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Inhibition of Inducible Nitric Oxide Synthase by *Agaricus bisporus* Extract in RAW 264.7 Macrophages

□ **Research Note** □

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Abstract

***Agaricus bisporus*, also known as white button mushroom, is one of the most popular mushrooms consumed in Korea. This mushroom contains high concentrations of flavanoids and exhibits antioxidant activity. In this study, we examined the effects of *Agaricus bisporus* ethanol extract (ABE) on lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 cells. Nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) protein levels were assessed in cells treated with 100 μM LPS in the presence or absence of ABE. 0.5 mg/mL of ABE suppressed NO production significantly. Moreover, ABE inhibited levels of iNOS protein. Taken together, these results suggest that ABE exerts anti-inflammatory activity in LPS-induced inflammation in RAW 264.7 cells.**

Key words: *Agaricus bisporus*, anti-inflammatory effect, nitric oxide, inducible nitric oxide synthase

INTRODUCTION

Inflammation, a protective response that occurs following trauma, infection, tissue injury, or noxious stimuli (1,2), results in the simultaneous induction of many physiological and immunological processes. An increased blood supply, enhanced vascular permeability, and migration of immune cells take place at the damaged site. Moreover, activated inflammatory cells secrete increased amounts of nitric oxide (NO), prostaglandins, and cytokines such as interleukin 1β, tumor necrosis factor-a, interferon gamma, and IL-10 (3).

NO is a short-lived molecule that mediates many biological activities, including vasoregulation, platelet aggregation, and neurotransmission (4). This important molecule is synthesized from L-arginine by various forms of nitric oxide synthase (NOS), such as inducible (iNOS), endothelial (eNOS), and neuronal (nNOS), with NADPH and oxygen as co-substrates (5). NO has also been associated with host defense mechanisms. Activated macrophages produce NO to mediate immune functions including antimicrobial and antitumor activities (6). Most importantly, iNOS is highly expressed in macrophages, which can lead to organ destruction in some inflammatory and autoimmne diseases (7). For this reason, inhibition of NO production has been identified as a promising therapy for the treatment for such diseases (8,9).

Lipopolysaccharide (LPS) is an outer membrane component of bacteria and a potent activator of monocytes and macrophages. LPS contributes to the pathophysiology of septic shock, as well as triggers the generation of reactive oxygen intermediates and the secretion of a variety of inflammatory mediators (10). Furthermore, stimulation of murine macrophages by LPS results in the expression of iNOS.

It is notable that increasing numbers of natural plant products are being used as medicine to prevent some acute and chronic diseases throughout the world. In particular, medicinal mushrooms such as Maitaki have been reported to possess immunostimulatory properties, and therefore have been commercially developed as dietary supplements. To date, relatively few studies have been carried out on the *Agaricus bisporus*, or white button mushroom. Some laboratories have reported the potential anti-cancer effects of the purified constituents of these mushrooms, including volatile compounds (11), decenoic acid (12), and lectin (13). Studies have also demonstrated that white button mushrooms display antioxidant activity as a result of their high concentration of flavanoids (14). However, the anti-inflammatory effect of the white button mushroom is still unknown. In the present study, we investigated the effects of *A. bisphorous* on LPS-induced inflammatory responses in the macrophage cell line RAW 264.7.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified essential medium, fetal bovine

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serum, penicillin, and streptomycin were obtained from Gibco/BRL Technologies Inc. (Gaithersburg, MD, USA). Anti-iNOS monoclonal and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Griess reagent, dithiothreitol, *Escherichia coli* LPS, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials

Agaricus bisphorous was supplied by Goodtrae in Buyeo-Gun, Chungnam, Korea. One kilogram of mushroom was extracted with 80% ethanol in 10 L at room temperature overnight. The yield was 9.34%. The *Agaricus bisphorous* extract (ABE) was concentrated on a rotary evaporator under reduced pressure and then freeze-dried.

Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the American Type Cultrue Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified essential medium supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere and those between passages five and twenty were used for experiments. RAW 264.7 cells were seeded onto 24-well plates at 5×10^4 cells per well, pretreated with 0.1 or 0.5 mg/mL ABE for six hr, and subsequently stimulated with 100 μM *E. coli* LPS for 24 hr.

Measurement of NO production

As a parameter of NO synthesis, the nitrite concentration was measured by the Griess reaction using supernatant from RAW 264.7 cultures as previously described (15). Briefly, 100 μL of cell culture supernatant was incubated with 100 μL Griess reagent (1:1 mixture of 0.1% N-(1-naphtyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate and absorbance at 570 nm was read with a microplate reader (Molecular Devices Co., Menlo park, CA, USA).

Western blotting analysis

Cells were harvested and lysed in RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitors. Total cellular protein (20 μg) was separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride, and then incubated overnight at 4°C with primary antibodies (1:1,000). Immunoreactive proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000) for one hr at room temperature, followed by incubation with enhanced chemiluminescent reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 4 software (San Diego, CA, USA). One-way ANOVA was used to compare quantitative data among groups. The Bonferroni post-hoc test was used if ANOVA determined significance to be $p < 0.05$.

RESULTS AND DISCUSSION

Inhibitory effect of ABE on LPS-induced NO production

To examine whether ABE regulates NO production, RAW 264.7 macrophages were incubated with 0.1 or 0.5 mg/mL ABE for six hr followed by treatment with 100 μM LPS for 24 hr. Then, NO in the culture medium was measured. The LPS-stimulated cells showed significant increases in nitrite accumulation which was inhibited by ABE (Fig. 1). Increased NO produced in response to bacterial LPS plays an important role in the inflammatory condition (16), often resulting in various inflammatory and autoimmune diseases. Thus, anti-inflammatory materials that exhibit an inhibitory effect on NO production could exert therapeutic effects in the treatment of septic shock and other inflammatory or infectious disorders (17). In this study, the ethanol extract

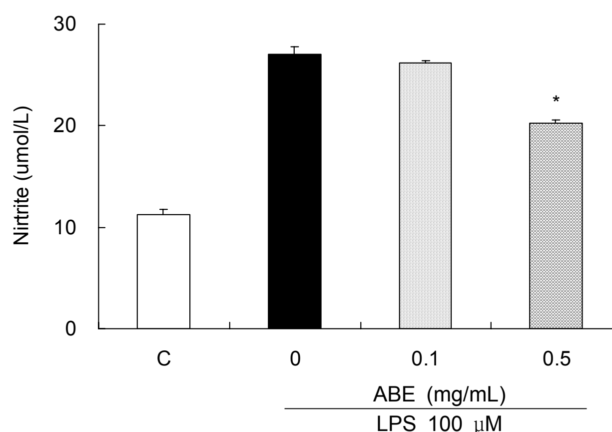

Fig. 1. Effect of ABE on NO production in RAW 264.7 macrophages stimulated by LPS. RAW 264.7 macrophages were incubated with the indicated concentrations of ABE and 100 μM LPS for 24 hr. Data represent means ± SD values from three independent experiments. $^*p < 0.05$ compared with the LPS treated cells.

of *Agaricus Bisphorus* significantly attenuated LPS-induced NO production, suggesting that this mushroom may possess anti-inflammatory activity.

Effect of ABE on LPS-induced iNOS protein expression

Western blot analyses were performed to determine the mechanism by which ABE inhibits NO production. iNOS protein was highly expressed after 24 hr stimulation with 100 μM LPS. Subsequent treatment with ABE decreased the level of iNOS protein dramatically in a concentration-dependent manner (Fig. 2A). Densitometry using the iSolution DT image acquisition and analysis program (IMT isolution, Vancouver, Canada) was performed to quantify the levels of protein, and band densities were expressed relative to control protein levels. This analysis confirmed that ABE significantly decreased levels of iNOS in LPS-induced RAW 264.7 cells (Fig. 2B). Reports have indicated that iNOS expression is regulated mainly at the transcriptional level (18). In addition, these studies suggest that NF-κB is activated by LPS treatment and induces *iNOS* gene expression. Therefore, to elucidate the exact mechanism by which ABE exerts its anti-inflammatory effects on LPS-induced iNOS expression, further investigation is needed.

In this study, we demonstrated that ABE inhibits the production of NO in LPS-stimulated macrophages. One possible pathway mediating this anti-inflammatory effect is through inhibition of iNOS expression. However, the active components in ABE that account for the anti-inflammatory activity remain to be investigated further.

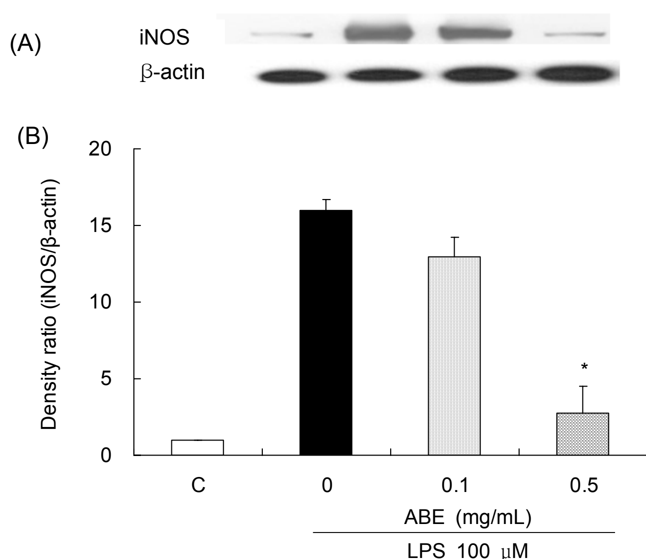

Fig. 2. Effect of ABE on iNOS protein expression in LPS stimulated RAW 264.7 macrophages. (A) The protein level of iNOS was determined by Western blotting. Equal loading of proteins was verified by β-actin blotting. (B) The density of bands were measured and the relative protein expression was shown. Data represent means±SD values from three independent experiments. $^{*}p<0.05$ compared with the LPS treated cells.

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