.

Western blot analysis: Murine macrophage RAW 264.7 cells (5×10^5 cells mL^{-1}) were seeded in DMEM medium in a 60-mm dish for 18 h, followed by treatment with LPS in the presence of various concentrations (12.5, 25, 50 µg mL^{-1}) of the PBE. After 24 h of incubation, the cells were washed twice with cold PBS. Whole cell lysates (30 µg for both iNOS & COX-2) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA). The membrane was incubated for 2 h with TTBS containing 1% bovine serum albumin (BSA) and then incubated with a specific primary antibody (iNOS & COX-2) at 4°C overnight. The membrane was washed 4 times with TTBS and incubated for 30 min with a peroxidase-conjugated secondary antibody at room temperature. Finally, the membrane was detected using the WEST-ZOL Western Blot Detection System (iNtRON, Gyeonggi, Korea).

Measurement of PGE_2: The RAW 264.7 cells (2.5×10^5 cells mL^{-1}) were cultured in 24-well plates for 18 h, followed by treatment with LPS in the presence of various concentrations (12.5, 25 50 µg mL^{-1}) of sample. After 24 h of incubation, PGE_2 in the culture supernatants was measured using an ELISA kit.

Cytokine assays: The amount of TNF-α and IL-6 in the cell culture supernatant and in serum was measured using an ELISA kit (R & D, Minneapolis, MI) as described previously (Yoon *et al*., 2009). RAW 264.7 cells were plated in a 12-well cell culture plate at a density of 2.5×10^5 cells/well in the presence of various concentrations of PBE (12.5, 25, 50 µg mL^{-1}) in 1 µg mL^{-1} LPS and incubated for 24 h. The culture supernatant was collected and assayed, according to the manufacturer's instruction, to determine the amount of TNF-α and IL-6 that had been released from the cells.

Statistical analysis: Each experiment was repeated at least three times and the results were expressed as mean±S.E. Statistical significances were compared between each treated group and analyzed by the Student's *t*-test. Data with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

A variety of seaweeds that have existed from antiquity to the present have played significant roles in drug discovery and development, especially anti-inflammatory agents against several diseases. Inflammation is a complex process regulated by a cascade of cytokines, growth factors, NO and prostaglandins produced by activated macrophages. During inflammation, macrophages play a central role in managing many different immunopathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as IL-1β, IL-6, NO, iNOS, COX-2 and TNF-α (Feldmann *et al*., 1991; Feldmann, 2008; Kim & Moudgil, 2008). Indeed, a number of inflammatory stimuli, such as LPS and pro-inflammatory

Fig. 1: Effect of PBE on nitric oxide production in LPS-stimulated RAW264.7 cells. The cells were stimulated with 1 μg mL^{-1} of LPS only or with LPS plus various concentrations (12.5, 25, 50 μg mL^{-1}) of PBE for 24 h. Nitric oxide production was determined by the Griess reagent method. Cell viability was determined from the 24-h culture of cells stimulated with LPS (1 μg mL^{-1}) in the presence of PBE. The data represent the mean ± SD of n=4. **P<0.05, **P<0.01* versus LPS alone. The PBE fraction was prepared as described in the Materials and methods section

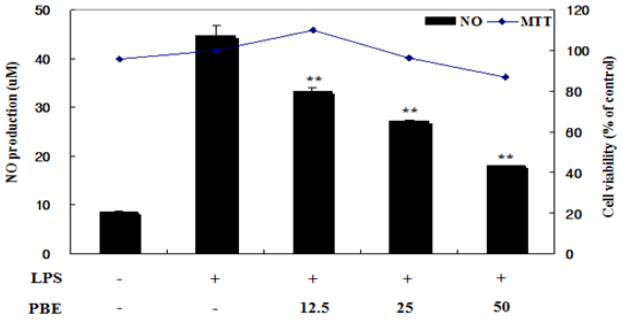
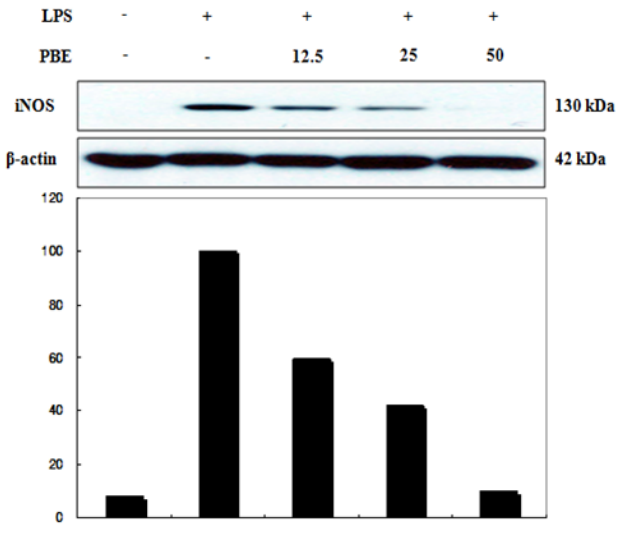
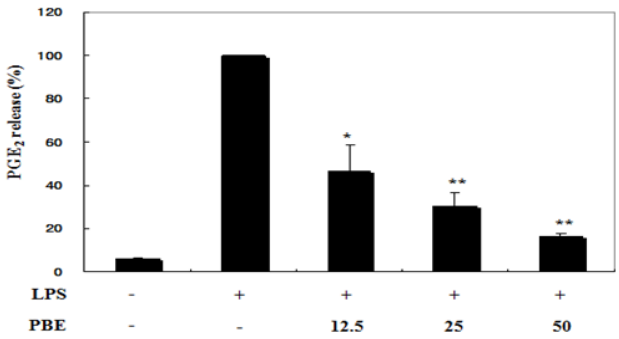


Fig. 2: Effect of PBE on the activation of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (5.0×10^5 cells per mL) were stimulated with LPS (1 μg mL^{-1}) in PBE (12.5, 25, 50 μg mL^{-1}) for 24 h. Whole-cell lysates (25 μg) were prepared and the protein lysates were subjected to 10% SDS-PAGE and expression of iNOS and β-actin were determined by Western blotting. The figure is representative of three similar experiments. The PBE fraction was prepared as described in the Materials and methods section.



cytokines, activate immune cells to up-regulate such inflammatory states. Therefore, these stimuli are useful targets in the development of new anti-inflammatory drugs and for the exploration of the molecular anti-inflammatory mechanisms of a potential drug (Zeilhofer & Brune, 2006; Jachak, 2007; Murakami & Ohigashi, 2007).

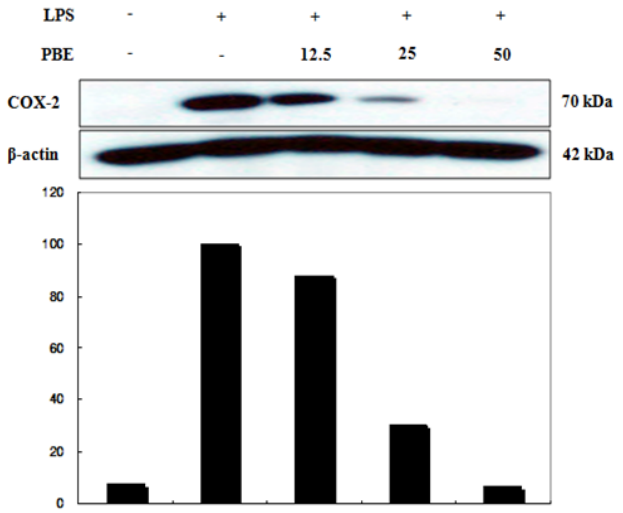
Fig. 3: Effect of PBE on PGE_2 production in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg mL^{-1} of LPS only or with LPS plus various concentrations (12.5, 25, 50 μg mL^{-1}) of PBE for 24 h. PGE_2 produced and released into the culture medium was assayed by ELISA method. The data represent the mean ± SD of n=4. **P<0.05, **P<0.01* versus LPS alone. The PBE fraction was prepared as described in the Materials and methods section



In this study, we prepared EtOAc extract from *P. binghamiae* (PBE) and examined its effects on LPS-induced inflammation of the RAW 264.7 murine macrophage cell line. First, we evaluated the cytotoxicity of PBE in RAW 264.7 cells by the MTT assay and it was observed that PBE did not affect cell viability below 50 μg mL^{-1}. The massive amounts of NO produced in response to bacterial LPS or cytokines play an important role during inflammation. To investigate the effect of PBE on NO production, we measured the accumulation of nitrite, a stable oxidized product of NO, in culture media. NO production was examined in RAW 264.7 cells stimulated with LPS in the presence or absence of PBE for 24 h. LPS (1 μg mL^{-1})-stimulated cells had significantly increased nitrite levels compared with the control. The NO production was inhibited by PBE treatment in a concentration-dependent manner (Fig. 1). PBE caused 59.9 % inhibition of NO production at a concentration of 50 μg mL^{-1}. Expression of iNOS protein was also barely detectable in the control but markedly increased 24 h after LPS (1 μg mL^{-1}) treatment (Fig. 2). Consistent with previous results, treatment with PBE resulted in a concentration dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells.

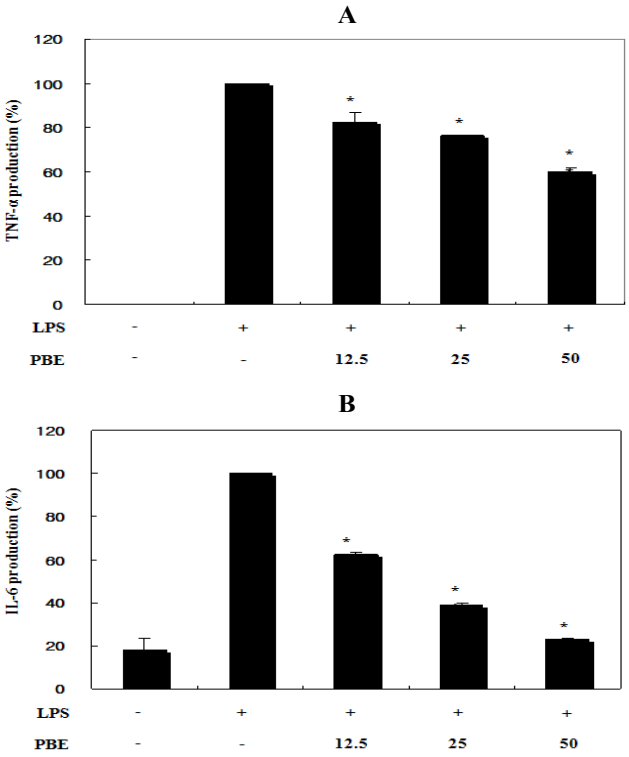
The production of PGE_2, TNF-α and IL-1β is a crucial part of the immune response to some inflammatory stimuli. Therefore, we examined the effects of PBE on PGE_2 production and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. The expression of PGE_2 production was measured in the medium of RAW 264.7 cells cultured with LPS (1 μg mL^{-1}) in the presence or absence of PBE. As shown in Fig. 3, PBE suppressed LPS-induced PGE_2 production in a concentration-dependent manner, exhibiting IC_{50} value of 9.3 μg/mL. We further evaluated the effect of PBE on LPS-induced COX-2 gene expression in

Fig. 4: Effect of PBE on the activation of COX-2 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (5.0×10^5 cells mL^{-1}) were stimulated with LPS (1 μg mL^{-1}) in PBE (12.5, 25, 50 μg mL^{-1}) for 24 h. Whole-cell lysates (25 μg) were prepared and the lysates were subjected to 10% SDS-PAGE and expression of COX-2 and β-actin were determined by Western blotting. The figure is representative of three similar experiments. The PBE fraction was prepared as described in the Materials and methods section



macrophages. The expression of COX-2 protein was measured in RAW 264.7 cells exposed to LPS (1 μg mL^{-1}) for 24 h. PBE effectively suppressed LPS-induced COX-2 expression (Fig. 4). The inhibitory effect of PBE on COX-2 expression suggests that this could be one of the mechanisms responsible for the anti-inflammatory action of PBE. To probe further that PBE inhibited the pro-inflammatory mediators, we also investigated the effect of PBE on LPS-induced TNF-α and IL-6 production by ELISA. As shown in Fig. 5, PBE inhibited LPS-induced TNF-α and IL-6 production in a concentration dependent manner. According to our results, PBE suppresses the production of NO, PGE_2, TNF-α and IL-1β, and decreases iNOS and COX-2 protein expression levels in LPS-induced RAW 264.7 cells. Although the anti-inflammatory effect of PBE was identified in this study, its mechanism of action was not determined. In particular, the potential inhibitory mechanisms toward nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) need to be further evaluated in future studies. NF-κB and MAPKs have been reported to be involved in maximal transcription of many cytokines, including TNF-α, IL-1, IL-6 and IL-8, which are thought to be important in the generation of acute inflammatory responses (Ghosh & Hayden, 2008; Wong & Tergaonkar, 2009). Therefore, it is possible that PBE may inhibit NF-κB and MAPKs activation induced by LPS. However, we cannot exclude the possible involvement of the inhibition of other transcription factors.

Fig. 5: Effect of PBE on the TNF-α (A) and IL-6 (B) production in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg mL^{-1} of LPS only or with LPS plus various concentrations (12.5, 25, 50 μg mL^{-1}) of PBE for 24 h. The TNF-α and IL-6 produced and released into the culture medium was assayed by the ELISA method. The data represent the mean ± SD of n=4. **p<0.05, **P<0.01* versus LPS alone. The PBE fraction was prepared as described in the Materials and methods section



In conclusion, the study demonstrates that PBE inhibits the production of NO, PGE_2, TNF-α and IL-1β in LPS stimulated macrophages. Among possible pathways that may regulate this anti-inflammatory effect, inhibiting iNOS and COX-2 expression via the NF-κB and/or MAPKs activation may be involved. Therefore, PBE may be a potential therapeutic drug for clinical use in inflammatory diseases.

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