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Research Note

Monoolein, isolated from *Ishige sinicola*, inhibits lipopolysaccharide-induced inflammatory response by attenuating mitogen-activated protein kinase and NF-κB pathways

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Abstract *Ishige sinicola* (*I. sinicola*) is an edible brown alga native to South Korea. In the present study, we screened the anti-inflammatory activity of monoolein isolated from *I. sinicola*. Monoolein pretreatment in lipopolysaccharide (LPS)-stimulated primary murine bone marrow-derived dendritic cells (BMDCs) showed strong dose-dependent inhibition of interleukin (IL)-12 p40, IL-6, and TNF-α cytokine production with IC_{50} values of 1.69±0.02, 6.87±0.37, and 5.19±0.56 μM, respectively. Pretreatment of monoolein attenuated the activation of MAPK and NF-κB pathways in the LPS-stimulated BMDCs by inhibiting the phosphorylation of p38, ERK1/2, JNK1/2, and IκBα. Furthermore, monoolein inhibited the production of NO and iNOS in RAW264.7 cells. Overall, our findings indicate that monoolein has a significant anti-inflammatory activity, and further studies regarding the potential of monoolein for medicinal use is warranted.

Keywords: *Ishige sinicola*, monoolein, pro-inflammatory cytokine, mitogen-activated protein kinase, NF-κB

Introduction

Inflammation plays a key role in the pathogenesis of numerous human diseases, such as cancer, chronic asthma, rheumatoid arthritis, and psoriasis (1). Toll-like receptor plays a critical role in triggering immune responses by sensing conserved microbial-derived macromolecules (2-4). Dendritic cells and macrophages are integral cellular elements of innate immune system and play a critical role in the recognition of pathogen-associated molecular pattern (2). Both these cell types express TLR4, which recognizes lipopolysaccharide (LPS). Dendritic cell stimulation by LPS leads to high production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-12, and IL-6 (5). Macrophages phagocytose pathogens and destroy it through reactive nitrogen intermediates (6). Nitric oxide synthase (NOS) causes the production of nitric oxide (NO) radical through the conversion of L-arginine to L-citrulline. NOS in macrophages is inducible (iNOS) and becomes active following infection, which causes the overproduction of NO. NO acts as a second messenger and is a major contributing factor during the inflammatory process (7).

Extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 kinase get activated through phosphorylation in response to extracellular signals and ultimately lead to the modulation of gene expression of various pro-inflammatory cytokines (8). NF-κB pathway plays a key role in the regulation of inflammation and immune response (2). Its activation requires phosphorylation and subsequent degradation of IκB that allows nuclear translocation of NF-κB, leading to the expression of certain pro-inflammatory cytokine genes (9).

Seaweeds are rich in bioactive compounds in the form of vitamins, phycobilins, polyphenols, carotenoids, phycocyanins, and polysaccharides, many of which are known to have advantageous applications in human health (10). *Ishige sinicola* (*I. sinicola*) is an edible brown alga and is widely distributed along the coast of Jeju Island in Korea (11). The extract of *I. sinicola* has inhibitory effects on NO production, obesity, and melanogenesis (12,13). Monoolein, isolated from the branches and leaves of *Ficus erecta,* has been reported to exhibit anti-oxidant and anti-tyrosinase activities (14).

The anti-inflammatory effect of monoolein, a bioactive component of *I. sinicola,* has not yet been reported. Therefore, the present study was carried out for the first time to isolate monoolein from *I. sinicola* and investigate its anti-inflammatory activity on LPS-induced inflammatory response in bone marrow-derived dendritic cell (BMDCs) and RAW264.7 cells.

Materials and Methods

Isolation of monoolein from *Ishige sinicola* *I. sinicola* was shade dried and cut into small pieces. The seaweed (500.0 g) was extracted with 70% aqueous ethanol to give a gummy extract (76.9 g). The extract was then fractionated, leading to *n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and water-soluble portions. A part of the EtOAc-soluble fraction (2.0 g) was subjected to medium pressure liquid chromatography through silica gel using *n*-hexane-EtOAc-methanol gradients to obtain 44 fractions. The fraction number 21 (80.0 mg, ~0.1% yield of ethanol extract) was determined to be a pure compound, monoolein. The chemical structure of monoolein was identified by spectroscopic methods, including ^{1}H and ^{13}C NMR measurements. The monoolein structure was also confirmed by comparing the obtained data to the literature values (12). The chemical structure of monoolein isolated from *I. sinicola* is shown in Fig. 1A.

Mice Six-week-old female C57BL/6 mice were used for the study. Approval for all animal procedures was obtained from Institutional Animal Care and Use Committee of Jeju National University (No. 2010-0028).

Cell cultures and measurement of cytokine and NO production For growth of BMDCs, 6-week-old female C57BL/6 mice were used as previously described (15). Briefly, bone marrow cells from the tibia and femur were grown in RPMI 1640 (BD, Grand Island, NY, USA) medium supplemented with 10% heat-inactivated FBS, 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA), and 3% J558L hybridoma cell culture supernatant containing granulocyte–macrophage colony-stimulating factor for dendritic cell generation. More than 95% of the population of cells was dendritic cells as determined by FACS analysis. BMDCs were seeded and treated with indicated doses of monoolein for 1 h and stimulated for another 18 h with LPS. Production of murine IL-12 p40, IL-6, and TNF-α in harvested supernatants were measured through enzyme linked immunosorbent assay (ELISA) (BD PharMingen, San Jose, CA, USA), according to the manufacturer's instructions. Production of NO in RAW264.7 cells and BMDCs treated with monoolein and stimulated with LPS was measured using Griess reagent (Promega, Madison, WI, USA) as previously described (15).

Western blot analysis To evaluate the level of proteins in whole-cell lysate, western blot analysis was performed using standard techniques as previously described (15). Briefly, BMDCs and RAW264.7 cells were seeded in 35-mm culture dishes (Corning, Corning, NY, USA) at 2×10^{6} cells per dish. The cells were treated or left untreated h with monoolein (10 μM) for 1 h and then stimulated with LPS at the specified time points. The supernatant was removed, and cells were lysed in lysis buffer (PRO-PREP lysis buffer, iNtRON Biotechnology, Seongnam, Korea). Cell lysate was subjected to western blot analysis as previously described (15).

Statistical analysis All experiments were conducted at least three times, and the data are reported as the mean±standard deviation of three independent experiments. One-way ANOVA was used for comparing the treated and control groups. A p-value of <0.05 was considered to be statistically significant.

Results and Discussion

Inhibitory effect of monoolein on the production of pro-inflammatory cytokine in LPS-stimulated dendritic cells Dendritic cells are major components of mammalian immune system and play a critical role in the production of pro-inflammatory cytokines (2). IL-12, IL-6, and TNF-α are the pro-inflammatory cytokines and have a critical role in inflammation-associated and autoimmune diseases (8). To determine its anti-inflammatory activity, monoolein was investigated for the inhibition of IL-12 p40, IL-6, and TNF-α production in LPS-stimulated BMDCs. Monoolein pretreatment exhibited strong inhibition of IL-12 p40, IL-6, and TNF-α production in the LPS-stimulated BMDCs (Fig. 1B). These results show that monoolein has an inhibitory effect on the production of cytokines in LPS-stimulated BMDCs. To confirm the anti-inflammatory activity of monoolein, effect of monoolein on cell viability of BMDCs was estimated through the MTT assay. The results demonstrate that cell viability was not affected by monoolein at the indicated doses (data not shown). It has previously been shown that IL-12 p40, IL-6, and TNF-α are linked to many inflammation-associated diseases, including rheumatoid arthritis, asthma, and psoriasis (6). Thus, compounds such as monoolein that abrogate the level of these pro-inflammatory cytokines could be possible candidates for the treatment of such inflammatory diseases. IL-12 p40 has various critical immunoregulatory activities and is a main cytokine in Th1-mediated autoimmune responses (16,17); therefore, the downregulation of unregulated IL-12 p40 production by monoolein may have the potential to ameliorate IL-12 p40-related autoimmune diseases. IL-6 and TNF-α have important physiological roles; however, dysregulated production of these cytokines has been associated with various inflammatory diseases (18,19). Thus, in the future, controlling the overproduction of IL-6 and TNF-α by monoolein might be helpful in ameliorating inflammation-associated diseases.

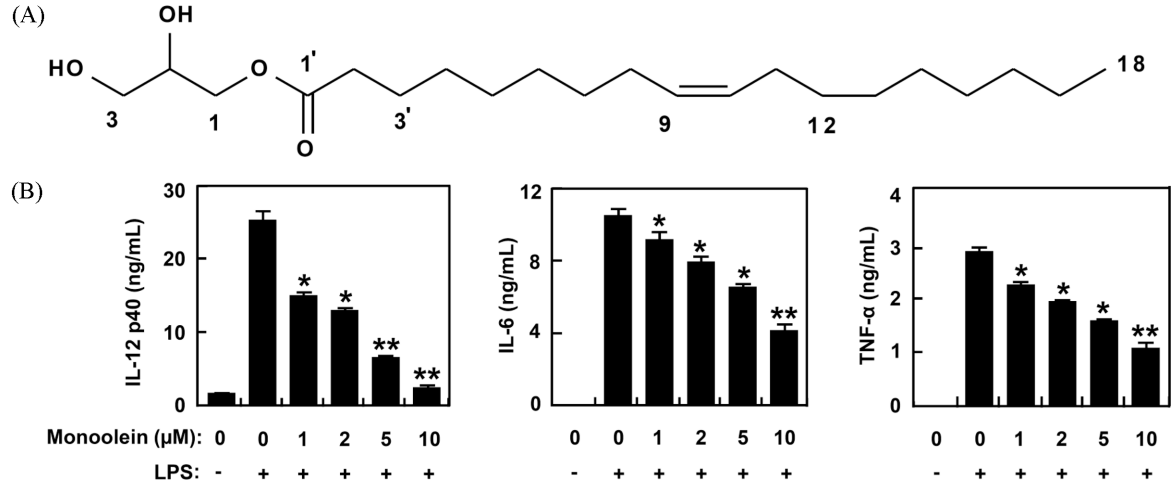

Fig. 1. Chemical structure of monoolein (A) and its inhibitory effect on IL-12 p40, IL-6, and TNF-α production in LPS-stimulated BMDCs (B). BMDCs were treated with monoolein at the indicated doses for 1 h before stimulation with LPS (10 ng/mL). Cytokines in culture supernatants were determined by ELISA. Data are representative of three independent experiments. *p<0.05, **p<0.01 vs. monoolein-untreated cells stimulated with LPS.

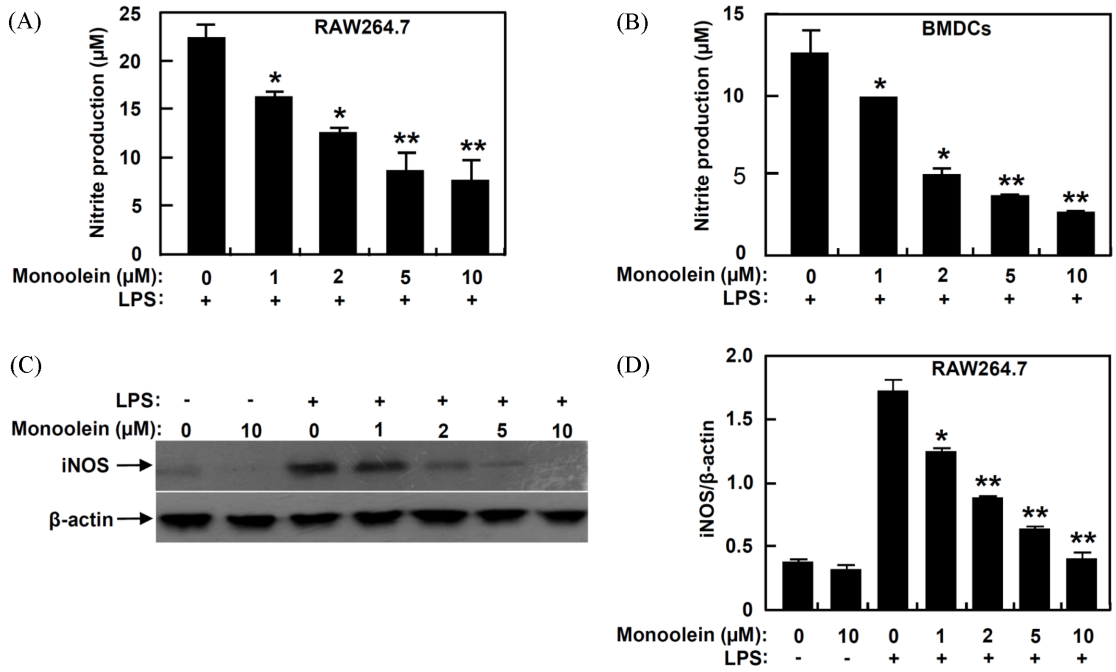

Fig. 2. Inhibitory effects of monoolein on production of NO in LPS-stimulated RAW264.7 cells and BMDCs and iNOS in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells and (B) BMDCs were treated with monoolein at the indicated doses for 1 h before stimulation with LPS (10 ng/mL). NO concentrations in culture supernatants were determined with Griess reagent. (C) RAW264.7 cells were pretreated or not treated with monoolein at the indicated doses for 1 h before stimulation with LPS. The protein levels of iNOS were determined using western blot analysis. β-Actin was taken as the loading control. (C) iNOS protein was quantified using scanning densitometry and band intensities were normalized by that of β-actin protein. Data are representative of three independent experiments. *p<0.05, **p<0.01 vs. monoolein-untreated cells in the presence of LPS.

Effect of monoolein on the production of NO in LPS-stimulated RAW264.7 cells and BMDCs and iNOS in LPS-stimulated RAW264.7 cells Macrophages and dendritic cells phagocytose pathogen and destroy it through reactive nitrogen intermediates, such as NO (7). NO plays a major role in inflammation (7). LPS stimulation causes the production of NO through iNOS (7). Therefore, we investigated whether monoolein inhibits the production of NO. RAW264.7 cells and BMDCs were pretreated with monoolein and then stimulated with LPS, and the quantity of NO release was evaluated. Monoolein pretreatment displayed a dose-dependent reduction in NO production in RAW264.7 cells (Fig. 2A) and BMDCs (Fig. 2B). iNOS is responsible for the high production of NO, causing cell injury and inflammation (7). To evaluate the effect of monoolein on the production of iNOS in LPS-stimulated RAW264.7 cells, western blot analysis was performed.

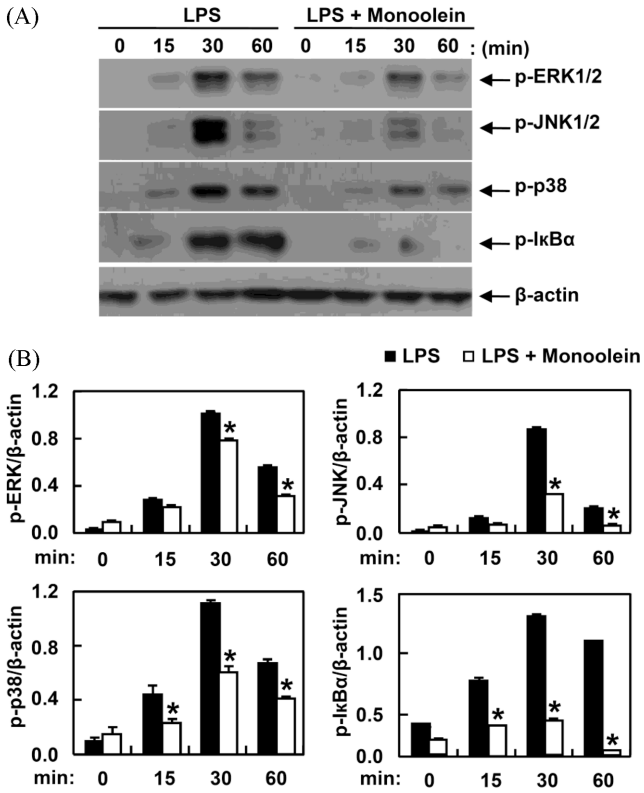

Fig. 3. Inhibitory effect of monoolein on phosphorylation of MAPK and IκBα in LPS-stimulated BMDCs. (A) BMDCs were treated with or without monoolein (10 μM) for 1 h and then stimulated with LPS (10 ng/mL) for the indicated time interval. The total cell lysate was obtained at the indicated time intervals. Western blot analysis was performed on the cell lysate to assess phosphorylation of ERK, JNK, p38, and IκBα. β-Actin was used as a loading control. (B) Phosphorylation of MAPKs and IκBα proteins were quantified using scanning densitometry and band intensities were normalized. Data are representative of three independent experiments. **p*<0.05 vs. monoolein-untreated cells stimulated with LPS.

Monoolein pretreatment substantially inhibited the production of iNOS in RAW264.7 cells (Fig. 2C and 2D). Thus, these results demonstrate that monoolein has significant inhibitory effect on the production of NO and iNOS. iNOS-derived NO overproduction is a ubiquitous mediator of a wide range of inflammatory conditions and reflects the degree of inflammation, which provides a measure of the inflammatory process (7). Overexpression of iNOS has been linked with different diseases, including arthritis, septic shock, and chronic inflammatory diseases (7). Thus, there is considerable evidence that inhibition of excessive iNOS-derived NO production will have anti-inflammatory effects. The present study proposes that monoolein has an inhibitory effect on the iNOS production and might be helpful in treating inflammatory diseases.

Effect of monoolein on the phosphorylation of MAPK and IκBα by LPS-stimulated BMDCs Stimulation of TLR4 by LPS elicits the activation of MAPK and NF-κB pathways, resulting in the production of pro-inflammatory cytokines (2,20). Therefore, through western blot analysis, we examined the effects on MAPK phosphorylation and NF-κB activation in LPS-stimulated BMDCs, with and without monoolein treatment (Fig. 3). Monoolein pretreatment in the presence of LPS showed strong inhibition of phosphorylation of ERK1/2, JNK1/2, and p38 (Fig. 3A and 3B). Activation of NF-κB pathway requires phosphorylation and subsequent degradation of IκBα that allows nuclear translocation of NF-κB (8). Activation of NF-κB pathway was indirectly analyzed through the phosphorylation of IκBα. Monoolein pretreatment strongly inhibited the phosphorylation of IκBα in LPS-stimulated BMDCs (Fig. 3A and 3B). Previous studies have shown that inhibitors that target MAPKs and NF-κB pathways exhibit anti-inflammatory activity (21). Therefore, these results demonstrate that monoolein can inhibit LPS-stimulated phosphorylation of ERK1/2, JNK1/2, p38, and IκBα and consequently inhibit MAPKs and NF-κB pathways.

In summary, we found for the first time that monoolein isolated from the brown alga *I. sinicola,* has an inhibitory effect on the production of pro-inflammatory cytokines and strongly inhibits MAPK and NF-κB pathways. Similarly, monoolein inhibited the production of NO and iNOS in LPS-stimulated RAW264.7 cells. Thus, monoolein exhibits anti-inflammatory activity. These data demonstrate that monoolein might be useful in the development of medicinal food supplements for the cure of inflammation-associated and autoimmune-related diseases. Thus, further studies are required on the detailed mode of action and *in vivo* efficacy of monoolein.

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Disclosure The authors declare no conflict of interest.

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