Sargassum Horneri (Turner) C. Agardh Regulates Neuroinflammation via Inhibition of LPS-stimulated Microglia Activation in Vitro and in Vivo

Jun Sik Lee (✉ junsiklee@chosun.ac.kr)

Chosun University College of Natural Science <https://orcid.org/0000-0003-1051-581X>

Jun Hwi Cho

Chosun University

Mi Eun Kim

Chosun University

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Abstract

Previously we reported that *Sargassum horneri* (Turner) C. Agardh (*S. horneri*) is a brown algae species that exerts anti-inflammatory activity toward murine macrophages. However, the anti-neuroinflammatory effects and the mechanism of *S. horneri* on microglia cells are still unknown. We investigated the anti-neuroinflammatory effects of *S. horneri* extract on microglia in vitro and in vivo. In present study, we found that *S. horneri* was not cytotoxic to BV-2 microglia cells, and it significantly decreased lipopolysaccharide (LPS)-induced NO production. Moreover, *S. horneri* also diminished the protein expression of iNOS, COX-2, and cytokines production including IL-1b, TNF-a, and IL-6 on LPS-stimulated microglia activation. *S. horneri* elicited anti-neuroinflammatory effects by inhibiting phosphorylation of p38 MAPK and NF-kB. In addition, *S. horneri* inhibited astrocytes and microglia activation in LPS-challenged mice brain. Therefore, these results suggested that *S. horneri* exerted anti-neuroinflammatory effects on LPS-stimulated microglia cells activation by inhibiting MAPK phosphorylation and NF-kB signaling.

1. Introduction

Neuroinflammation is an essential event to enable recovery from innate immune responses following events such as nervous tissue damage or microbial infection. It starts with signals such as brain damage from infection, trauma, and/or autoimmunity (19). Neuroinflammation can also be chronic. In neuroinflammation, it plays an important role in cells or molecules, and many molecules such as microglia, pattern-recognition receptors and cytokines (25). These neuroinflammation mediators damage the blood-brain-barrier (BBB) of the central nervous system (CNS) (3). Microglia secrete anti-inflammatory factors or mediators that affect BBB integrity (10). When microglial cells are continuously activated, immune cells become concentrated in the brain (23). Microglial cells are the representative immune cells of the CNS (17) and the major effector cells that mediate neuroinflammation. They are responsible for phagocytosis in the brain where they account for 10–15% of brain cells. Activated microglia secrete inflammatory cytokines in several neuroinflammatory diseases (22). They can actively change their morphology by reacting to infection conditions or signals. Microglia maintain small size in their resting inactive state, but morphology changes in response to damage or pathogens, and they migrate through chemotaxis to become phagocytic cells (24). Activated microglia phagocytose apoptotic cells by releasing pro-inflammatory and other molecules (7). Neurons that have undergone apoptosis secrete various factors such as soluble factors and extracellular membrane proteins, which in turn induces microglial activity. This process is known as the self-propelling cycle (2). Murine microglial BV-2 cells involved in the inflammatory response stimulate Toll-like receptor-4 in response to lipopolysaccharide (LPS) and activate mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) (4). Once activated, these signaling pathways produce pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) and stimulate the expression of enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) to produce a variety of inflammatory molecules such as nitric oxide (NO) (9). Although these inflammatory mediators are needed to remove viruses and tumors,

their overproduction can cause serious damage to tissues and nerves and lead to chronic inflammatory diseases (4). Inhibiting inflammatory mediator production may be one way to combat excessive inflammation. *Sargassum horneri* (Turner) C. Agardh (*S. horneri*) is a brown algae found in coastal areas of Asia such as Korea, China, Japan that has various effects in the body (21, 14). Studies suggest that *S. horneri* can help prevent osteoporosis and control cholesterol, blood pressure, and hyperlipidemia (26, 27). Cosmetics and functional foods containing *S. horneri* are known to have anti-aging, anti-allergic, anti-acne, and whitening effects (12, 8). We previously reported that anti-inflammatory effects of ethanolic extract from *S. horneri* on LPS-induced macrophage activation (14). However, no study has assessed its effects on neuroinflammation mediated by murine microglia. Here we investigated the anti-neuroinflammatory activity of *S. horneri* in the setting of LPS-induced inflammation.

2. Materials And Methods

2.1. Reagents

The compound Griess reagent, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), TRI Reagent were purchased Sigma Chemical (St. Louis, MO, USA). The antibody Anti-p-p65, anti-p65, anti-i-NOS, anti-COX-2, anti-p-p38, anti-p-ERK, anti-p-JNK, anti-JNK were purchased Santacruz (Santa, CA, USA), and anti-ERK, anti-p38 were purchased Cell signaling (Cell signaling, MA, USA).

2.2. Cell Culture and Extration of Sample

Murine microglia cells, BV-2, were cultured. And cells were cultured at 37°C in the presence of 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 200 IU/ml penicillin, 200 µg/ml streptomycin. *S.horneri* was reconstituted in ethanol and then diluted to the desired concentration in DMEM (final ethanol concentration 0.3%). Control groups without *S.horneri* used 100% ethanol solvent.

2.3. Cell viability Assay

Cell viability was measured by Annexin V/PI staining. The BV-2 cells (2×10^6 cells/well) were seeded in 6-well culture plates with DMEM. Cells were treated with the various concentrations *S. horneri* (0 ~ 300 µg/ml) and incubated in 37°C for 24 h. After 24 h, the cells were detached and centrifuged at 20°C and 2000 rpm for 3 min. Wash 2 ~ 3 times with warm PBS, staining using Annexin V/PI Kit, and measure using FACS (Fluorescence activated cell sorter).

2.4. NO Assay

BV-2 cells (3×10^4 cells/well) were seeded in 96-well culture plate in DMEM. Cultured cells were pretreated with various concentration of *S.horneri* (0 to 300 µg/ml) for 2 h and then cells were incubated for 22 h in absence or presence of LPS (200 ng/ml). After incubation, the cultured medium was mixed with an equivalent volume of Griess Reagent and incubated for 15 min at room temperature. After 15 min incubation, absorbance was measured using an ELISA microplate reader at 540 nm of absorbance.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

BV-2 cells (3×10^4 cells/well) were seeded in 96-well culture plate. Cells were pretreated with various concentration of *S.honeri* (0 to 300 µg/ml) for 2 h, and then cells were incubated for 22 h in absence or presence of LPS (200 ng/ml). After incubation, supernatant was used for samples and the quantification of TNF- α IL-6 release was measured by Mouse IL-6, TNF- α ELISA MAX Deluxe Sets (BioLegend, CA, USA), according to manufacturer's protocol. Briefly, standards and samples were incubated for 1 h and Avidin-HRP bind to detection antibody. To visualization, substrate solution was added to each well, and then the reaction was stopped by stop solution (2 N H₂SO₄). Absorbance was measured by ELISA microplate reader at 405 nm wavelength.

2.6. Reverse Transcription (RT)-PCR

BV-2 cells (5×10^5 cells/well) were seeded in 12-well culture plate in DMEM. Cultured cells were pretreated with various concentrations of *S.honeri* (0 ~ 300 µg/ml) for 2 h and then cells were incubated for 6 h in absence or presence of LPS. After incubation, the cells were collected by centrifugation and total RNA was isolated from *S.honeri*-treated cells using TRIZOL reagent according to manufacturer's protocol. To synthesize cDNA, 0.1 µg of total RNA was primed with oligo dT and reacted with mixture of dNTP, M-MLV RTase and reaction buffer (Promega, WI, USA). To measure the mRNA level of inflammatory cytokines including IL-1 β , TNF- α , IL-6, and i-NOS, COX-2, we designed the primers for target genes (Bioneer, Daejeon, Korea). And cDNA was amplified using Gene Atlas G02 gradient thermal cycler system (Astec Fukuoka, Japan) e-Taq DNA Polymerase kit (Solgent, Daejeon, Korea) and the primers. And then, PCR products were visualized by fluorescent dye and UV transilluminator.

2.7. Western Blot Analysis

BV-2 cells (2×10^6 cells/dish) were seeded in 60 mm culture dish in DMEM. Cultured cells starved 4 h in serum free media DMEM and exposed to LPS (200 ng/ml) in the absence or presence of 300 µg/ml of *S.honeri* pretreatment. Following 15, 30 and 45 min of incubation at 37°C, cells were washed twice with cold PBS and lysed with modified RIPA buffer containing (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 g/mL leupeptin, 1 µg/mL pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride) for 30 min at 4°C. Lysates were cleared by centrifuging at 14000 X g for 15 min at 4°C. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL, USA).

Equivalent amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) transfer membrane. The membrane was placed into a blocking solution (5% skim milk and 2% BSA) at room temperature for 1h. After blocking, anti-ERK1/2, anti-phospho-ERK1/2 (p-ERK), anti-p38, anti-phospho-p38 (p-p38), anti-NF- κ B (NF- κ B-p65), and anti-phospho-NF- κ B-p65 antibodies overnight (Santa Cruze Biotechnology, CA, USA) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Santa Cruze Biotechnology) were used as the secondary antibodies. Band detection was

performed using the enhanced chemiluminescence (ECL) detection system and exposed to radiographic film. Pre-stained blue markers were used for molecular weight determination.

2.8. In vivo experiment

All experiments were approved and performed in accordance with the regulations of the Chosun University Care and Use Committee (IACUC, 201901). Mice were housed in a temperature-controlled (21–23°C) room under a 12:12 h light/dark cycle and provided food and water *ad libitum*. C57/BL6 (8 weeks) male mice were randomly divided into 3 groups of five mice per group (Control, LPS, LPS + *S. honeri*). *S. honeri* was dissolved in sterile saline and pre-administered by intraperitoneal injection (i.p.) (10 and 20 mg/kg) for 3 days prior to LPS injection. Also, LPS group was injected intraperitoneally (1 mg/kg) for 24 h. Mice brain samples sacrificed after performed CO₂ euthanasia and analyzed.

2.9. Immunohistochemistry

The mice were intra-cardially perfused with 10 mM phosphate-buffered saline (PBS) and subsequently 4 % paraformaldehyde. The brain was extracted and post-fixed in 4 % paraformaldehyde for 24 h at 4 °C. Fixed brain was transferred to 30 % sucrose solution. These brain samples were embedded in OCT for frozen sections, and then coronally sectioned at 40 µm using a freezing microtome (MICROM, Walldorf, Germany). Brain sections were washed and blocked with blocking solution for 30 min at room temperature. The sections were incubated with anti-ionized calcium-binding adapter molecule 1 (Iba-1, microglia marker) antibody and glial fibrillary acidic protein (GFAP, astrocytes marker) (Wako Chemical USA, Inc.; Richmond, VA, USA) in TBS-TS at 4 % overnight. Brain sections were washed with TBS and incubated with anti-mouse IgG labeled with Alexa Fluor 488 and 568 for 3 h at room temperature, respectively. Confocal fluorescence images were acquired FV10i fluoview confocal microscope (Olympus; Tokyo, Japan).

2.10. Statistical Analysis

The results are presented as the mean ± standard deviation. The data were analysis of variance (ANOVA) followed by Scheffe's post-hoc test using SPSS. The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. *S. honeri* inhibits NO production in LPS-stimulated BV-2 microglial cells

We confirmed effect of *S. honeri* on cell viability and NO regulation. Annexin V/PI staining showed that *S. honeri* was not cytotoxic at doses up to 300 µg/ml (Fig. 1A). To determine the effect of *S. honeri* on NO production, BV-2 microglial cells were pretreated with *S. honeri* for 2 h and then stimulated with LPS (200 ng/ml). NO production was increased in LPS compared to control, but NO levels decreased in a dose-

dependent manner with *S. honeri* treatment (0-300 µg/ml) (Fig. 1B). These results indicate that *S. honeri* effectively inhibits NO production in LPS-stimulated BV-2 microglia.

3.2. *S. honeri* reduced LPS-induced iNOS and COX-2 protein expression

Since *S. honeri* inhibits NO production, we assessed its impact on protein expression of iNOS, which is a pro-inflammatory enzyme producing NO, and cyclooxygenase (COX)-2. Western blot analysis was performed to confirm that *S. honeri* inhibits the expression of pro-inflammatory enzymes. BV-2 microglial cells were pretreated for 2 h with *S. honeri* and then stimulated with LPS (200 ng/ml) for 22 h. As shown in Fig. 2, protein levels of iNOS and COX-2 were also decreased in a BV-2 microglial cells pretreated *S. honeri* (0-300 µg/ml) for 2 h, even though expression of both proteins were stimulated by LPS in dose-dependent manners. These results indicate that *S. honeri* inhibits protein expression of iNOS and COX-2 in LPS-stimulated BV-2 microglial cell.

3.3. *S. honeri* reduced neuroinflammatory factors in LPS-stimulated BV-2 microglial cells

We next used RT-PCR to measure mRNA levels of the pro-inflammatory substances IL-6, TNF-α, and IL-1β and determined whether their expressions were inhibited by *S. honeri*. BV-2 microglial cells were pretreated for 2 h with *S. honeri* and then stimulated with LPS (200 ng/ml) for 6 h. As shown in Fig. 3A, expression of pro-inflammatory genes such as IL-6, TNF-α, and IL-1β were increased in LPS-stimulated BV-2 microglial cells, and *S. honeri* dose-dependently decreased mRNA expression.

To determine whether levels of IL-6, TNF-α, and IL-1β secreted by BV-2 microglial cells were decreased by *S. honeri*. IL-6, TNF-α, and IL-1β are pro-inflammatory cytokines expressed by BV-2 microglial cells following exposure to LPS. The results confirmed that IL-6 and TNF-α produced from LPS-stimulated BV-2 microglial cells were significantly decreased by pretreatment with *S. honeri* (Fig. 3). We therefore suggest that IL-1β, IL-6, and TNF-α production can be inhibited by *S. honeri*, suggesting that the compound has anti-neuroinflammatory effects.

3.4. *S. honeri* inhibited phosphorylation of p38 MAPK, ERK, MAPK, and NF-κB

MAPKs such as p38 MAPK, JNK, ERK plays important roles in regulating cell growth, division, stress, and cytokine-mediated cellular responses; they are involved in signal pathways that regulate inflammatory mediators through transcription factor activation. To clarify how *S. honeri* inhibits inflammatory mediators, we confirmed that it inhibited LPS-induced MAPK activation signaling pathways. p38 MAPK and ERK phosphorylation were increased in BV-2 microglial cells stimulated by LPS for 15, 30, or 45 min, but this was inhibited by *S. honeri* treatment. Interestingly, p-p38 MAPK phosphorylation was significantly decreased by *S. honeri* treatment (Fig. 4A). NF-κB is a transcription factor that regulates the intracellular

synthesis of various molecules including the expression of inflammatory cytokines and iNOS. So, we asked whether the anti-neuroinflammatory activity of *S. honeri* was due to inhibited NF- κ B activity. As shown in Fig. 4B, phosphorylation of p65 was increased in the nucleus of LPS-stimulated BV-2 cells after 15, 30, and 45 min, but p65 phosphorylation was decreased in the nucleus by *S. honeri* treatment. This suggests that *S. honeri* strongly inhibited NF- κ B migration to the nucleus upon activation. Collectively, these results indicate that the inhibitory effect of the *S. honeri* on pro-inflammatory cytokines production in LPS-induced BV-2 cells is mediated by p38 MAPK, ERK, and NF- κ B signaling.

3.5. *S. honeri* attenuate astrocyte and microglia activation in the LPS-administrated mouse brain

To confirm anti-neuroinflammatory activity of *S. honeri* in brain-resident glial cells, LPS-induced astrocyte and microglia activation were examined in mouse brain. GFAP (astrocyte marker) and Iba-1 (microglia marker) was highly expressed in hippocampus in LPS-injected groups compared with control groups. As shown in Fig. 5, LPS-induced astrocyte activation in hippocampus was significantly decreased by 20 mg/kg of *S. honeri* administration. Moreover, LPS-induced microglia activation in hippocampus was significantly decreased by *S. honeri* administration in a dose-dependent manner. These data suggested that *S. honeri* attenuates the LPS-induced neuroinflammatory response by inhibiting astrocyte and microglia activation.

4. Discussion

S. honeri is a seaweed that exerts various effects in the body. Previous studies reported that *S. honeri* can preventing osteoporosis and help control cholesterol, blood pressure, and hyperlipidemia. Cosmetics and functional foods containing *S. honeri* are known to have anti-aging, anti-allergic, anti-acne, whitening, and anti-inflammatory effects (12, 21, 14). However, no mechanism has been described for how it could regulate murine microglia activity. In present study, we investigated the anti-neuroinflammatory effect of *S. honeri* on LPS-stimulated microglia activation. As shown in Figs. 1 and 2, stimulation of the murine microglia cell line BV-2 enhanced iNOS and NO expression. NO is a highly reactive factor involved in vasorelaxation, neurotransmission, and cell-mediated immune responses (28, 20, 18). NO production mediates the inflammatory response and exerts cytotoxic activity against external pathogens. LPS-induced BV-2 microglial cells act as inflammatory mediators downstream of NO to induce pro-inflammatory cytokines such as IL-6, IL-1 β , and TNF- α ; they also enhance inflammation by secreting COX-2 (14, 11). Moreover, NO overexpression aggravates various inflammatory diseases. Therefore, substances that inhibit inflammatory mediators that produce NO and pro-inflammatory cytokines may be useful for treating various immune diseases. We found that *S. honeri* treatment significantly inhibited NO production and iNOS expression on LPS-stimulated microglial cells in a dose-dependent manner (Fig. 1B and 2). These results indicated the effect that *S. honeri* could suppress neuroinflammation because it reduced the production of NO.

In previous studies, activated microglia induces the production of neuroinflammatory factors such as IL-1 β , IL-6, and TNF- α , can induce neuroinflammatory responses (11). Therefore, we investigated the amount of inflammatory factors produced by the *S. horneri* treatment. As shown in Fig. 3, gene expression and production levels of IL-1 β , IL-6, and TNF- α were increased by LPS treatment, but decreased in a concentration dependent manner by *S. horneri* treatment in LPS-stimulated BV2 microglial cells.

MAPKs include JNK, ERK, and p38 MAPK, and play important roles in various cellular processes such as cell differentiation, proliferation, and inflammation (15). Inactive MAPK remains in the cytosol and it activated by phosphorylation in response to LPS or other stimuli, at which point it translocates to the nucleus and stimulates cytokine production (13). In this study, *S. horneri* pretreatment reduced phosphorylated ERK and p38 MAPK in LPS-stimulated BV-2 microglial cell activation (Fig. 4A).

NF- κ B play important roles in inflammatory reactions and in various immune responses, tumorigenesis, and autoimmune diseases (5, 6, 16). NF- κ B is present in the cytoplasm in an inactive state bound to I κ -B α , but in response to external stimuli such as LPS, I κ -B α is phosphorylated, and the separated NF- κ B translocates to the nucleus where it is involved in the production of NO and pro-inflammatory cytokines (1). As shown in Fig. 4B, we found that NF- κ B migration into the nucleus was strongly decreased following pretreatment with *S. horneri*.

Microglia and astrocyte activation is important in neuroinflammatory responses. It also induces in various CNS injuries such as Alzheimer's and Parkinson's disease (11).

Therefore, in order to determine the inhibitory effect of *S. horneri* on neuroinflammation, we analyzed microglia and astrocyte activation by *S. horneri* administration in LPS-challenged mice. As shown in Fig. 5, we found that microglia and astrocyte were activated in the group treated with LPS. However, microglia and astrocyte activation were significantly inhibited in the 20 mg/kg *S. horneri* pretreatment group. This result suggests that *S. horneri* has a preventive effect on neuroinflammation.

Collectively, our results show that *S. horneri* attenuated neuroinflammation via the activation of MAPK and NF- κ B pathways including ERK and p38 MAPK in LPS-stimulated BV-2 cells, thereby inhibiting the production of NO and the pro-inflammatory cytokines IL-6 and TNF- α . These findings indicate that *S. horneri* may be an effective treatment for neuroinflammatory diseases.

Declarations

Author Contributions

ME Kim, JH Cho, and JS Lee conceived and designed the experiments; ME Kim, and JH Cho performed experiments and analyzed the data. ME Kim and JS Lee wrote the paper.

Funding Information

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Competing Interests

The authors declare no competing interests

Availability of Data and Materials

The dataset used and/or analyzed during the current study are available from corresponding author on reasonable request.

Ethics Approval

All experiments were approved and performed in accordance with the regulations of the Chosun University Care and Use Committee (IACUC, 201901).

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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Figures

Figure 1

Anti-neuroinflammatory effect of *S. honeri* on NO production in LPS-stimulated BV-2 microglial cells. Microglial cell viability was determined by MTT assay after *S. honeri* treatment with BV-2 microglial cells (A). After *S. honeri* treatment of BV-2 microglial cells stimulated with LPS (200 ng/ml), NO production ability was determined by Griess assay (B). The result is representative of repeated three independent

experiments. Experimental results were indicated as mean ($\pm$ SE). **p < 0.001, significantly different from LPS-treated group; ## p < 0.001, significantly different from LPS-untreated group.

Figure 2

Effect of *S. honeri* on protein expression of iNOS and COX-2 in LPS-stimulated BV-2 microglial cells. *S. honeri* was pretreated with BV-2 microglial cells 2 hr, and then incubated with 200 ng/ml LPS for 22 hr. iNOS and COX-2 protein expression was determined by Western blot. The result is representative of

6 hr. RNA was isolated and analyzed by RT-PCR. IL-1 β , TNF- α , and IL-6 gene expression was confirmed, respectively (A). The cells were pre-treated with *S. honeri* (25, 50, 100, 200, and 300 μ g/ml) for 2 h, followed by treatment of LPS (200 ng/ml) for 24 h; the levels in the supernatants were measured by ELISA. Each bar indicates the mean $\pm$ SE of four independent experiments. **p < 0.001, significantly different from LPS-treated group; ## p < 0.001, significantly different from LPS-untreated group.

Figure 4

S. honeri attenuated LPS-induced MAPK phosphorylation and NF-κB translocation. Cells were pre-treated with 300 μg/ml of *S. honeri* for 2 h, followed by treatment of LPS (200 ng/ml) for 15, 30, 45 min. The expression of phosphorylated proteins was measured by Western blot. The total extracts and nucleus extracts were assayed with antibodies specific for p-ERK, p-38 MAPK, and p-p65 (NF-κB). Total form and β-actin was used as internal control for total fraction. The blots are representative of three independent experiments (A and B). Each bar indicates the mean ± SE of four independent experiments. **p < 0.001, significantly different from LPS-treated group; ## p < 0.001, significantly different from LPS-untreated group.

Figure 5

honeri inhibited LPS-induced astrocyte and microglia activation in brain hippocampus. C57BL/6 mice were randomly divided into four groups. The control group was challenged the same amount of solvent i.p. (n=5). The treatment group was administered LPS (1 mg/kg) and *S. honeri* (10 and 20 mg/kg) as followed in vivo study design. Brain sections were immunostained with anti-GFAP (Green) or anti-Iba-1

(Red) antibody as described in the Materials and Methods section. (Scale bar = 100 μm). The result is representative of repeated three independent experiments.