Differential Modulation of Lipopolysaccharide-Induced Inflammatory Cytokine Production by and Antioxidant Activity of Fomentariol in RAW264.7 Cells

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Abstract Medicinal mushrooms have been used worldwide to treat cancer and modulate the immune system. Over the last several years, there has been increasing interest in isolating bioactive compounds from medicinal mushrooms and evaluating their health beneficial effects. *Fomes fomentarius* is used in traditional oriental medicine and is known to possess antioxidant, anti-inflammatory, antidiabetic, and antitumor effects. In the present study, we isolated fomentariol from *Fomes fomentarius* and investigated its anti-inflammatory effect in murine macrophages (RAW264.7 cells) stimulated with lipopolysaccharides. Fomentariol inhibited the production of nitric oxide and intracellular reactive oxygen species triggered by lipopolysaccharides. Interestingly, fomentariol differentially regulated cytokine production triggered by lipopolysaccharides. Fomentariol effectively suppressed the production of interleukin-1β and interleukin-6 but not tumor necrosis factor-α. The inhibitory effect of fomentariol against nitric oxide, interleukin-1β, and interleukin-6 production was possibly mediated by downregulation of the extracellular signal-regulated kinase signaling pathway. Taken together, our results suggest that fomentariol differentially modulated inflammatory responses triggered by lipopolysaccharides in macrophages and is one of the bioactive compounds that mediate the physiological effects of *Fomes fomentarius*.

Keywords ERK signaling pathway, *Fomes fomentarius*, Fomentariol, LPS-induced inflammatory responses, RAW264.7 cells

Inflammation is an initial step in the host defense mechanisms against microbial infections and plays a role in the restoration and regeneration of damaged tissues. Macrophages are an essential component of the innate immunity and mediate inflammatory responses by recognizing pathogens and producing pro-inflammatory mediators. Nitric oxide (NO) and reactive oxygen species (ROS) are produced by activated macrophages and exert strong antimicrobial activities inside cells [1]. Macrophages also produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which then modulate the activation and differentiation of T and B cells, which are major players in the pathogen-specific adaptive immunity [2]. However, hyperactivation of inflammatory responses mediated by macrophages is closely related to various diseases such as autoimmune and metabolic [1, 2]. Therefore, targeting macrophages is well accepted as a promising therapeutic intervention to treat and prevent numerous diseases associated with inflammation.

Medicinal mushrooms have been widely used in traditional oriental medicines for years. The anti-inflammatory activities of crude extracts of medicinal mushrooms have been demonstrated in previous studies using [3-7]. Recently, considerable efforts have been focused on the isolation of single compounds with potent and unique biological activities [8-11]. *Fomes fomentarius* is a bracket fungus commonly known as horse’s hoof fungus, which has been used as a traditional medicine in China and Korea for centuries to treat various disease conditions including cancers and disorders.
of the gastrointestinal tract [12]. Recent studies demonstrated that water and methanol extracts of \textit{F. formetarius} exerted antidiabetic and anti-inflammatory/antinociceptive activities, respectively [13, 14]. However, the bioactive compounds mediating with these activities were unknown. Recently, we reported that 9-oxo-10E,12E-octadecadienoate (FF-8) isolated from the methanol extract of \textit{F. formetarius} (MEFF) exerted anti-inflammatory activity by suppressing signal transducer and activator of transcription 3 (STAT3) activation. To the best of our knowledge, this is the first study demonstrating the anti-inflammatory activity of this compound. During our investigation of the bioactive constituents of wild mushrooms, fomentariol was isolated from the methanolic extract of the fruiting body of \textit{F. fomentarius}. Fomentariol, a benzotropolone derivative, was previously isolated from \textit{F. fomentarius} and \textit{F. somentarius}, and was synthesized [15, 16]. Despite its interesting structural skeleton, reports of its biological activity are limited. Therefore, in this study, we investigated its anti-inflammatory activity using the RAW264.7 murine macrophage cell line.

**MATERIALS AND METHODS**

**Chemicals and antibodies.** Lipopolysaccharide (LPS; \textit{Escherichia coli} 0111:B4), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, dimethyl sulfoxide (DMSO), 2,7'-dichlorofluorescein diacetate (DCFH-DA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The primary antibodies used were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) except for phospho-p65 (Cell Signaling, Danvers, MA, USA), phospho-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I\(\kappa\)B\(\alpha\); Cell Signaling), and \(\beta\)-actin (AbFrontier Company, Seoul, Korea). The secondary antibodies were purchased from Thermo Scientific (Logan, UT, USA). All other chemicals were obtained from Sigma-Aldrich Corp. unless otherwise stated.

**Isolation and purification of fomentariol.** The fruiting body of \textit{F. fomentarius} (fresh weight 7.1 kg) was ground and extracted twice with methanol at room temperature. The methanolic extract was partitioned consecutively between hexane, chloroform, ethyl acetate, butanol, and water. The hexane-soluble portion was concentrated under reduced pressure, subjected to silica gel column chromatography, and eluted stepwise using a hexane:ethyl acetate gradient (100:1 to 1:1, v/v). An active fraction was chromatographed using a Sephadex LH-20 column eluted with a chloroform:methanol solution (1:1, v/v), followed by preparative high-performance liquid chromatography with a reverse-phase column and elution with 75% aqueous methanol to obtain the active compound. The chemical structure of the active compound was determined as fomentariol using extensive one- and two-dimensional nuclear magnetic resonance spectroscopy and mass measurements (Fig. 1A). The spectroscopic data were well matched to those previously reported in the literature [16, 17].

![Fig. 1. Effect of fomentariol on cell viability. A, Structure of fomentariol. RAW264.7 cells were pretreated with fomentariol for 2 hr and stimulated with lipopolysaccharide (LPS; 500 ng/mL) for additional 24 hr; B, Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; C, Levels of nitric oxide in culture supernatants were measured using Griess reaction assay; D, Cell lysates were prepared for western blot analysis using a specific inducible nitric oxide synthase (iNOS) antibody as described in the Materials and Methods. \(\beta\)-Actin was the internal control. ND, not detectable. Data are mean \(\pm\) SEM of three independent experiments; \(*p < 0.01\) and \(**p < 0.001\) compared with LPS alone.](image-url)
Cell culture. Murine RAW264.7 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution (all from Thermo Scientific). The cells were maintained at 37°C in a 5% CO₂, humidified atmosphere and experiments were conducted on cells at approximately 70–80% confluence.

Cell viability. The cells were pretreated with formentariol for 2 hr, stimulated with LPS for an additional 24 hr, and then viability was measured using an MTT assay [17]. This assay is based on the reducing activity of mitochondria in living cells, which convert formazan from the oxidized (soluble) to the reduced (insoluble) form. The MTT solution (0.5 mg/mL) was added to each well of a culture plate, and after 3 hr, the medium was discarded. The formazan formed in each well was dissolved in DMSO. The optical density was measured, which is proportional to living cells and expressed as a percentage of control.

Determination of NO. Cells were seeded at a density of 5 x 10^4 cells per well in 96-well plates and pretreated with varying concentrations of FF-8 for 2 hr followed by treatment with LPS (500 ng/mL) for 24 hr in the presence of FF-8. Cell culture supernatants were collected and mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethlenediamide) at room temperature for 10 min. The absorbance was measured at 570 nm using a microplate reader (BioTek, Winooski, VT, USA); the nitrite level was calculated from a sodium nitrite standard curve, and the NO concentrations in the supernatant were determined by comparison with the standard curve.

Measurement of intracellular ROS. Incorporation of DCFH-DA into cells leads to its conversion to 2,7-dichlorofluorescein (DCF) by an oxidative processes. Therefore, this method is widely used to evaluate the intracellular redox status. RAW264.7 cells were preincubated with or without formentariol for 2 hr and then continuously stimulated with LPS (500 ng/mL) for 24 hr. After washing thrice with phosphate-buffered saline, the cells were treated with serum-free medium containing H_2DCFDA 20 μM (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C in the dark, washed again with cold phosphate-buffered saline three times, and then the DCF fluorescence was detected using a fluorescence microscope (Carl Zeiss, Jena, Germany) and Accuri flow cytometer (BD Biosciences, San Diego, CA, USA).

Measurement of cytokine production. The levels of TNF-α and IL-6 production in the cell culture supernatant were measured using respective ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Immunoblotting. For immunoblotting, cell lysates were prepared using the M-PER mammalian protein extraction reagent containing a phosphatase/protease cocktail inhibitor (Thermo Scientific). Protein concentrations were determined, and 50 μg of protein from each sample was resolved using SDS-PAGE, transferred to polyvinylidene fluoride membranes (Amersham, Bucks, UK), which were blocked with 5% skim milk (Difco, Detroit, MI, USA). Then, the membranes were probed with primary antibodies against inducible nitric oxide synthase (iNOS) and the phosphorylated or total nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)/IkBα and mitogen-activated protein kinases (MAPK) namely, extracellular signal-regulated kinase (ERK), p38, and c-Jun terminal kinase (JNK) at 4°C overnight. Species-appropriate horseradish peroxidase-conjugated IgGs were used as secondary antibodies. Finally, proteins were visualized using a SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific) and protein bands were detected using the LAS 4000 mini luminescent image analyzer (GE Health-Care, Buckinghamshire, UK).

Statistical analysis. Values are expressed as the mean ± SEM. The data analysis was carried out using the GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test was used to compare means between groups. Differences with a p < 0.05 were considered statistically significant.

RESULTS

Fomentariol suppressed NO production by inhibiting iNOS expression. The RAW264.7 cell line innately exhibits the characteristics of monocytes and macrophages [18]. Therefore, this cell line is commonly used to study molecules that modulate macrophage functions. Here, we first tested whether fomentariol affected cell viability using an MTT assay and Fig. 1B shows that it did not induce cytotoxic activity at the concentrations (10 and 20 μg/mL) tested, regardless of LPS pretreatment. Then, we determined whether formentariol affected NO production in LPS-stimulated RAW264.7 cells. The nitrite concentrations of the cell culture supernatants were measured using the Griess reagent as an indicator of the NO levels. While treatment with formentariol alone did not activate RAW264.7 cells to produce NO, stimulation with LPS remarkably increased the cellular level of NO. When cells were treated with fomentariol for 2 hr prior to LPS stimulation for 24 hr, the LPS-induced NO production was significantly reduced dose-dependently manner as shown in Fig. 1B. To determine whether fomentariol reduced NO production by modulating the level of iNOS, we evaluated its protein expression using immunoblotting. Similar to NO production, iNOS protein expression was not induced by fomentariol but significantly increased following LPS treatment. Furthermore, pretreatment with formentariol efficiently downregulated iNOS expression...
in RAW264.7 cells stimulated with LPS, which correlated with the reduced level of NO production.

**Fomentariol suppressed ROS production.** The levels of ROS were evaluated using fluorescence microscopy in the absence or presence of fomentariol in RAW264.7 cells (Fig. 2A). Without LPS treatment, ROS was not detected inside the cells but following LPS treatment, a strong fluorescence signal induced by an increase in ROS production was observed. Fomentariol treatment significantly suppressed ROS production triggered by LPS. Next, the suppressant effect of fomentariol on LPS-induced ROS production was further confirmed using flow cytometric analysis. RAW264.7 cells were pretreated with fomentariol and then stimulated with LPS for 24 hr. As shown in Fig. 2B, LPS induced the production of ROS as detected by the rightward shift in the fluorescence signal production, and this effect was counteracted by fomentariol pretreatment dose-dependently. The statistical analysis of the mean fluorescence intensity is shown in the right panel of Fig. 2B. A higher concentration (20 μg/mL) of fomentariol lowered the level of ROS close to that of the unstimulated cells.

**Fomentariol differentially modulated cytokine production.** To further analyze the anti-inflammatory effect of fomentariol, we determined its effect on the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 using an ELISA. Cells pretreated with fomentariol without LPS did not show an increase in the secretion of any of the cytokines analyzed in this study (Fig. 3) while those stimulated with LPS produced significant amounts. Interestingly, fomentariol pretreatment showed differential effects on cytokine production. While there was no difference in the TNF-α level of LPS-stimulated cells with and without pretreated with fomentariol, pretreatment with fomentariol significantly suppressed IL-1β and IL-6 in LPS-stimulated cells by approximately 60% and 30%, respectively.

**Fomentariol suppressed ERK1/2 signaling.** Cytokine production is regulated by various signal transduction...

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**Fig. 2.** Effect of fomentariol on reactive oxygen species production. RAW264.7 cells were preincubated with or without fomentariol for 2 hr and then activated with lipopolysaccharide (LPS, 500 ng/mL) for 24 hr. Serum-free medium containing H₂DCFDA 20 mM (Invitrogen) was added to cells, followed by 30 min incubation (37°C). 2,7-Dichlorofluorescein fluorescence was evaluated using fluorescence microscopy (A) and flow cytometric analysis (B). Mean fluorescence intensities measured using flow cytometer are shown as bar graph (C). Data are mean ± SEM of three independent experiments; **p < 0.01 compared with LPS alone.**
Fig. 3. Effect of fomentariol on pro-inflammatory cytokine production. RAW264.7 cells were preincubated with or without fomentariol for 2 hr and then activated with lipopolysaccharide (LPS; 500 ng/mL) for 24 hr. Cell culture supernatants were collected for cytokine analysis. Tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B), and IL-6 (C) were measured using ELISA. ND, not detectable. Data are mean ± SEM of independent three experiments; *p < 0.05 and ***p < 0.001 compared with LPS alone.

Fig. 4. Effect of fomentariol on lipopolysaccharide (LPS)-induced nuclear factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase signaling pathways. RAW264.7 cells were pretreated with fomentariol for 2 hr followed by stimulation with LPS (500 ng/mL) for 15 or 30 min. Levels of phospho- and total proteins of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBa), p65 (A), extracellular signal-regulated kinase (ERK), p38, and c-Jun terminal kinase (JNK) in cell lysates were determined using specific antibodies (B). β-Actin was the internal control. A representative blot from three independent experiments is shown. Bar graphs represent relative densities normalized to total ERK or JNK (C). Data are shown as mean ± SEM of three independent experiments; *p < 0.05 compared with LPS alone.
pathways, and previous studies have demonstrated that compounds with anti-inflammatory activity often interfere with the NF-κB and MAPK pathway [4, 5, 9, 11, 19]. To determine the specific signaling pathway targeted by fomentariol, cells were pretreated with fomentariol and then stimulated with LPS for a short time. Analysis of the cell lysates to evaluate the activation of signaling molecules in the NF-κB and MAPK pathway revealed no differences between untreated and treated cells in levels of NF-κB pathway molecules (Fig. 4A). Similarly, the levels of phosphorylation of p38 were not changed following fomentariol treatment. In contrast, activation of the ERK1/2 and JNK signaling pathway was significantly inhibited by fomentariol (Fig. 4B and 4C), indicating that these pathways could have been the targets of fomentariol, which altered IL-1β and IL-6 but not TNF-α expression.

**DISCUSSION**

Previous studies demonstrated that the extracts of *F. fomentarius* possessed various biological activities. For example, Aoki *et al.* [20] reported that polysaccharides from *F. fomentarius* inhibited the viral infection of tobacco, bell pepper, and tomato plants. In addition, the melanin-glucan complex isolated from *F. fomentarius* was also suggested to possess anti-infective activities against *Helicobacter pylori, Candida albicans, Herpes vulgaris*, I, and human immunodeficiency virus 1 zmb in another study [21]. The anti-inflammatory effect of MEFF is well demonstrated by Park *et al.* [14] who discovered that it effectively suppressed LPS-mediated inflammatory responses including the production of NO, prostaglandin E2, and TNF-α via downregulation of the NF-κB pathway in the RAW264.7 murine macrophages cell line [14]. However, the bioactive compounds responsible for these anti-inflammatory activities were not identified until we reported that FF-8 suppressed the LPS-induced activation of macrophages. In the present study, we identified fomentariol as a constituent that contributes to the anti-inflammatory effect of MEFF along with FF-8. However, in contrast to FF-8 that inhibited the phosphorylation of STAT3 triggered by LPS, fomentariol exerted its suppressive effect on the activation of the MAPK pathway.

Toll-like receptors (TLRs) are the most studied pathogen-associated molecular pattern receptors, which play essential roles in initiating innate immune responses against bacteria and viruses. TLR4, which recognizes LPS, is highly expressed by macrophages, dendritic cells, and monocytes [1]. When gram-negative bacteria are encountered, LPS present in the outer membrane binds to TLR4 on the surface of macrophages. Then, activated macrophages trigger inflammatory responses to exert innate immune responses, followed by acquired immune responses driven by T and B cells. However, excessive or uncontrolled activation of macrophages is associated with chronic inflammatory diseases [2]. Therefore, targeting TLR4 signaling to dampen this deleterious immune response is considered as a therapeutic approach for various diseases involving dysregulated macrophage activation. We found that fomentariol, a benzotropolone derivate, suppressed the activation of macrophages induced by LPS, indicating that this compound possesses anti-inflammatory activity.

NO, a free radical that was initially considered a vasodilator, is involved in the pathogenesis of numerous diseases by mediating inflammatory responses. The production of NO is catalyzed by iNOS, which converts arginine into NO and citrulline [22]. Here, we found that fomentariol partially suppressed NO production by inhibiting the expression of iNOS in macrophages. In addition to NO, ROS is generated by LPS-stimulated macrophages and is involved in the tissues injury induced by excessive inflammatory responses as well as in the destruction of pathogens inside macrophages [23]. In our study, we observed that fomentariol treatment apparently blocked ROS generation and restored the balance of the intracellular redox state by attenuating the oxidative stress. Oxidative stress caused by abnormal production of ROS is known to play a crucial role in the pathogenesis of metabolic diseases such as type 2 diabetes and atherosclerosis [24]. Based on our results, it would be interesting to investigate whether fomentariol also ameliorates oxidative stress caused by the accumulation of lipids or high glucose levels, which are involved in type 2 diabetes [25].

Various cytokines are produced by macrophages following LPS stimulation and initiate inflammatory responses. Among them, TNF-α, IL-1β, and IL-6 are major players in the inflammatory process [22]. In the present study, we found that fomentariol-treated macrophages produced a significantly lower amount of IL-1β and IL-6 than the untreated macrophages did. Furthermore, the degree of inhibition was greater with IL-1β than it was with IL-6. Interestingly, TNF-α, a potent inflammatory cytokine, was not affected by fomentariol pretreatment of LPS-stimulated macrophages. These results indicate that fomentariol does not completely block the LPS-mediated activation of macrophages, which would lead to a complete downregulation of all inflammatory cytokines as well as NO and ROS. Instead, fomentariol differentially affects the production of specific inflammatory cytokines to varying degrees. Several studies have demonstrated that docosahexaenoic acid (DHA) also differentially modulates the expression of cytokines [19, 26, 27]. For example, a recent study by Honda *et al.* [27] demonstrated that DHA significantly reduced the mRNA expression of IL-6 but not TNF-α [27]. Taken together, our results suggest that fomentariol modulates LPS-mediated inflammatory responses by targeting IL-6 and IL-1β but not TNF-α.

Following ligation of LPS by TLR4, a series of intracellular signaling pathways are activated, which ultimately induce the transcription/translation of inflammatory cytokines. To elucidate the underlying molecular mechanisms by which fomentariol exerts anti-inflammatory activity, its influence
on the activation of the NF-κB pathway was evaluated in macrophages challenged with LPS. As shown in Fig. 4A, fomentariol did not have any effects on the phosphorylation of p65 and its inhibitor, IkBα. This finding indicates that the suppressive effects of fomentariol against NO, IL-6, and IL-1β are not due to impaired activation of NF-κB signaling. Activation of the MAPK pathway, which consists of p38, ERK, and JNK pathways, is also triggered by LPS and mediates the transcription of various cytokines and chemokines. Here, we found that fomentariol suppressed the phosphorylation of both ERK and JNK while it did not affect p38. These results suggest that these two signaling pathways are possible targets of fomentariol and could be involved in mediating the reduction in levels of NO, IL-6, and IL-1β.

Taken together, the results presented in this study demonstrate that fomentariol differentially modulated the production of inflammatory cytokines and exerted suppressive effects on NO and ROS. These effects may be mediated, at least in part, by the suppression of ERK and JNK signaling pathways. Our findings highlight the potential of fomentariol as a novel therapeutic agent for treating inflammatory diseases. Further in vivo studies are essential to confirm the therapeutic or preventive effect of fomentariol in animal diseases models.

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