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Communication

Acrosorium polyneurum Extract Inhibits the LPS-Induced Inflammatory Response by Impairing the MAPK and NF-κB Pathways

Zahid Manzoor[1,2], Irshad Ali[1,2], Doobyeong Chae[1,2] and Young-Sang Koh[1,2*]

[1]Department of Microbiology and Immunology, School of Medicine and Brain Korea 21 PLUS Program, Jeju National University, Jeju; [2]Institute of Medical Science, Jeju National University, Jeju, Korea

Marine algae exhibit broad spectrum anti-bacterial and anti-inflammatory activities. *Acrosorium polyneurum* (*A. polyneurum*) is a marine red alga and belongs to the family Delesseriaceae. The present research evaluates the anti-inflammatory effects of *A. polyneurum* extract (APE) on pro-inflammatory cytokine production. APE demonstrated substantial inhibitory effects on production of pro-inflammatory cytokine in bone marrow-derived macrophages (BMDMs). APE pre-treatment in the lipopolysaccharide (LPS)-stimulated BMDMs exhibited a robust inhibitory effect on production of interleukin (IL)-12, IL-6 and tumor necrosis factor (TNF)-α. It revealed a robust inhibitory effect on phosphorylation of ERK1/2, JNK1/2 and p38. APE also showed remarkable inhibitory effect on phosphorylation and degradation of IκBα. Furthermore, APE pre-treatment demonstrated substantial inhibition of LPS-induced production of nitric oxide and inducible nitric oxide synthase. Collectively, these data suggest that APE has a noteworthy anti-inflammatory property and deserve further studies concerning its potential use as a medicinal agent for inflammation-related disorders.

Key Words: *Acrosorium polyneurum*; Mitogen-activated protein kinase; NF-κB; Nitric oxide; Pro-inflammatory cytokine

INTRODUCTION

Pattern recognition receptors (PRRs) are basically highly conserved proteins, expressed by the cells of innate immune system, and involved in recognition of pathogen-associated molecular patterns (PAMPs), which are exclusively present in microorganisms (1~3). Among various PRRs, toll-like receptors (TLR) are well studied and play critical roles in recognizing various types of PAMPs and managing proper immune response (4~6).

TLRs are the main regulator of innate host immune response against harmful stimuli (1). For example, TLR4 expressed in macrophages recognizes lipopolysaccharide (LPS) and results in activation of different signaling cascades, such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathway, leading to pro-inflammatory cytokine production (7).

Phytochemicals have been major bioresources and explored well for medicinal use (8). Now scientists are exploring natural products from marine bioresources. A large number of biomaterials for good bioactivities have been documented, but much still remains to be explored for high nutritional value and pharmaceutical potential (9). Red algae

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*Corresponding author: Young-Sang Koh. Department of Microbiology and Immunology, Jeju National University School of Medicine, 102 Jejudaehakno, Jeju 63243, Korea.
Phone: +82-64-754-3851, Fax: +82-64-702-2687, e-mail: yskoh7@jejunu.ac.kr
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are known as oldest multicellular plants and most diverse eukaryotes (10). Red algae are the largest species among algae, providing a good resource for medicinal food and helpful in different ways in food industry. For example, carrageenans are sulphated polysaccharides, extracted from red algae and widely used in the food industry for gelling or stabilizing activities. Several red algae are good source of food and some are used in preparing agars (11). Extracts from marine algae have been reported to exhibit broad spectrum anti-bacterial, anti-inflammatory and antioxidant activities (12, 13). *Acrosorium polyneurum* (*A. polyneurum*), a marine red alga, belongs to the family Delesseriaceae and is distributed on the seashores of Korea. During ongoing research to evaluate the biological effects of various marine alga extracts, in the present study, *A. polyneurum* extract (APE) was studied for the anti-inflammatory properties for the first time. The effect of APE has not been studied regarding its impact on primary murine macrophages. Thus, in the present study we report for the anti-inflammatory effects of APE on LPS-stimulated bone marrow-derived macrophages (BMDMs) and RAW 264.7 cells.

MATERIALS AND METHODS

Preparation of *A. polyneurum* extract

Thalli of *A. polyneurum* were collected on Jeju Island, Korea. The material for extraction was cleaned, dried at room temperature and fine powder was made by grinding. The dried alga was extracted as described previously (13). The evaporated ethanol extract was suspended in water.

Mice

Six-week-old female C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) for BMDMs. All animal procedures were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of Jeju National University, Jeju, Korea (#2010-0028).

Cell cultures and measurement of cytokine and nitric oxide production

Bone marrow cells were differentiated in DMEM (Gibco, Grand Island, NY, USA) medium containing macrophage colony-stimulating factor for BMDMs generation as described previously (14, 15). For BMDMs, on day 6 of incubation, the cells were harvested and seeded in 48-well plates at a density of 1×10^5 cells/0.5 ml and, then, treated with the APE for 1 hour (h) before stimulation with LPS (10 ng ml^{-1}). Supernatants were harvested 18 h after stimulation. The concentrations of murine interleukin (IL)-12 p40, IL-6 and tumor necrosis factor (TNF)-α in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (BD PharMingen, San Jose, CA, USA; R&D system, MN, USA), according to the manufacturer's instructions. Production of nitric oxide (NO) in RAW-264.7 cells treated with APE and stimulated with LPS was measured with Griess reagent system (Promega, Madison, WI, USA) as previously described (16).

Cell viability assay

To assess cell viability, the standard procedure of 3-(4,5-dimethyl-2,5 thiazolyl)-2,5 diphenyl tetrazolium bromide (MTT) assay was used as described previously (17).

Western blot analysis

Western blot analysis was performed using standard techniques as previously described (18, 19). Briefly, BMDMs and RAW264.7 cells were dispensed to 60-mm culture dishes (Nunc, Roskilde, Denmark) at 4×10^6 cells per dish and cultured for 24 h at 37℃. The cells were pre-treated with or without APE (25 μg ml^{-1}) for 1 h before treatment with LPS (10 ng ml^{-1}) at the indicated time points. The cells were collected and, then, lysed in lysis buffer (PRO-PREP lysis buffer, iNtRON Biotechnology, South Korea). A protein sample (30 μg) was subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated with 1/1,000-diluted rabbit polyclonal antibodies that specifically recognize phospho-p44/

42 (p-ERK1/2), p44/42 MAPK, phospho-p38, p38 MAPK and phospho-SAPK/JNK, SAPK/JNK, IκBα (Cell Signaling Technology, Danvers, MA, USA), inducible nitric oxide synthase (iNOS) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membrane was incubated with a horseradish peroxidase-linked goat anti-rabbit IgG (Cell Signaling Technology), and immunoactive bands were detected as previously described (19).

Statistical analysis

All experiments were executed at least 3 times. The data are shown as the mean ± the standard deviation (SD) of 3 independent experiments. One-way ANOVA (SPSS program; IBM, Armonk, NY, USA) was used for comparison between treated and control groups. $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of APE on pro-inflammatory cytokine production in LPS-stimulated BMDMs

Cytokines are required to regulate host responses to inflammation and macrophages are the key producers of various cytokines (1). BMDMs express TLR4 that recognizes LPS and leads to the production of cytokines (20). Therefore, we determined the anti-inflammatory activity of APE by testing its inhibitory effects on IL-12, IL-6 and TNF-α production in LPS-stimulated BMDMs. The maximal concentration of APE for treating cells was assessed by MTT assay, and as a result, APE had no effect on the cell viability at indicated concentrations (data not shown). LPS induced a substantial increase of pro-inflammatory cytokine production in BMDMs. APE pre-treatment profoundly inhibited pro-inflammatory cytokine production in the LPS-stimulated BMDMs (Fig. 1). These results show that APE had an inhibitory effect on production of cytokines in LPS-stimulated BMDMs. IL-12 has various critical immunoregulatory activities and is a main cytokine in Th1-mediated autoimmune responses, therefore downregulation of unregulated IL-12 production by APE may have potential to ameliorate IL-12-related autoimmune diseases (21, 22). IL-6 and TNF-α have important physiological roles, however; dysregulated production of these cytokines has been associated with various

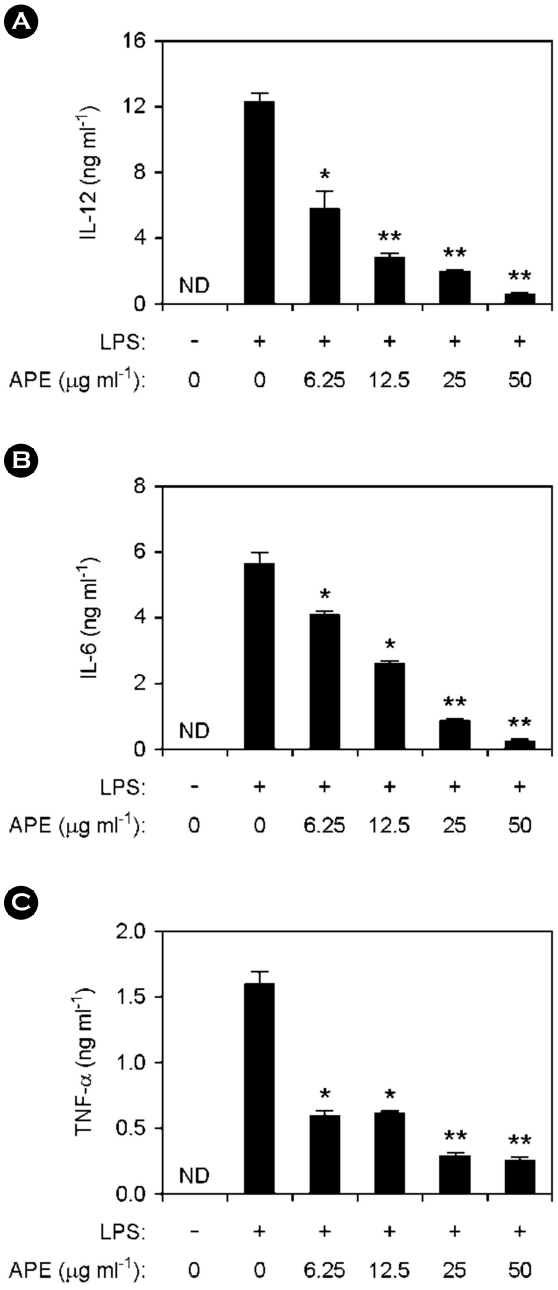

Figure 1. Inhibitory effects of *Acrosorium polyneurum* Extract (APE) on cytokine production in LPS-stimulated BMDMs. (A-C) Before stimulation with LPS (10 ng ml^{-1}), BMDMs were treated with APE at various doses as shown for 1 h and cytokines levels were assessed by ELISA. ND, not detectable; APE, *A. polyneurum* extract. *$p < 0.05$, **$p < 0.01$ vs. APE-untreated cells in the presence of LPS.

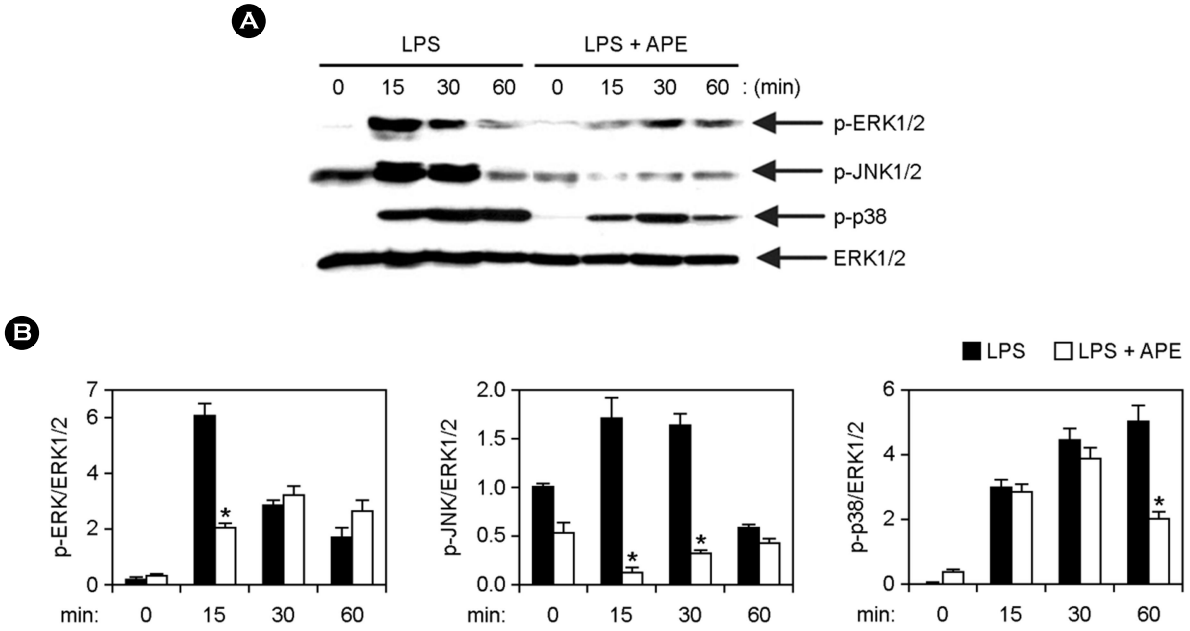

Figure 2. Inhibitory effects of APE on phosphorylation of MAPKs by LPS-stimulated BMDMs. (A) Cells were pre-treated with or without APE (25 μg ml^{-1}) for 1 h before stimulation with LPS (10 ng ml^{-1}). Total cell lysate was obtained at various time intervals as shown. Western blot analysis was done on the cell lysate to evaluate phosphorylation of ERK1/2, JNK1/2 and p38. Total ERK1/2 MAPK was taken as the loading control. (B) Phosphorylation of MAPKs protein was quantified using scanning densitometry. *$p < 0.05$ vs. APE-untreated cells in the presence of LPS.

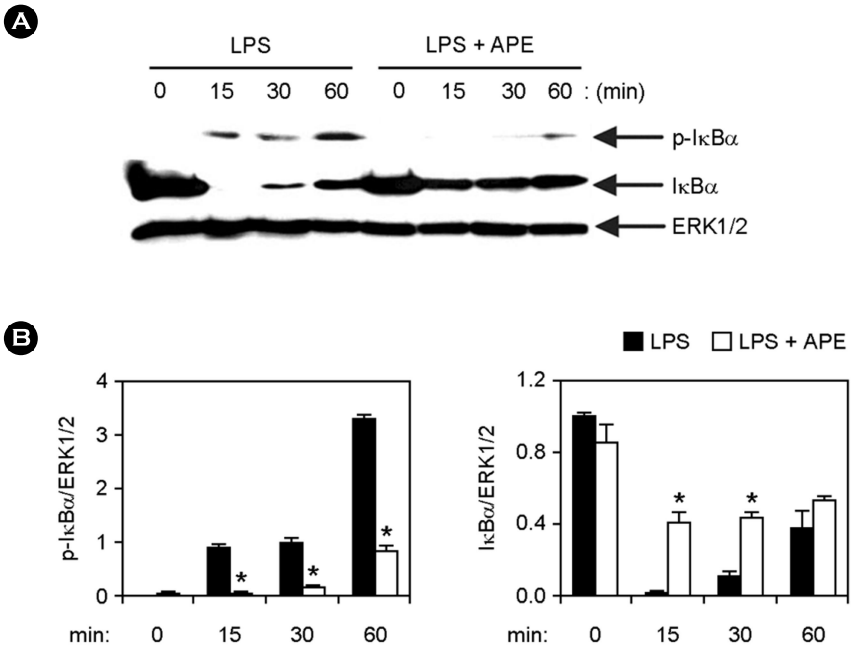

Figure 3. Inhibitory effects of APE on NF-κB activation by LPS-stimulated BMDMs. (A) Cells were treated as described in Fig. 2A, and Western blot analysis was performed. (B) Scanning densitometry was performed as described in Fig. 2B. *$p < 0.05$ vs. APE-untreated cells in the presence of LPS.

inflammatory diseases (23, 24). Thus in future, controlling the over production of IL-6 and TNF-α by APE might be helpful in ameliorating inflammation-associated diseases such as autoimmune and autoinflammatory diseases.

Effects of APE on the phosphorylation of MAPKs and activation of NF-κB by LPS-stimulated BMDMs

Recognition of LPS by TLR4 results in activation of different intracellular signaling pathways including MAPK and NF-κB, and leads to the production of cytokines (1, 25). Henceforth, we studied the effects on MAPK phosphorylation and NF-κB activation in LPS-stimulated BMDMs, with and without APE treatment by Western blot analysis (Fig. 2 and 3). Stimulation of BMDMs with LPS resulted in phosphorylation of ERK1/2, JNK1/2 and p38 MAPKs. APE pre-treatment in the presence of LPS displayed robust inhibition of MAPKs phosphorylation (Fig. 2A, B). Together, these data suggest that APE can inhibit LPS-stimulated ERK1/2, JNK1/2 and p38 phosphorylation in BMDMs.

Stimulation of TLR4 leads to phosphorylation of IκB. The degradation of phosphorylated IκB results in translocation of NF-κB to the nucleus and facilitates its binding to the target promoter sites (1). Stimulation of BMDMs with LPS resulted in phosphorylation of IκBα (Fig. 3). In the presence of LPS, APE pre-treatment showed substantial inhibition of IκBα phosphorylation (Fig. 3A and B). Activation of NF-κB was also evaluated indirectly through the degradation of IκBα. LPS-stimulation resulted in degradation of IκBα within 15 min of stimulation (Fig. 3A and B). APE pre-treatment inhibited IκBα degradation, and as a consequence is likely to inhibit the activation of NF-κB in LPS-stimulated BMDMs (Fig. 3A and B). Collectively, these data propose that APE inhibited the NF-κB activation. Taken together, these findings advocate that inhibition of LPS-stimulated pro-inflammatory cytokines production by APE may associate with blockage of the MAPK and NF-κB-dependent pathway.

Effect of APE on the production of NO and iNOS in LPS-stimulated RAW264.7 cells

RAW 264.7 cells express TLR4 that recognizes LPS and leads to the production of NO (26, 27). Therefore, the effect of APE on production of NO in LPS-induced RAW 264.7 cells was tested with Griess assay. To measure the cell viability at the same time, colorimetric MTT assay was used, and as a result, APE had no adverse effect on the viability of cells at the indicated doses (data not shown). LPS induced a significant increase of NO production in RAW264.7 cells. APE pre-treatment strongly inhibited NO production in the

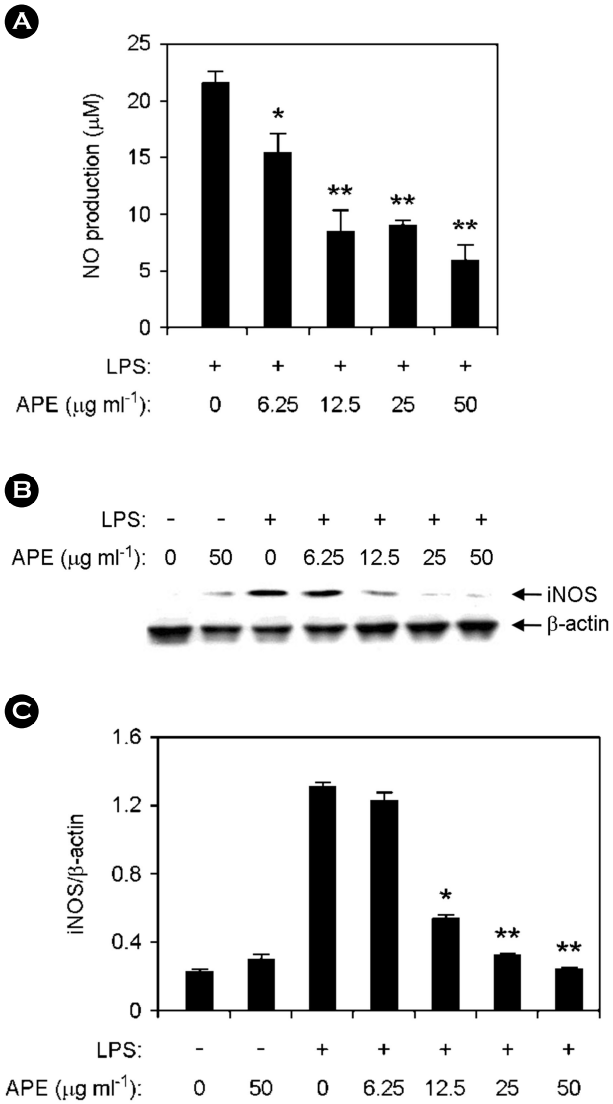

Figure 4. Inhibitory effects of APE on the production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated or not treated with APE at various doses as shown for 1 h before stimulation with LPS (10 ng ml^{-1}). (A) The NO production was investigated by Griess assay. (B) The protein levels of iNOS were measured by Western blot analysis and β-actin was used as the loading control. (C) For quantification of iNOS protein expression scanning densitometry was used and normalized by that of β-actin. *$p < 0.05$, **$p < 0.01$ vs. APE-untreated cells in the presence of LPS.

LPS-stimulated RAW264.7 cells (Fig. 4A). We performed Western blot analysis to explore the effect of APE on the production of iNOS in LPS-stimulated RAW264.7 cells. LPS-stimulation exhibited a strong increase in the iNOS production in RAW264.7 cells (Fig. 4B and C). However, APE pre-treatment significantly inhibited iNOS expression in LPS-stimulated RAW264.7 cells (Fig. 4B and 4C). Over-expression of iNOS has been linked with different diseases, including arthritis, septic shock and chronic inflammatory diseases (27). The present study proposes that APE has an inhibitory effect on the iNOS production and might be helpful in treating inflammatory diseases.

In conclusion, we established that APE had an inhibitory effect on the pro-inflammatory cytokines production by diminishing MAPKs and NF-κB signaling pathways. APE also showed inhibition of NO production by down regulating iNOS expression and, thus, deserves further study regarding its potential use as a medicinal agent. In addition, these results suggest that APE might be helpful in the treatment of inflammation-associated diseases. Hence, further studies are mandatory regarding comprehensive analysis and detailed mode of actions of the pure active components of APE.

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