Armillariella mellea Shows Anti-inflammatory Activity by Inhibiting the Expression of NO, iNOS, COX-2 and Cytokines in THP-1 Cells

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Abstract: Armillariella mellea (AM), also known as Mi-Huan-Ku, a popular medicinal fungus used in the traditional Chinese medicine for treating headache, neurasthenia and insomnia. In the present study, our aim was to determine the effects of aqueous (AAM) and ethanol (EAM) extracts of A. mellea on lipopolysaccharide (LPS)-induced inflammatory response by measuring the inducible nitric oxide synthase (iNOS), cyclooxygenase-1 and -2 (COX-1 and COX-2) protein expression, cytokines (TNF-α, IL-4 and IL-8) formation, nitric oxide (NO) release and prostaglandin (PGE₂) production in human monocytic (THP-1) cells. At concentration of 100 µg/ml, EAM, but not AAM, effectively protected against LPS-induced cell death in THP-1 cells. At concentrations of 10~100 µg/ml, EAM showed a potent anti-inflammatory activity as demonstrated by a dose-dependent inhibition of LPS (1 µg/ml)-induced release of NO and PGE₂, and significantly decreased the transcription of proinflammatory cytokines. EAM at 100 µg/ml significantly blocked the LPS induction of iNOS and COX-2 expression, but not COX-1. Therefore, the protective effect of EAM against LPS-induced inflammatory mediators release could explain, at least in part, its effectiveness in alleviating certain inflammatory related diseases.

Keywords: Armillariella mellea; Anti-inflammatory; iNOS; COX-1; COX-2; Cytokines.

Introduction

Chronic inflammations and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. Among the proinflammatory enzymes, the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), which are responsible for increasing the levels of nitric oxide (NO) and prostaglandins (PGE₂), are...
known to be involved in various chronic diseases including multiple sclerosis, Parkinson’s and Alzheimer’s diseases, and colon cancer (Heiss et al., 2001). Lipopolysaccharide (LPS), which is a component of the cell wall of gram-negative bacteria, can activate certain cellular signals of macrophages, hepatocytes and monocytes during inflammation and infection (Ganey et al., 2001; Bitler et al., 2005). Several isoforms of NOS exist and fall into three major classes, i.e. inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Many studies have shown that the chronic phase of inflammation is closely associated with an increase in iNOS activity (Miller and Grisham, 1995). NO synthesized by iNOS has been implicated as a mediator of rheumatoid arthritis and other autoimmune diseases (Yui et al., 1991). In addition, prostaglandins (PGE₂) are key inflammatory mediators that are converted from arachidonic acid by the cyclooxygenase.

There are two isoforms of cyclooxygenase, namely cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is a constitutively expressed form in normal physiological functions, whereas COX-2 is induced in response to inflammatory signals such as cytokines and bacterial endotoxin LPS (Feng et al., 1995; Smith and Langenbach, 2001). Furthermore, COX-2 is responsible for the elevated production of PGE₂ during inflammation (Mitchell et al., 1995; Crofford et al., 2000). After drug treatment, the process of gene transcriptions for inflammatory proteins, such as TNF-α, IL-4, IL-6, and IL-8 etc was inhibited in LPS-induced human monocytic cells (Hart et al., 2000; Szczepanik et al., 2001).

Armillariella mellea (AM; also known as Mi-Huan-Ku), a popular medicinal fungus in Asia, belong to the family of Tricholomataceae. Its growth has a strong association with Gastrodia elata (also known as Tian Ma of family Orchidaceae), a slow growing and expensive herb in the traditional Chinese medicine. Recently, biologists in mainland China have intensively carried out experiments to cultivate AM in view of replacing the supply of G. elata (Gao et al., 2001). Preliminary studies also showed that AM possesses similar pharmacological properties as G. elata. Traditionally, it is used for treating geriatric patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in limbs, and infantile convulsion (Yang et al., 1984).

At present, scientific information on chemical and biological properties of AM is limited; many of its therapeutic properties remain in the stage of traditional beliefs. In chemical studies, fruiting bodies of AM was reported to contain armillaramide (Gao et al., 2001), melleolides K, L and M (Momose et al., 2000), and fibrinolytic metalloprotease (Kim and Kim, 1999). However, the mechanism of the anti-inflammatory response after AM treatment has not been clarified. Hence in this study, our aim was to investigate the anti-inflammatory property of AM and its mechanism(s) of action in vitro.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO), penicillin, streptomycin, anti-β actin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS, which
was obtained from *E. coli* strain O55:B5) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from GIBCO BRL (Gaithersburg, MD, USA). The ELISA kit used for the determination of prostaglandin **E**₂ (**PGE**₂) was purchased from Alexis Biochemicals (Ann Arbor, MI, USA). Human TNF-α, IL-4 and IL-8 ELISA kits were obtained from Ray Biotech Inc. (Norcross, GA, USA). The anti-COX-1, anti-COX-2, and anti-iNOS bodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse IgG antibody was obtained from Promega (Madison, WI, USA). All other chemicals and reagents used were of analytical grade.

**Preparation of Extracts**

The cultured AM mycelia were obtained from Kang Jian Biotech Corp. (Nantou Hsien, Taiwan). They were dried and ground to powdered-form, and then kept in an air-tight plastic bag until use.

To prepare the aqueous extract, 100 g of AM powder was extracted with 1 L of boiling water for 1 hour. The extract was filtered with filter paper (Advantec No. 1, Japan) while the residue was re-extracted under the same conditions twice. The filtrates were combined, concentrated and then lyophilized. For the ethanol extract, it was prepared by soaking 100 g of AM powder with 1 L of ethanol (95 %) at room temperature for 6 days. After filtering the extract with filter paper, the filtrate collected was concentrated and lyophilized. The dried powdered-extract was stored at 4°C until use. The average yield obtained for aqueous AM extract (AAM) and ethanol AM extract (EAM) was about 24% and 20%, respectively.

**Cell Culture**

THP-1, a human monocytic cell line, was obtained from the American Type Culture Collection (ATCC No. TIB-202; Manassas, VA, USA). Cells were grown in 90% DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. They were maintained at 37°C in a humidified atmosphere of 5% **CO**₂.

**MTT Assay for Cell Viability**

Cytotoxicity studies were performed in 96-well plates. THP-1 cells were cultured at 1 × 10⁵ cells per well containing 100 µl of RPMI-1640 medium. After an overnight incubation, cells were treated with LPS (1 µg/ml) or LPS plus AM extracts at various concentrations (10, 50 and 100 µg/ml), whereas the control group was treated with 0.1% DMSO. The cultures were further incubated for 24 hours. Cells were washed once before adding 50 µl of FBS-free medium containing MTT (5 mg/ml). After 4 hours of incubation at 37°C, the medium was discarded and the formazan blue formed in the cells was dissolved in DMSO. The optical density was measured at 550 nm.
Nitrite Determination

After incubating the cells with either LPS (1µg/ml) or LPS plus various concentrations of EAM (10, 50 and 100 µg/ml) for 24 hours, the supernatant was removed from the cultures. The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction (Heiss et al., 2001). Briefly, 100 µl of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), and then incubated at room temperature for 10 min, the absorbance was measured at 550 nm using an ELISA reader (Anthos Labtec Instruments, Eugendorf, Austria). The amount of nitrite was determined by using a sodium nitrite standard curve.

PGE₂ Assay

The prostaglandin E₂ (PGE₂) level in the culture medium was quantified by using ELISA kits and conducted according to the manufacturer instructions.

Measurement of Cytokines

Proinflammatory cytokines (TNF-α, IL-4 and IL-8) were measured by using ELISA kits and carried out according to the manufacturer instructions.

Western Immunoblot Analysis

Western blot analysis of iNOS, COX-1 and COX-2 were carried out by employing their respective antibodies. Cellular proteins from the control, 100 µg/ml EAM, 1 µg/ml LPS alone or 1 µg/ml LPS plus 100 µg/ml EAM treated samples were isolated in lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and 120 mM sodium chloride) containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 1 µg/ml aprotonin (Sigma Chemical Co., St. Louis, MO, USA). The samples were centrifuged at 10,000 x g for 10 min. Equal amounts of lysate protein (50 µg/lane) were then loaded onto SDS-polyacrylamide gels and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking the nonspecific binding sites with 5% (w/v) skim milk in 0.1% (v/v) Tween 20 containing phosphate buffered saline (PBST) for 1 hour at room temperature, the membrane was incubated for 1 hour with the specific primary antibodies, namely anti-iNOS (1:500), anti-COX-1 (1:500), anti-COX-2 (1:500), and anti-β-actin (1:5000). Antibody recognition was detected with an anti-mouse secondary antibody linked to the horseradish peroxidase. Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham, Aylesbury, UK). The expression of β-actin was used as a control.
Statistical Analysis

Data were presented as means ± standard deviations (SD) from three independent experiments. Values were evaluated by one way ANOVA, followed by Duncan’s multiple range tests using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Control and treatment groups were compared by student’s t-test. Differences were considered significant when p < 0.05.

Results

Effect of AM Extracts on the THP-1 Cell Viability

In order to determine whether AAM and EAM extracts cause toxicity on THP-1 cells, the MTT assay was performed. Figures 1A and 1B showed that THP-1 cell numbers were affected after 24 hours of treatment with AAM (10–100 µg/ml) and EAM (10 and 50 µg/ml) in the presence or absence of LPS (1 µg/ml). EAM (100 µg/ml) more effectively protected cells from the cell death induced by LPS, as shown by a better percentage of cell viability (98.5 ± 1.66%) than AAM (77.5 ± 2.69%). EAM was used in detailed mechanistic studies of the anti-inflammatory property.

Effect of EAM on NO Production

To investigate the effect of EAM on NO production, we measured the accumulation of nitrite, the stable end product of NO, in the culture using Griess reagent. THP-1 cells were stimulated with LPS (1 µg/ml) for 24 hours to evoke nitric oxide (NO) synthesis. As shown in Table 1, co-incubation of cells with EAM (10, 50 and 100 µg/ml) and LPS resulted in a dose-dependent reduction of NO formation. Co-treatment of cells with 100 µg/ml EAM and LPS produced a similar level of NO in the medium as that of the control (Table 1).

Effect of EAM on PGE$_2$ Synthesis

PGE$_2$ release is an important indicator of inflammatory response in cells. An increase in PGE$_2$ production (39.23 ± 2.98 ng/ml) was shown in samples after 24 hours of LPS (1 µg/ml) treatment. Co-treatment of cells with LPS and different concentrations of EAM (10, 50 and 100 µg/ml) significantly suppressed the LPS-induced PGE$_2$ production (Table 1). The greatest inhibition of LPS-induced PGE$_2$ production was noted in the 100 µg/ml EAM treated group.

Attenuation of LPS-Induced Cytokines Formation

To determine the effect of EAM on the production of proinflammatory cytokines such as TNF-α, IL-4 and IL-8, THP-1 cells were treated with control (0.1% DMSO), LPS (1 µg/ml) alone, and LPS (1 µg/ml) plus various concentrations of EAM (10, 50 and
Table 1. Effects of Ethanol A. mellea Extract (EAM) on LPS (1 µg/ml)-Induced Nitrite Oxide (NO) and PGE₂ Production

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nitrite Oxide (µM)</th>
<th>PGE₂ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.70 ± 0.24⁴</td>
<td>5.50 ± 0.98⁴</td>
</tr>
<tr>
<td>LPS</td>
<td>41.28 ± 1.22²</td>
<td>39.23 ± 2.98²</td>
</tr>
<tr>
<td>LPS + 10 µg/ml EAM</td>
<td>27.95 ± 2.37²</td>
<td>32.47 ± 2.98²</td>
</tr>
<tr>
<td>LPS + 50 µg/ml EAM</td>
<td>19.02 ± 1.02²</td>
<td>21.00 ± 2.94²</td>
</tr>
<tr>
<td>LPS + 100 µg/ml EAM</td>
<td>14.97 ± 1.73²</td>
<td>6.90 ± 0.30²</td>
</tr>
</tbody>
</table>

Each data represents the mean ± SD of three independent experiments. Values within the same column with the different superscript letters were significantly different at p < 0.05 as analyzed by Duncan’s multiple range tests.

Figure 1. Effects of aqueous (AAM) and ethanol (EAM) extracts of A. mellea on the THP-1 cell viability. Cells were treated with 0, 10, 50 and 100 µg/ml (A) AAM and (B) EAM in the presence or absence of LPS (1 µg/ml) for 24 hours. Values represent the mean ± SD of three independent experiments. The asterisk indicates a significant difference from the control group (0.1% DMSO) as analyzed by Student’s t-tests (* p < 0.05).
A. MELLEA SHOWS ANTI-INFLAMMATORY ACTIVITY IN THP-1 CELLS

Table 2. Effects of Ethanol A. mellea Extract (EAM) on LPS (1 µg/ml)-Induced Cytokine Formation

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TNF-α (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>139.1 ± 0.10^e</td>
<td>84.6 ± 3.4^e</td>
<td>161.3 ± 7.4^e</td>
</tr>
<tr>
<td>LPS</td>
<td>3594.2 ± 13.0^a</td>
<td>1362.7 ± 36.6^a</td>
<td>2498.0 ± 28.6^a</td>
</tr>
<tr>
<td>LPS + 10 µg/ml EAM</td>
<td>2444.2 ± 18.5^b</td>
<td>1276.3 ± 31.3^b</td>
<td>1510.7 ± 29.2^b</td>
</tr>
<tr>
<td>LPS + 50 µg/ml EAM</td>
<td>845.7 ± 14.5^c</td>
<td>791.7 ± 31.1^c</td>
<td>630.0 ± 51.0^c</td>
</tr>
<tr>
<td>LPS + 100 µg/ml EAM</td>
<td>269.7 ± 23.4^d</td>
<td>229.3 ± 21.3^d</td>
<td>308.0 ± 16.1^d</td>
</tr>
</tbody>
</table>

Each data represents the mean ± SD of three independent experiments. Values under the same cytokine with the different superscript letters were significantly different at p < 0.05 as analyzed by Duncan’s multiple range tests.

Figure 2. Effects of EAM on the expression of iNOS, COX-1 and COX-2 proteins in THP-1 cells. Cells were incubated with 100 µg/ml EAM in the presence or absence of LPS (1 µg/ml) for 24 hours. Protein (50 µg) from each sample was resolved on 10% SDS-PAGE, and then performed on Western blotting. β-actin was used as a control.

Figure 2. Effects of EAM on the expression of iNOS, COX-1 and COX-2 proteins in THP-1 cells. Cells were incubated with 100 µg/ml EAM in the presence or absence of LPS (1 µg/ml) for 24 hours. Protein (50 µg) from each sample was resolved on 10% SDS-PAGE, and then performed on Western blotting. β-actin was used as a control.

100 µg/ml) for 24 hours. The cytokine levels in the media were measured by ELISA kits. LPS-treated cells showed a marked up-regulation of TNF-α, IL-4 and IL-8 production, however, they were inhibited by pretreatment with EAM in a dose-dependent pattern (Table 2). A similar trend of responses was also noted in the experiments by using lower doses of LPS (i.e. 20 ng/ml and 0.5 µg/ml).

Effect of EAM on the Expression of iNOS, COX-1 and COX-2

To further investigate the role of EAM on anti-inflammation, THP-1 cells were stimulated with the control (0.1% DMSO), 1 µg/ml LPS alone, 100 µg/ml EAM alone, and 1 µg/ml
LPS plus 100 µg/ml EAM respectively for 24 hours. Western blotting analysis indicated that the expression of iNOS, COX-1 and COX-2 were up-regulated after LPS stimulation. Co-treatment of cells with LPS plus EAM for 24 hours significantly inhibited iNOS and COX-2 expressions, but the protein level of COX-1 was not affected (Fig. 2).

**Discussion**

The present study demonstrated that EAM is an effective inhibitor of LPS-induced NO generation, cytokine (TNF-α, IL-4 and IL-8) secretion, PGE₂ production, iNOS and COX-2 expressions in THP-1 cells. Inflammation is a complex process, which involves numerous mediators of cellular and plasma origins. Studies indicated that β-amyloid peptides (Szczepanik et al., 2001) and hydrolyzed olive vegetation water (Bitler et al., 2005) exhibited anti-inflammatory activities in human THP-1 cells. In this study, we showed that EAM possesses potent anti-inflammatory activity on the same cells.

LPS injection stimulated plasma and tissue cytokine formation, which leads to a markedly enhanced expression of iNOS with consecutive generation of large amounts of NO in plasma and tissue (Landry and Oliver, 2001; Höcherl et al., 2002). A high level of NO produced by iNOS has been defined as a cytotoxic indicator in inflammation and endotoxemia (Kroncke et al., 1997). NO and cytokines stimulate the expression of iNOS and COX-2, which lead to an enhanced formation of PGE₂. These molecules have been implicated as important mediators in the process of inflammation (Smith et al., 2000; Ahmad et al., 2002). The present results show that LPS induced a dramatic increase in NO and PGE₂ production in THP-1 cells. However, co-treatment of cells with LPS and EAM significantly reduced the levels of NO and PGE₂. The levels of these mediators in the cells co-treated with EAM at concentration of 100 µg/ml, were no different from that of the control group, suggesting that EAM is an effective inhibitor of LPS-induced NO generation and PGE₂ production.

Like NO, PGE₂ is a pleiotropic mediator produced at inflammatory sites by COX-2 that gives rise to pain, swelling and stiffness (Seibert et al., 1994). Inhibitors of iNOS and COX-2 have been considered as potential effective therapeutics for preventing inflammatory diseases (Surh et al., 2001). In this study, EAM was shown to suppress the expression of COX-2 and iNOS but not COX-1. This finding suggests that the anti-inflammatory property of EAM could be mediated through the iNOS and COX-2 expression.

Previous studies have shown that proinflammatory cytokines such as TNF-α, IFN-γ, IL-4, IL-6 and IL-8 are involved in the development of various inflammatory lesions (Szczepanik et al., 2001; Aceves et al., 2004; Bitler et al., 2005). These cytokines were also shown to mediate the process of tissue responses in different phases of inflammation. Thus, drugs that inhibit the production of these cytokines may serve as important medicines in controlling inflammation (Hart et al., 2000; Juergens et al., 2004). In this study, EAM significantly inhibited TNF-α, IL-4 and IL-8 expression in THP-1 cells stimulated by LPS. Studies with lower doses of LPS (i.e. 20 ng/ml and 0.5 µg/ml) also showed that EAM significantly reduced the excretion of these cytokines (data not shown). These findings further support that EAM possesses potent anti-inflammatory activity.
In the search of new anti-inflammatory drugs, recent strategies have been concentrated on a selective inhibition of COX-2 enzymatic activity while not affecting COX-1 activity. Interestingly, we demonstrated that EAM possesses inhibitory effect on COX-2 but not COX-1 expression. Although nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutic agents for inflammation and pain, they are believed to possess potential gastrointestinal and renal side-effects resulting from inhibiting the enzyme activity of housekeeping COX-1. With its selective inhibition on COX-2 activity, the use of EAM for preventing inflammatory related diseases could avoid such adverse effects.

In conclusion, we have demonstrated that EAM possesses potent anti-inflammatory activity. It prevented cytokines formation, NO generation, PGE$_2$ production, iNOS and COX-2 expression in the LPS-treated THP-1 cells. These findings are the first pharmacological evidences on the anti-inflammatory of Armillariella species. The present results have also revealed the potential application of _A. mellea_ extract in treating inflammatory related diseases.

Acknowledgments

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References


