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RESEARCH ARTICLE

Benzyl alcohol derivatives from the mushroom *Hericium erinaceum* attenuate LPS-stimulated inflammatory response through the regulation of NF-κB and AP-1 activity

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Abstract

On the search for anti-inflammatory compounds from natural Korean medicinal sources, a bioassay-guided fractionation and chemical investigation of the MeOH extract from the fruiting bodies of *Hericium erinaceum* resulted in the isolation and identification of five benzyl alcohol derivatives (**1–5**). In this study, their anti-inflammatory effects on lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators were examined using RAW 264.7 macrophage cells. The structures of isolates were identified by comparing their spectroscopic data with previously reported values. The analysis of their inhibitory activities on LPS-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in RAW 264.7 macrophage cells showed that erinacerin B (**2**) and hericenone E (**4**) decreased the levels of NO and PGE_2 production in a concentration-dependent manner. Next, this study was performed to examine their mechanism of action on the regulation of NO and PGE_2 production. Compounds **2** and **4** were found to block the LPS-induced phosphorylation of two major inflammatory transcription factors, NF-κB (p65/p50) and AP-1 (c-Jun and c-Fos). Taken together, these results suggest that down-regulation of LPS-induced NO and PGE_2 production by compounds **2** and **4** is mediated through the modulation of NF-κB and AP-1 activation in macrophage cells. These results impact the development of potential health products for preventing and treating inflammatory diseases.

Keywords

Erinacerin B, hericenone E, *Hericium erinaceum*, nitric oxide, prostaglandin E_2, RAW 264.7 macrophage cells

History

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Introduction

Nitric oxide (NO) is a signaling molecule synthesized by the enzyme nitric oxide synthase (NOS) from the oxidation of L-arginine, and is involved in a number of physiological and pathophysiological processes such as immune and inflammatory responses and neuronal transmission in the brain[1]. Three forms of NOSs have been identified[2], and two of them (cNOS; neuronal and endothelial NOS) are constitutive forms, which produce low concentration of NO that is essential for maintaining tissue homeostasis. On the other hand, an inducible form of NOS (iNOS) produces high concentration of NO which is involved in various pathological conditions. The iNOS expression is induced in response to various pro-inflammatory stimuli, including lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interferon-γ, prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6), and is associated with various chronic inflammatory diseases[3,4]. Since overproduced NO is responsible for inflammation, discovery of agents for inhibition of NO production has been attractive target for alleviating or treating chronic inflammatory diseases.

Hericium erinaceum (Hericiaceae) (also called Lion's Mane Mushroom) is an edible and medicinal mushroom used in Korea, Japan and China for treating dyspepsia, gastric ulcer and enervation[5]. Previous phytochemical studies on this mushroom resulted in the isolation of unique metabolites, including hericenones A-J and hericerin[6–9]. In addition, pharmacological studies with some of these isolated compounds reported to have important biological activities, including cytotoxicity, stimulation of nerve growth factor (NGF)-synthesis, suppression of endoplasmic reticulum stress and inhibitory of pollen growth[6–9]. Recently, we reported the isolation of a new isoindolinone alkaloid, together with nine known compounds and their cytotoxic activities[10]. However, to the best of our knowledge, anti-inflammatory constituents from *H. erinaceum* in LPS-treated RAW264.7 macrophages have not yet been reported, although it was reported that its extract showed anti-inflammatory properties by suppressing LPS-induced pro-inflammation gene activation in RAW264.7 macrophages[11]. Here, we investigated anti-inflammatory

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metabolites from *H. erinaceum* using the bioactivity-guided isolation techniques and their mode of action on the inhibition of LPS-induced NO and PGE_2 production.

Materials and methods

Materials

Analytical-grade solvents (SAMCHUN CHEMICALS, Seoul, Korea) were used for extraction, fractionation and isolation. HPLC grade solvents were purchased from Burdick & Jackson and Fisher Scientific. LPS (*E. coli* 0111:B4) and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Standard compounds [BAY11-7082 (purity >95%), an IκB kinase (IKK) inhibitor, and U0126 (purity >95%), an extracellular signal-regulated kinase (ERK) inhibitor] were obtained from Calbiochem (La Jolla, CA). A GFP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Foetal bovine serum and RPMI 1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All of the other chemicals were purchased from Sigma Chemical Co.

Mushroom materials

H. erinaceum (Hericiaceae) was purchased from Pochun Mushroom Development Co., Ltd. at Pochun-gun in Gyunggi-Do province, Korea, in July 2010. A voucher specimen (SKKU-2010-07) of the mushroom has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation

Partially dried fruiting bodies of *H. erinaceum* (5.0 kg) were extracted twice with 80% MeOH at room temperature. The resultant 80% MeOH extracts (500 g) were suspended in distilled water (10 L) and successively partitioned with *n*-hexane (800 mL × 3 times), CH_2Cl_2 (800 mL × 3 times), EtOAc (800 mL × 3 times) and *n*-BuOH (800 mL × 3 times), yielding residues weighing 63.3, 4.5, 2.0 and 17.5 g, respectively. Each fraction was evaluated for an inhibitory effect on NO production and cell viability in LPS-treated RAW264.7 cells. The *n*-hexane-soluble and CH_2Cl_2-soluble fractions displayed strong suppressive effects on NO production in the macrophage cells.

The *n*-hexane-soluble fraction (60 g) was chromatographed on a silica gel column using a gradient solvent system of *n*-hexane-EtOAc (50:1 to 1:1) to yield five fractions (fr. A-E). Fraction E (10 g) was separated over RP-C_{18} silica gel using a gradient solvent system of MeOH-H_2O (1:9–1:0). According to TLC analysis, three sub-fractions (fr. E1-E3) were collected. Fraction E1 (1 g) was separated using a reverse phase RP-C_{18} silica gel column (a gradient solvent of 40–70% MeOH/H_2O) and purified further by semi-preparative reverse-phase HPLC using a solvent system of 60% MeOH/H_2O to obtain compound **1** (38 mg). HPLC apparatus (Gilson, Middletone, WI) consisted of a vacuum degasser, a quaternary pump, a photodiode array detector, an auto injector and a column compartment with a thermostat. Fraction E3 (1 g) was applied to RP-C_{18} silica gel column (a gradient solvent of 50–70% MeOH/H_2O) and purified further by semi-preparative reverse-phase HPLC using a solvent system of 50% MeOH/H_2O to give compound **3** (36 mg).

The CH_2Cl_2-soluble fraction (4 g) was chromatographed over a silica gel column with a gradient solvent system of MC-MeOH (50:1–1:1) to afford five fractions (fr. A-E). Fraction B (1 g) was separated using RP-C_{18} silica gel using a gradient solvent system of MeOH-H_2O (1:9–1:0). According to TLC analysis, five sub-fractions (fr. B1-B5) were obtained. Fraction B4 (0.2 g) was separated using a reverse-phase RP-C_{18} silica gel column (a gradient solvent of 50–70% MeOH/H_2O) and purified further by semi-preparative reversed-phase HPLC using a solvent system of 50% MeOH/H_2O to yield compound **2** (4 mg). Fraction E (0.9 g) was subjected to RP-C_{18} silica gel column chromatography using a gradient solvent system of MeOH-H_2O (1:9–1:0) to obtain two sub-fractions (fr. E1-E2). Fraction E1 (0.3 g) was separated over RP-C_{18} silica gel column chromatography (a gradient solvent of 40–70% MeOH/H_2O) and purified further by semi-preparative reversed-phase HPLC using a solvent system of 60% MeOH/H_2O to yield compounds **4** (7 mg) and **5** (50 mg). The tested compounds were demonstrated to be pure as evidenced by NMR and HPLC analyses (purity ≥95%).

Cell culture

RAW264.7 cells were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a 5% CO_2 atmosphere. For each experiment, the cells were detached with a scraper. Examination of cell densities of 2×10^6 cells/mL revealed that the proportion of dead cells was consistently <1% using a Trypan blue dye exclusion assay as the criteria for viability.

Determination of NO and PGE_2 production

After pre-incubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, cells were pre-treated with MeOH extract, its sub-fractions, or the isolated compounds for 30 min. The cells were then further incubated with LPS (1 µg/mL) for 24 h. The inhibitory effects of MeOH extract, sub-fractions or the isolated compounds on NO and PGE_2 production were determined using the Griess reagent as described previously[12,13].

Cell viability

After pre-incubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, cells were treated with MeOH extract or the isolated compounds and incubated for 24 h. The cytotoxic effects of MeOH extract and the isolated compounds were evaluated by a conventional MTT assay, as reported previously[14]. Three hours prior to the termination of the cell cultures, 10 µL of a MTT solution (10 mg/mL in phosphate-buffered saline, pH 7.4) was added, and the cells were continuously cultured until termination of the experiment. The incubation was halted by the addition of 15% sodium dodecyl sulfate into each well to solubilize the produced formazan[15]. The absorbance at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader.

Immunoblot analysis

HEK293 cells (5×10^6 cells/mL) were lysed in buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium or thovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 1 mM benzimidine and 2 mM hydrogen peroxide) for 30 min at 4 °C under rotation. The lysates were clarified by centrifugation at $16\,000 \times g$ for 10 min at 4 °C. The soluble cell lysates were then immunoblotted, and the total or phospho-levels of NF-κB or AP-1 family proteins were visualized as previously reported[16].

Statistical analysis

All of the data are presented as the mean ± SEM of three different experiments performed in triplicate. For statistical comparisons, we used analysis of variance with Scheffe's *post hoc* test and a Kruskal–Wallis/Mann–Whitney test. A p-value < 0.05 was considered to be statistically significant. All of the statistical tests were performed using SPSS (SPSS Inc., Chicago, IL).

Results

Isolation and identification of active compounds from *H. erinaceum*

Partially dried fruiting bodies of *H. erinaceum* were extracted twice with 80% MeOH at room temperature. The resultant MeOH extracts showed inhibitory activity on NO production in LPS-treated RAW264.7 macrophages (Figure 1), as reported previously.[11] The MeOH extracts were suspended in distilled water and successively partitioned with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. Each fraction was evaluated for an inhibitory effect on NO production and cell viability in LPS-treated RAW264.7 cells. The *n*-hexane-soluble fraction showed moderate inhibitory activity against NO production without significant cell death in the macrophage cells, and the CH_2Cl_2-soluble fraction displayed potent suppressive effect on NO production (the fraction had lower cell viability at concentration of 300 μg/mL) (data not shown). On the other hand, the EtOAc-soluble and *n*-BuOH-soluble fractions had no inhibitory effect. Therefore, we investigated the *n*-hexane-soluble and CH_2Cl_2-soluble fractions in order to identify the active constituents responsible for anti-inflammatory effect. The active fractions were further subjected to repeated column chromatography, followed by semi-preparative HPLC purification to afford five benzyl alcohol derivatives (**1–5**) (Figure 2). Their structures were identified as

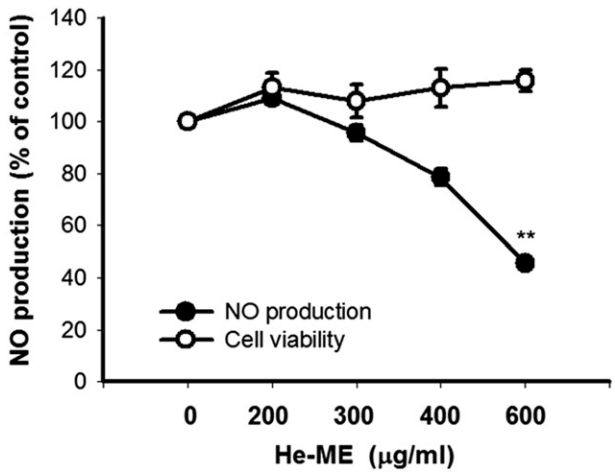
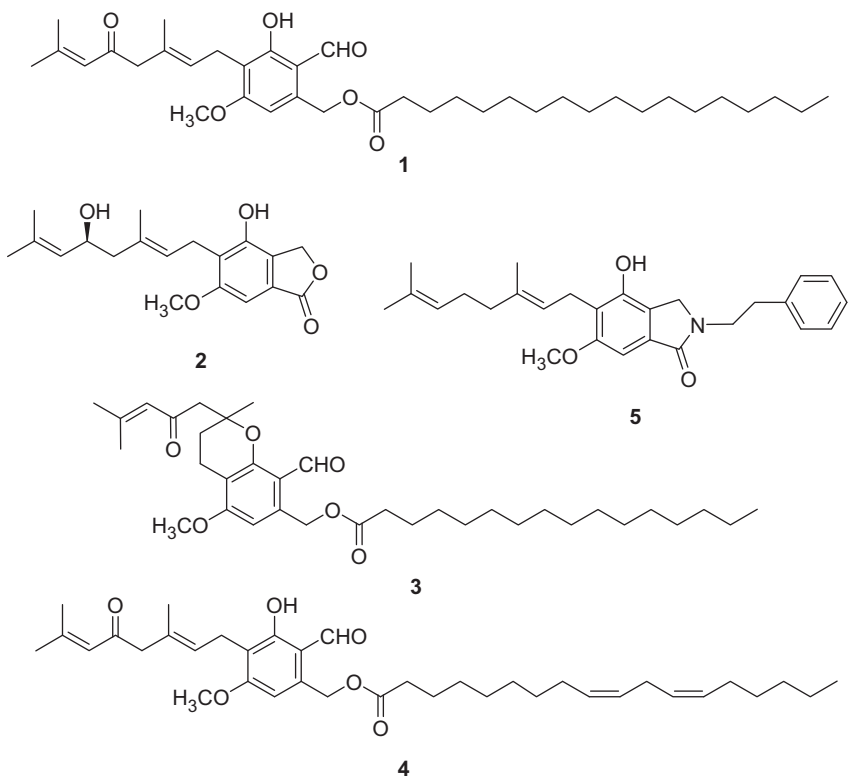


Figure 1. Effects of the MeOH extract of *H. erinaceum* on production of NO. The levels of NO were determined by the Griess assay or EIA from culture supernatants of RAW264.7 cells treated with MeOH extract (0–600 μg/mL). **: $p < 0.01$ compared with control or normal.

Figure 2. Chemical structures of compounds **1–5**.



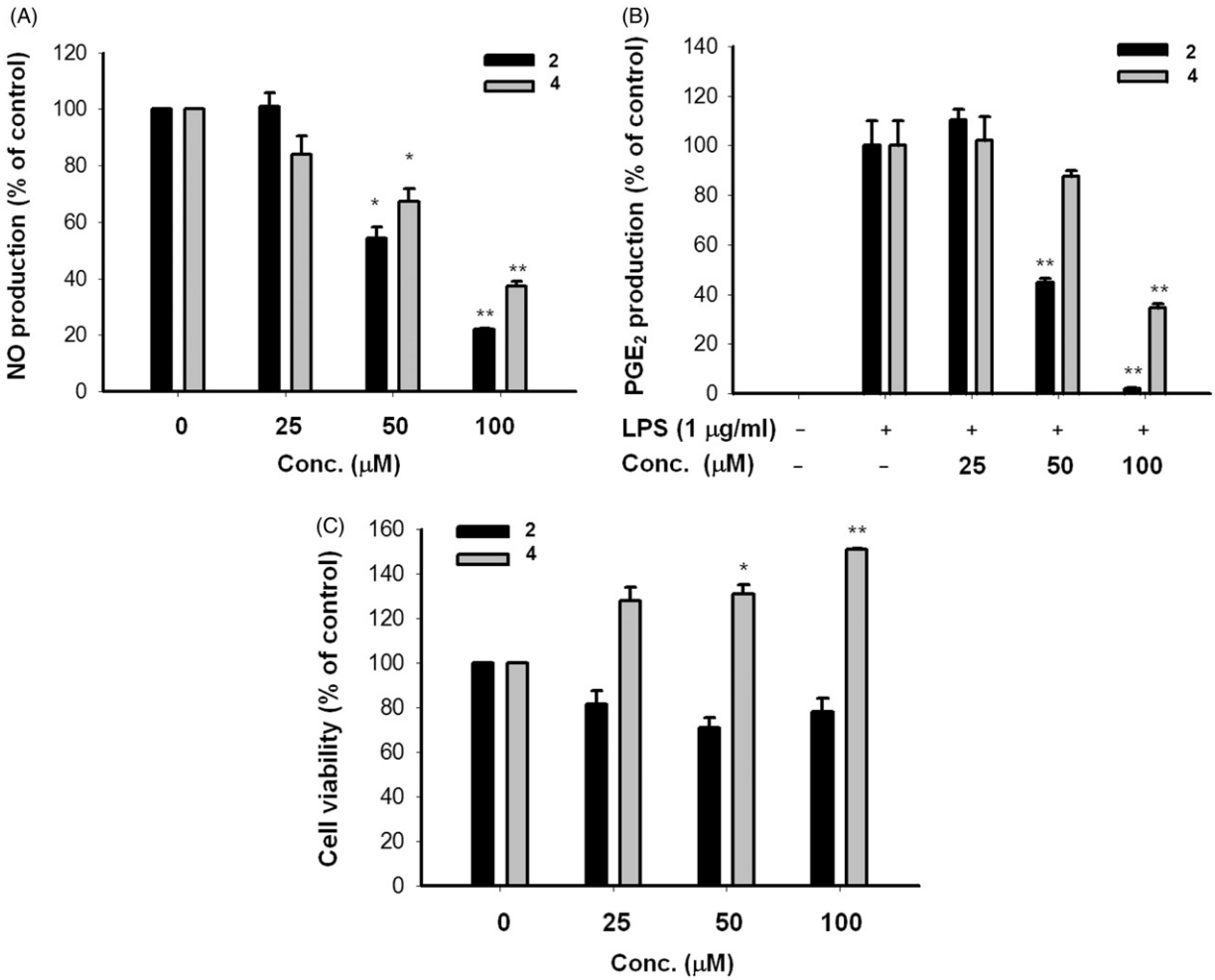


Figure 3. Effects of the compounds **2** and **4** on production of NO and PGE_2 in LPS-induced macrophage cells. (A) Effect of the compounds **2** and **4** on LPS-induced NO production in macrophages. (B) Effect of the compounds **2** and **4** on LPS-induced PGE_2 production in macrophages. The levels of NO and PGE_2 were determined by the Griess assay from culture supernatants of RAW264.7 cells treated with compounds (**2** and **4**) and LPS (1 μg/mL) for 24 h. (C) Cell viability of RAW264.7 cells treated with compounds (**2** and **4**) was determined by an MTT assay. *: $p < 0.05$ and **: $p < 0.01$ compared with control or normal.

hericenone D (**1**)[7], erinacerin B (**2**)[17], hericenone F (**3**)[18], hericenone E (**4**)[7], and isohericerin (**5**)[10] by comparing their spectroscopic data with previously reported values.

Effects on the isolated compounds on LPS-induced NO production

Primarily, we evaluated the anti-inflammatory activities of the isolated compounds (**1–5**) in LPS-treated RAW264.7 macrophages by measuring the production level of NO, which is a representative inflammatory mediator released from activated macrophages[19]. The treatment of compounds **2, 4** and **5** clearly inhibited the LPS-induced NO production at a concentration of 100 μM (data not shown). However, a significant effect on cell viability was observed in the tested concentration of compound **5** as determined by MTT assay[14,15], indicating that the inhibitory effect of **5** on NO production was mediated by its cytotoxicity.

Effects on the compounds 2 and 4 on LPS-induced NO and PGE_2 production

Next, in accordance with our finding, the effects of the compounds **2** and **4** on production of NO and PGE_2 in LPS-induced macrophage cells were examined. Treatment of LPS-stimulated cells with compounds **2** and **4** decreased the levels of NO and PGE_2 production in a concentration-dependent manner (Figure 3A and 3B). The IC_{50} values of the inhibitory of NO and PGE_2 production were summarized in Table 1. Cell viability of the macrophages was not affected by adding up to 100 μM of compounds **2** and **4** (Figure 3C).

Table 1. IC_{50} values for the inhibitory effects on NO and PGE_2 production of compounds **2** and **4**.

	IC_{50} value (μM)[a]	
Compound	NO	PGE_2
2	67.36 ± 2.53	60.10 ± 0.42
4	78.95 ± 4.73	85.20 ± 0.30

[a]IC_{50} value of each compound was defined as concentration (μM) causing 50% inhibition of NO and PGE_2 production in macrophage cells.

Effects of the compounds 2 and 4 on the phosphorylation of NF-κB and AP-1 subunits

To briefly understand the molecular mechanism of the active compounds **2** and **4**, we next examined if any transcription factors were inhibited by the compounds using immune

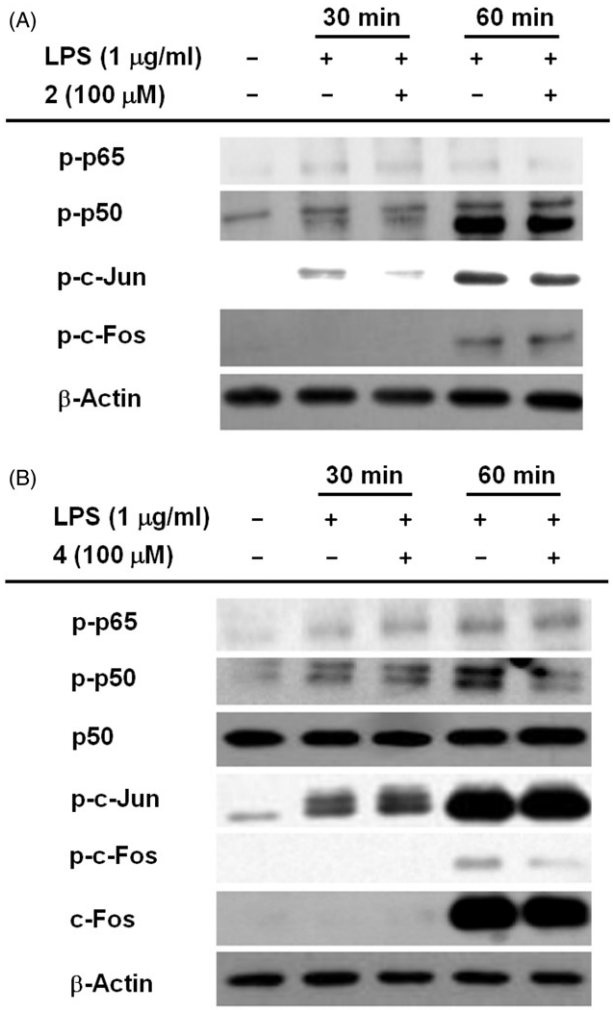


Figure 4. Effects of compounds **2** and **4** on the phosphorylation of NF-κB and AP-1 subunits in LPS-treated RAW264.7 cells. (A and B) Phospho-proteins or the total levels of p50, p65, c-Jun, c-Fos and β-actin from cell lysates were determined by phospho-specific or the total protein antibodies.

blotting analysis with whole cell lysates[16]. NF-κB (p65 and/or p50) and AP-1 (c-Jun and c-Fos) are regarded as two of the major transcription factors involved in inflammatory responses[20,21]. Therefore, we investigated whether the compounds were capable of suppressing the activation of NF-κB and AP-1 by measuring their active forms using phospho-specific antibodies, which is implicated in the transcriptional regulation of inflammatory mediators in LPS-stimulated RAW264.7 cells. As shown in Figure 4(A) and 4(B), the compounds **2** and **4** blocked the phosphorylation of NF-κB and AP-1 in a time-dependent manner. Particularly, compound **2** significantly reduced the abundance of LPS-induced phospho-c-Jun increased by treatment at 30 min as well as LPS-induced phospho-p65 at 60 min (Figure 4A). Similarly, compound **4** remarkably diminished the abundance of phospho-p50 and phospho-c-Fos at 60 min under the same conditions (Figure 4B).

Effects of the BAY11-7082 (BAY) and U0126 (U0) on production of NO and PGE_2

To confirm whether the suppression of NF-κB or AP-1 is indeed associated with inhibition of LPS-induced NO and

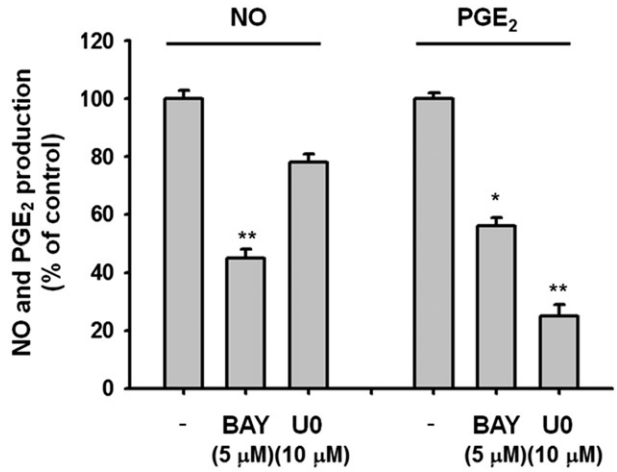


Figure 5. Effects of the BAY11-7082 (BAY), an IKK inhibitor and U0126 (U0), an ERK inhibitor on production of NO and PGE_2 in LPS-induced macrophage cells. The levels of NO and PGE_2 were determined by the Griess assay from culture supernatants of RAW264.7 cells treated with the compounds (BAY, 5 μM; U0, 10 μM) and LPS (1 μg/mL) for 24 h. *: $p<0.05$ and **: $p<0.01$ compared with control or normal.

PGE_2 production, the effects of the BAY11-7082 (BAY), an IKK inhibitor and U0126 (U0), an ERK inhibitor on production of NO and PGE_2 were examined in LPS-induced macrophage cells. Interestingly, Figure 5 strongly indicated that NF-κB inhibition by BAY11-7082 (BAY) suppressed both NO and PGE_2 production, while AP-1 inhibition by U0126 (U0) was linked to blockade of PGE_2 release.

Discussion

H. erinaceum is an edible mushroom, and its fruiting bodies are well known as a traditional medicine or foods in Korea, Japan and China. Even though this mushroom has attracted much interest due to its folk usage as a remedy for dyspepsia, gastric and duodenal ulcers[5], the constituents of this mushroom responsible for anti-inflammatory activity and its underlying mechanism have been still unclear.

Therefore, in this study, we examined the effects of the compounds isolated from the active fractions of MeOH extract on the production of inflammatory mediators, NO and PGE_2 in LPS-induced RAW264.7 macrophages. We demonstrated that compounds **2** and **4** suppressed levels of NO and PGE_2 production in a concentration-dependent manner. This result strongly suggested that compounds **2** and **4** may have important anti-inflammatory properties, and the compounds can explain the immunopharmacological effects of CH_2Cl_2-soluble fraction as active components. Interestingly, no published studies have described the anti-inflammatory activities of compounds **2** and **4**, although it has been reported that compound **4** is able to prevent impairments of spatial short-term and visual recognition memory induced by amyloid β (25-35) peptide without NGF-inducing activity[22].

Besides, our results demonstrated that NF-κB and AP-1 are major contributing transcription factors in the suppression of inflammatory mediators, NO and PGE_2 by the compounds **2** and **4**. In fact, it is widely accepted that the phosphorylation of NF-κB, which results in nuclear translocation and activation, is regulated by loops linked to protein tyrosine

kinases (Syk and Src), phosphatidylinositol-3 kinase (PI3K), phosphorrinositide-dependent kinase 1 (PDK1)/AKT, and IKK/IκB-α[16,20,21,23]. Likewise, IL-1 receptor-associated kinases (IRAKs), transforming growth factor-β-activated kinase1 (TAK1) and mitogen-activated protein kinase (ERK, p38 and c-Jun N-terminal kinase) are known to regulate the activation of AP-1[16,20,21,23].

NF-κB is well known to be a major transcription factor for regulating the expression of inflammation-associated enzyme and cytokine genes, such as iNOS, COX-2 and TNF-α[24]. In un-stimulated cells, NF-κB subunits (p65 and/or p50) form a complex with an inhibitory factor IκB-α and are inactivated in the cytosol. Upon stimulation of pro-inflammatory signals, including LPS, IκB-α is phosphorylated by IKK and inactivated through ubiquitin-mediated degradation. Free NF-κB subunits (p65 and/or p50) are translocated into the nucleus and act as a transcription factor. In addition to NF-κB, c-Jun and c-Fos are also activated and involved in the LPS-induced iNOS expression in macrophage cells[24]. Treatment with erinacerin B (**2**) and hericenone E (**4**) inhibited LPS-induced NF-κB and AP-1 activations in macrophage cells (Figure 4A and 4B). Thus, erinacerin B (**2**) might regulate anti-inflammatory activity through modulating LPS-induced NO and PGE_2 production with the mechanism of the inhibition of nuclear translocation of NF-κB p65 subunit and c-Jun, while p50 and c-Fos might be associated with the inhibition of NO and PGE_2 production by hericenone E (**4**).

We also confirmed that NF-κB inhibition by BAY11-7082 (BAY) suppressed both NO and PGE_2 production and AP-1 inhibition by U0126 (U0) induced the blockade of PGE_2 release. Hence, these results indicated that the suppression of NF-κB or AP-1 by the compounds **2** and **4** could affect their anti-inflammatory features. Nonetheless, knowing the specific pathways or enzymes targeted by the compounds could be more important work for understanding the molecular mechanism of compounds **2** and **4**. Therefore, relevant works will be continued in future studies.

In conclusion, the present findings suggested for the first time that erinacerin B (**2**) and hericenone E (**4**) from the active fraction of *H. erinaceum* are potential inhibitors of LPS-induced diverse pro-inflammatory mediators such as NO and PGE_2 in macrophage cell through the inhibition of NF-κB (p65/p50) and AP-1 (c-Jun and c-Fos) phosphorylation. Thus, the compounds **2** and **4** may have therapeutic potential for the modulation and regulation of macrophage activation and may provide a potential therapeutic approach for inflammatory diseases.

Acknowledgements

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Declaration of interest

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