JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Inhibitory Effect of a Glycoprotein Isolated from Golden Oyster Mushroom (*Pleurotus citrinopileatus*) on the Lipopolysaccharide-Induced Inflammatory Reaction in RAW 264.7 Macrophage

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ABSTRACT: Mushrooms have become an important source of natural antitumor, antiviral, antibacterial, immunomodulatory, and anti-inflammatioy agents. Golden oyster mushroom, *Pleurotus citrinopileatus*, is a common mushroom in oriental countries for human consumption. The present study investigated the anti-inflammatory reaction of the bioactive nonlectin glycoprotein (PCP-3A) isolated from the fresh fruiting body of this mushroom. Western blot analysis on LPS-induced iNOS, COX-2, and NF-κB expressions in RAW 264.7 cells as affected by PCP3-A was performed to elucidate the mechanism of NO and PGE2 reduction. The results showed that PCP-3A failed to affect RAW 264.7 viability at a concentration up to 6.25 μg/mL, but inhibited LPS (1 μg/mL)-induced expression, and that PCP-3A inhibited the production of NO and PGE2 in LPS-activated macrophages via the down-regulation of certain pro-inflammatory mediators, including iNOS and NF-κB.

KEYWORDS: mushroom, glycoprotein, RAW 264.7 cell, PCP-3A

INTRODUCTION

Macrophage activation is crucial in the progression of multiple inflammatory diseases through the release of inflammatory mediators such as prostaglandins, cytokines, and nitric oxide (NO).[1] Among the inflammatory responses, the iNOS mRNA and iNOS protein activity levels in macrophages are increased upon stimulation by lipopolysaccaride (LPS) or cytokines. Macrophages may then secrete more NO as a consequence.[2]

Nuclear transcription factor κB (NF-κB) is a key transcriptional factor involved in regulating the expression of pro-inflammatory mediators, including cytokines and chemokines, thereby playing a critical role in mediating inflammatory responses.[3] NF-κB is a dimeric transcription factor consisting of homo- or heterodimers of Rel-related proteins.[4] In the inactive state, NF-κB resides in the cytoplasm and forms a multiprotein complex with an inhibitory subunit IκB. On activation by external stimuli, the inflammatory signal converges on and activates a set of IκB kinases known as the IκB kinase (IKK) complex.

Mushrooms have been used in Asian countries for medicinal and tonic purposes for a long time.[5] Oyster mushrooms (*Pleurotus* spp.) are widespread throughout the hardwood forests of the world. Many bioactive components derived from *Pleurotus* are known as antitumor agents, cholesterol reducers, or antioxidative agents[6] and have also been shown to modulate the immune system.[7] Our recent investigations revealed the antitumor property of *Pleurotus citrinopileatus*.[8] The present paper focuses on the anti-inflammatory activities of this mushroom. The aim of the study was to examine the putative anti-inflammatory effect of glycoprotein PCP-3A from *P. citrinopileatus* on macrophage RAW 264.7. The effect of this glycoprotein on NO and PGE2 production and iNOS, NF-κB, and COX-2 expressions as well as their mechanisms of action would also be elucidated.

MATERIALS AND METHODS

Isolation of PCP-3A. PCP-3A is purified from the crude extract of *P. citrinopileatus* through ion-exchange and gel filtration column chromatography as previously reported.[8]

Cell Culture. RAW 264.7 cells (murine) were obtained from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) and cultured in a 5% CO_2 atmosphere at 37 °C with Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin−streptomycin, and 1% glutamine. The cells were plated in 10 cm dishes, 6- or 96-well, allowed to grow for 24 h, and then treated with PBS as the control, and sample with or without LPS. All of these treatments were carried out in serum-free conditions.

MTT Assay for Cell Viability. RAW 264.7 macrophages were added into a 96-well plate at a density of 5×10^4 cells/well and allowed to grow for 24 h in DMEM medium. Subsequently, the culture medium was discarded, replenished with a mixture of LPS (1 μg/mL) and various concentrations of PCP-3A, and then incubated for another 24 h. Filtered methylthiazole tetrazolium (MTT) solution in serum-free DMEM was added to each well (0.5 mg MTT/mL). The cells were incubated at 37 °C for 2 h. Unreacted dye was then removed. The insoluble MTT formazan crystals were allowed to dissolve in DMSO at room temperature for 15 min, and the absorbance (at 570 nm) of each sample was measured. The viability of the samples was calculated using the following equation:

$$\text{viability (\%)} = (\text{absorbance sample})/(\text{absorbance control}) \times 100\%$$

Received: April 4, 2011
Revised: May 29, 2011
Accepted: May 31, 2011
Published: May 31, 2011

dx.doi.org/10.1021/jf201335g | *J. Agric. Food Chem.* 2011, 59, 7092–7097

NO and PGE2 Formation. RAW 264.7 macrophages were treated in a manner similar to that described for cell viability. In brief, cells were incubated with medium alone, or with LPS and the sample, for 24 h. To each well was added 100 μL of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water] and then incubated for 15 min in the dark. The total amount of nitrite present was calculated on the basis of the absorbance of the sample at 570 nm. The cells were incubated with LPS (1 μg/mL) alone as the positive control and incubated with PBS as the blank control. The amount of NO synthesized in response to LPS stimulation was calculated using the following equation:

$$\text{NO synthesis (\%)} = [(\text{absorbance of PCP-3A-treated sample} - \text{absorbance of PBS-treated sample}) / (\text{absorbance of LPS-treated sample} - \text{absorbance of PBS-treated sample})] \times 100\%$$

Prostaglandin E2 (PGE2) was measured using a PGE2 EIA monoclonal kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. Briefly, 50 μL aliquots of diluted spent supernatant were added in a 96-well goat anti-mouse IgG coated plate and then incubated for 18 h at 4 °C. Each well in the incubated plate was washed with the commercial wash buffer, 200 μL of Ellman's reagent (Cayman Chemical) was added, and then the palte was shaken for 90 min in the dark to develop the color. The amount of PGE2 was calculated on the basis of a PGE2 standard curve.

Cytoplasmic and Nuclear Extracts. Separation of nuclear and cytoplasmic fractions was conducted using NE-PER1 nuclear and cytoplasmic extraction reagents (Thermo Scientific). RAW 264.7 macrophages were plated at a density of 2×10^5 cells/well and allowed to grow for 48 h. The cells were treated with different concentrations of PCP-3A with or without 1 mg/mL LPS for 24 h. A 200 μL aliquot of 0.25% trypsin solution was added to each well. The cell suspension in each well was transferred into a 1.5 mL microcentrifuge tube and then centrifuged at 500*g* for 3 min to discard the supernatant. The cell pellet was treated with 200 μL of ice-cold cytoplasmic extraction reagent I in a tube, vortexed for 15 s, and chilled on ice for 10 min; 11 μL of ice-cold cytoplasmic extraction reagent II was added, and the pellet was vortexed again for 5 s and then chilled on ice for 1 min. The chilled tube was vortexed for 5 s and then centrifuged at 18000*g* for 5 min at 4 °C. The supernatant (cytoplasmic extract) was immediately transferred to a prechilled 1.5 mL tube. The pellet was resuspended in 100 μL of nuclear extraction reagent, vortexed for 15 s, and then chilled on ice for 40 min with 15 s of vortexing every 10 min. The mixture was centrifuged at 16000*g* for 10 min at 4 °C to collect the supernatant (nuclear extract). Both cytoplasmic and nuclear extracts were stored at −80 °C until used for Western blot analysis.

Western Blotting. RAW 264.7 macrophage cells were treated in a manner similar to that described for examining NO formation and cell viability. The treated cells were washed twice with ice-cold PBS, lysed in extraction lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40, and 10 μg/mL leupeptin], and then centrifuged at 10000*g* for 30 min at 4 °C. The cytosolic fraction (supernatant) proteins were measured using the Bradford assay, with bovine serum albumin (BSA) as the standard. Total cytosolic extracts (500 μg of protein/mL), cytoplasmic extracts (300 μg of protein/mL), and nuclear extracts (100 μg of protein/mL) were separated on 8% sodium dodecyl sulfate (SDS)−polyacrylamide mini-gels for iNOS, COX-2, p65, p50, and β-actin (mouse monoclonal antibody) detection and then transferred to an Immobilon polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in a 5% skim milk for 1 h at room temperature and then incubated overnight at 4 °C with the indicated primary antibodies (at a 1:1000 dilution). After hybridization with primary antibodies, the membrane was washed with phosphate-buffered saline Tween 20 (PBST) three times, incubated with a horseradish peroxidase (HRP)-labeled secondary antibody overnight at 4 °C, and then washed with PBST three times. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech).

Transfection of RAW 264.7 Macrophage with pNF-κB-Luciferase Vector. RAW 264.7 macrophage cell line was transiently transfected with a pNF-κB-luciferase vector or pTAL-Luc vector (Clontech, Palo Alto, CA) using TransFast transfection reagent following the manufacturer's protocol. The pNF-κB-luciferase vector contained the firefly luciferase gene from *Photinus pyralis*, whereas the pTAL-Luc vector served as a negative control. Briefly, 5×10^4 cells were plated in a 24-well plate and allowed to grow to 80−90% confluency for 24 h. The transfection reagent was resuspended in 400 μL of nuclease-free water on the day before transfection and stored at −20 °C overnight. To each well was added 200 μL of DNA-Transfast reagent mixture; the plate was incubated for 1 h at 37 °C, 1 μL of complete growth medium was added, and then incubation was continued for 48 h at 37 °C for transfection.

Measurement of Luciferase Activity. After transfection, cells were treated with different concentrations of PCP-3A ranging from 0 to 6.25 μg/mL and 1 μg/mL LPS for 24 h. Luciferase activity from the cells was measured using a luciferase assay system following the manufacturer's protocol and a Femtomaster FB 12 luminometer (Zylux Corp., St. Louis, MO). Briefly, the luciferase activity was measured by adding 20 μL of cell lysate in 100 μL luciferase assay reagent followed with immediate flash reading using a Femtomaster luminometer. The enzyme activity was normalized on the basis of protein content as determined by Bio-Rad protein assay.

Statistical Analysis. The results were expressed as the mean ± standard deviation (SD). Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by Duncan's multiple-comparison test. Differences were considered to be significant when the *p* values were <0.05.

RESULTS AND DISCUSSION

Macrophages play an important role in both host-defense mechanisms and inflammation.[9,10] The overproduction of inflammatory mediators by macrophages has been implicated in several inflammatory diseases and cancers.[11] The PCP-3A isolated from *P. citrinopileatus* demonstrated the antiproliferative effect against human myeloid leukemic U937 cells.[8] In the present study, we monitored the effect of PCP-3A on the viability of RAW 264.7 cells by MTT assay to evaluate the cytotoxicity of this glycoprotein. No cytotoxicity on RAW 264.7 cells was observed at different concentrations of PCP-3A ranging from 0 to 6.25 μg/mL (Figure 1).

Inflammation is inherent to the pathogenesis of a variety of diseases. Macrophages are known to be among the critical immune cells in the regulation of inflammatory responses. Activated macrophages secrete a number of different inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), reactive oxygen species (ROS), PGE2, and NO.[12−14] We evaluated the capability of PCP-3A to inhibit the production of inflammatory mediators NO and PGE2 in the present study. The results show that LPS induced a dramatic increase in NO and PGE2 productions in RAW 264.7 cells (Figure 2). However, cells treated with LPS and PCP-3A together significantly reduced the levels of NO and PGE2. The level of NO production in the cells treated with PCP-3A alone at

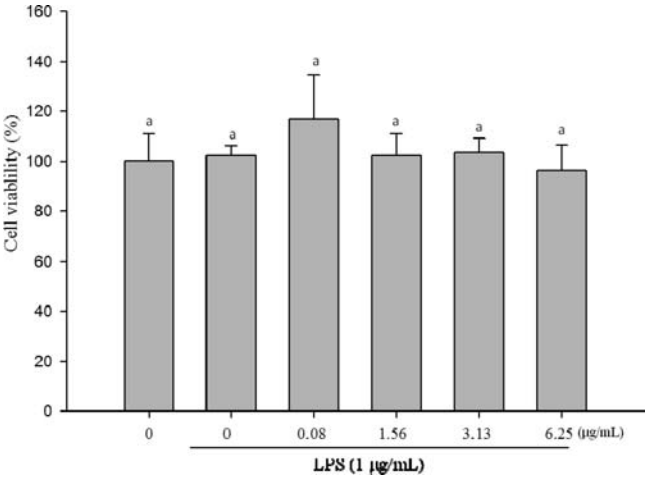

Figure 1. Effect of PCP3-A on the cell viability of LPS-induced RAW 264.7 cells. The cells (2×10^5/mL) were tested after incubation with PCP-3A (0−6.25 μg/mL) and LPS (1 μg/mL) for 24 h at 37 °C. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

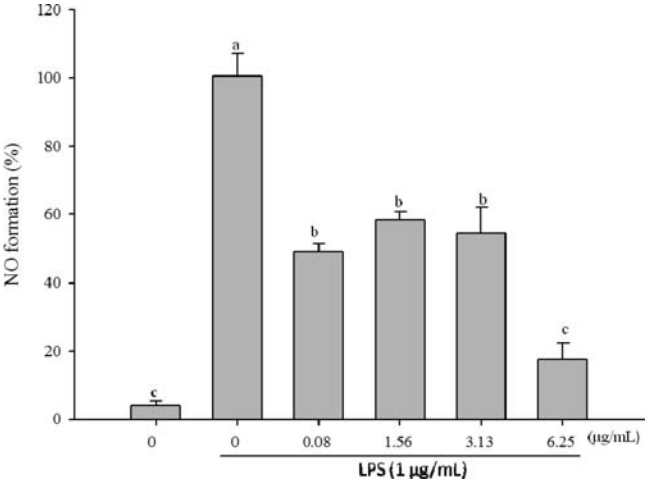

Figure 2. Effect of PCP3-A on LPS-induced NO formation in RAW 264.7 macrophages. The cells (2×10^5/mL) were treated with different concentrations (0−6.25 μg/mL) of PCP-3A with or without 1 μg/mL LPS (negative control) for 24 h at 37 °C before the analysis. Accumulated NO in the culture medium was evaluated by Griess test. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

the concentration of 6.25 μg/mL was not significantly different from that of the control group, suggesting that PCP-3A is an effective inhibitor against LPS-induced NO production.

Our results provided evidence of the effect of PCP-3A in reducing the production of LPS-induced inflammatory mediators in macrophages. NO, as a mediator of inflammation, can be induced by LPS or immunological stimuli (e.g., IFN-γ).[15] The small amount of NO produced by constitutive NOS, including endothelial NOS and neural NOS, is important in physiological homeostasis, whereas the large amount of NO produced by inducible NOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation. The excessive production of NO by macrophages or other cells exposed to

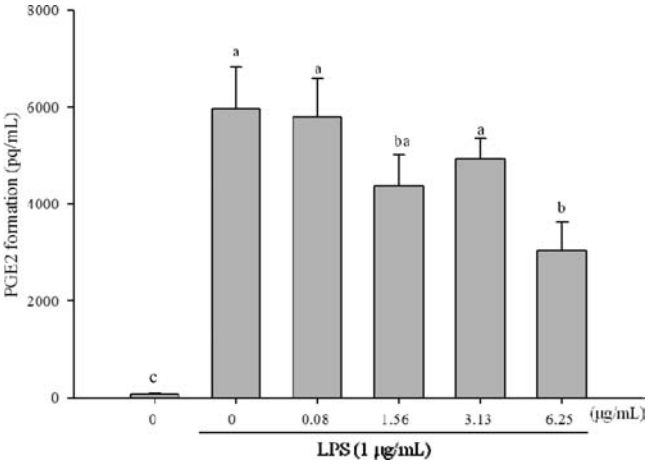

Figure 3. Effect of PCP3-A on LPS-induced PGE2 formation in RAW 264.7 macrophages. The cells (2×10^5/mL) were treated with different concentrations (0−6.25 μg/mL) of PCP-3A with or without 1 μg/mL LPS (negative control) for 24 h at 37 °C before the analysis. Accumulated PGE2 in the culture medium was evaluated by EIA monoclonal kit. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

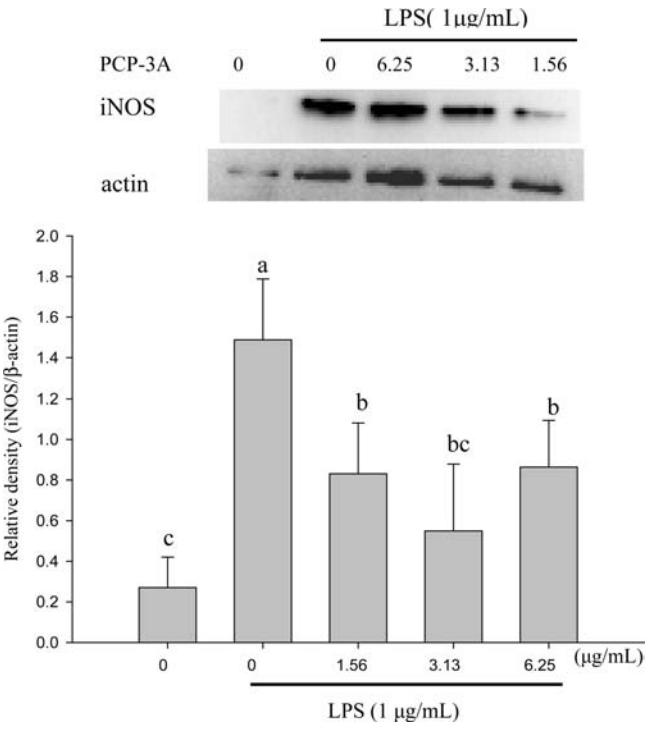

Figure 4. Effect of PCP-3A on LPS-induced iNOS protein expression in RAW 264.7 macrophages. The cells (2×10^5/mL) were incubated with various concentrations of PCP-3A and 1 μg/mL LPS for 24 h at 37 °C before the analysis. The expressions of iNOS and β-actin were detected by Western blotting using specific antibodies. Each sample contained 30 μg total protein. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

endotoxin may contribute to septic shock, cerebral injury, myocardical ischemia, local or systemic inflammatory disorders, diabetes, and other diseases.[14,16]

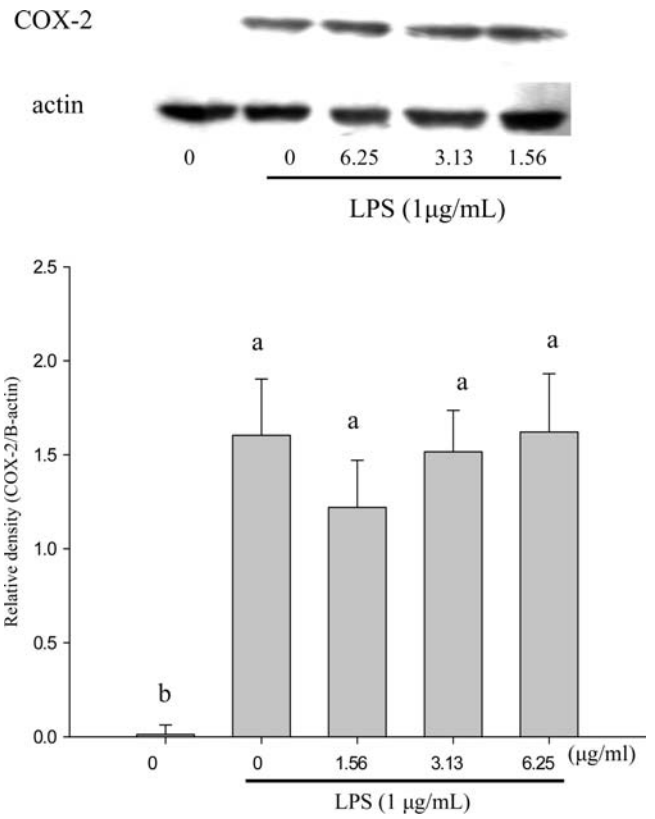

Figure 5. Effect of PCP-3A on LPS-induced COX-2 protein expressions in RAW 264.7 cells. The cells (2×10^5/mL) were incubated with various concentrations of PCP-3A and 1 μg/mL LPS for 24 h at 37 °C before the analysis. The expressions of COX-2 and β-actin were detected by Western blotting using specific antibodies. Each sample contained 30 μg of total protein. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

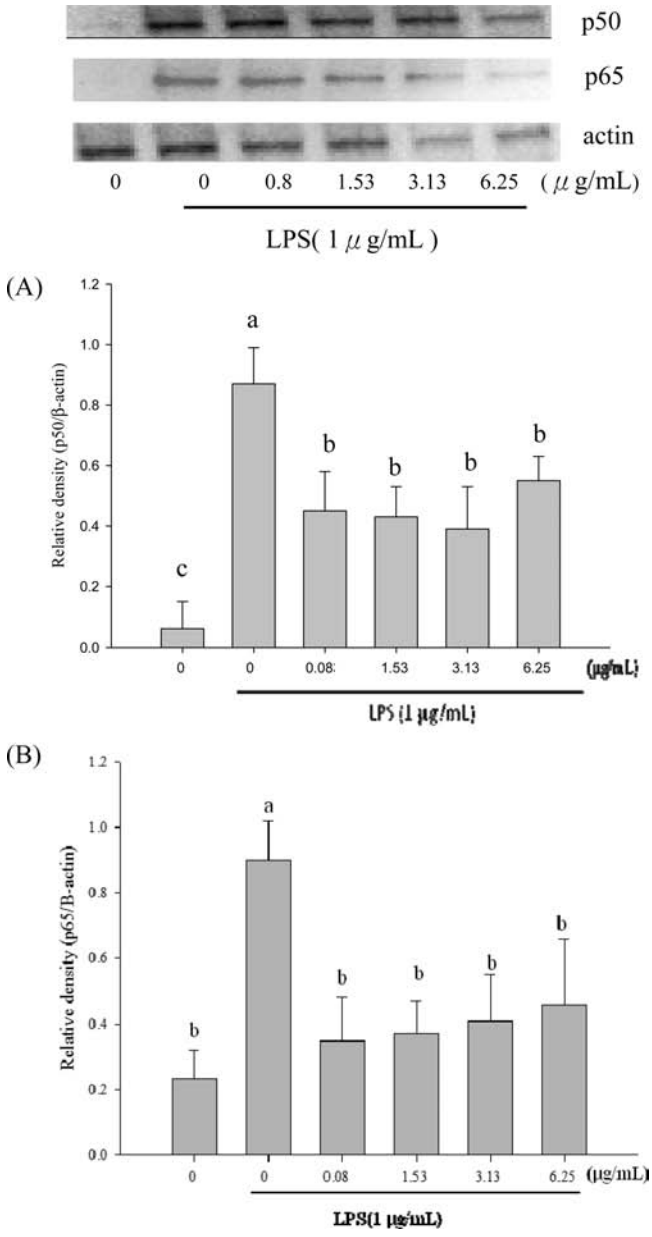

Figure 6. Effect of PCP-3A on the cytoplasmic expressions of p50 (A) and p65 (B) in LPS-induced RAW 264.7 macrophages. The cells (2×10^5/mL) were placed in 6-well plates and incubated with PCP-3A (0, 0.08, 1.53, 3.13, and 6.25 μg/mL) and LPS (1 μg/mL), or with PBS as the control, for 24 h at 37 °C before the analysis. The protein content in the cytoplasmic extract was evaluated by NE-PER Cytoplasmic Extraction Reagent. The amounts of NF-κB, p50, and p65 in the cytoplasmic extract were evaluated by Western blotting. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

Chronic inflammation causes the up-regulation of several pro-inflammatory proteins, including iNOS and COX-2, in the affected tissues. The overproduction of NO and PGE2 plays an important role in the process of macrophage activation and is associated with acute and chronic inflammations.[17] The inhibition of NO and PGE2 production by the down-regulation of iNOS and COX-2 in inflammatory cells is often employed in the evaluation for anti-inflammatory effect of biomaterials. In the present study, the anti-inflammatory effect of PCP-3A is proved by its effectiveness in the reduction of NO and PGE2 productions at a high concentration (6.25 μg/mL) (Figures 2 and 3).

LPS may bind to toll-like receptor-4 (TLR4), activate a number of transcription factors including AP-1 and NF-κB, and enhance the expression of a number of inflammatory genes, such as TNF-α, IL-6, iNOS, and COX-2.[18] The effect of PCP-3A on iNOS and COX-2 expressions was determined to further elucidate the anti-inflammatory mechanism of this glycoprotein. The treatments of RAW 264.7 macrophages with various concentrations of PCP-3A (0.08, 1.53, 3.13, and 6.25 μg/mL) markedly suppressed iNOS expression (Figure 4). PCP-3A down-regulated NO production and iNOS expression (Figures 2 and 4). NO is synthesized from L-arginine by nitric oxide synthase (NOS).[14] Our results indicate that PCP-3A is able to down-regulate NO production through the down-regulation of iNOS expression. The above-mentioned findings support an anti-inflammatory mechanism for PCP-3A in macrophages similar to that reported for the extracts of *Inonotus xeranticus*,[19] *Phellinus linteus*,[20] *Antrodia camphorata*,[22] *Armillariella mellea*,[21,23] and other mushrooms, involving the suppression in NO production as a consequence of the inhibition on iNOS expression. PCP-3A decreased the production of PGE2 while maintaining the expression of COX-2 (Figure 5), suggesting the possibility for this glycoprotein to inhibit COX-2 enzyme activity.

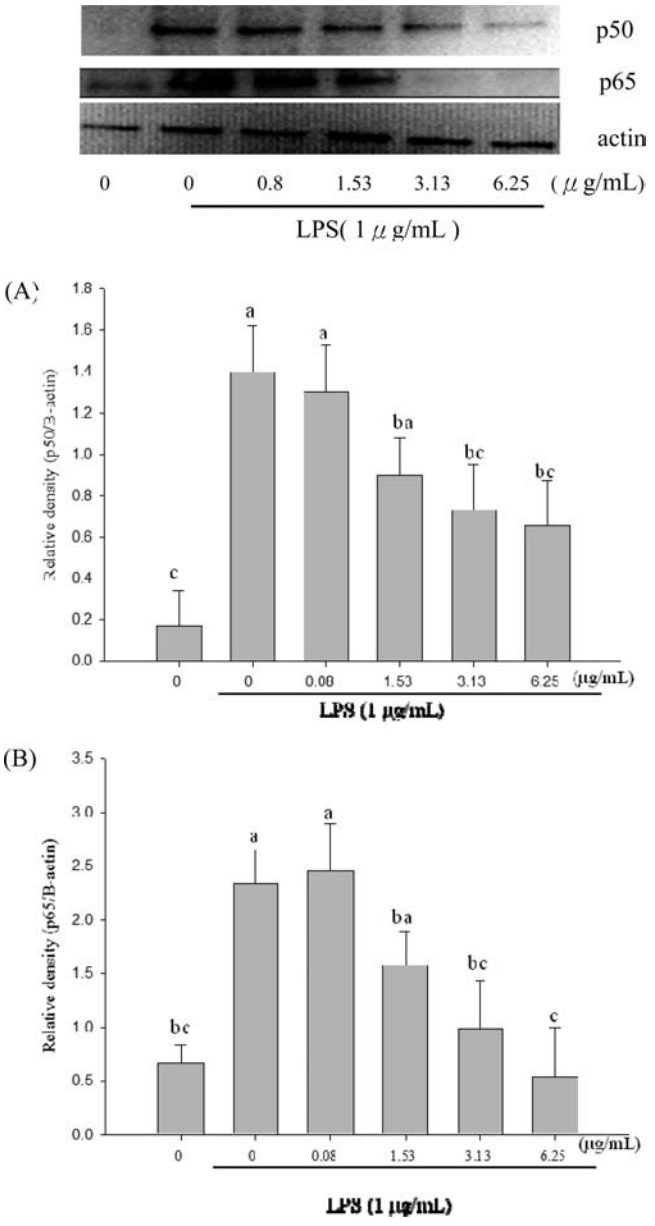

Figure 7. Effect of PCP-3A on the nuclear expressions of p50 (A) and p65 (B) in LPS-induced RAW 264.7 macrophages. The cells (2×10^5/mL) were placed in 6-well plates and incubated with PCP-3A (0, 0.08, 1.53, 3.13, and 6.25 μg/mL) and LPS (1 μg/mL), or with PBS as the control, for 24 h at 37 °C before the analysis. The protein content in the nuclear extract was evaluated by NE-PER Nuclear Extraction Reagent. The amounts of NF-κB, p50, and p65 in the nuclear extract were evaluated by Western blotting. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

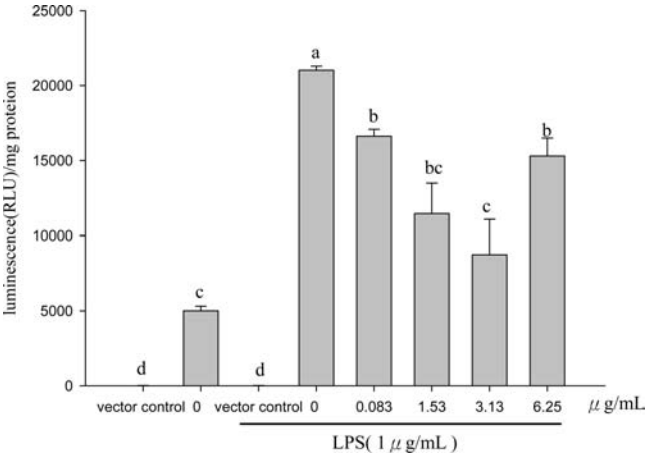

Figure 8. Effect of PCP-3A on NF-kB transactivation in LPS -induced RAW 264.7 macrophages. RAW 264.7 cells (2×10^5/mL) in 6-well plates were cotransfected with NK-κB reporter luciferase plasmids for 24 h at 37 °C. The transfected cells were treated with PCP-3A (0.83, 1.53, 3.13, and 6.25 μg/mL) and LPS (1 μg/mL) or PBS for 24 h at 37 °C. Luciferase activities were evaluated using a Dual-Luciferase Reporter Assay System. The results from three replicated experiments are expressed as the mean ± SD. Values having different letters are significantly different ($p < 0.05$).

It is known that NF-κB plays an essential role in the expression of genes involved in immune and inflammatory responses.[23] NF-κB is also known to be a major transcription factor to regulate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, and COX-2.[24,25] In the present study, we examined the protein levels of p50 and p65 in nuclear and cytoplasm extracts because these two proteins are the major components of NF-κB activated by LPS-stimulated macrophages.[26] We found that the treatment with PCP-3A (0−6.25 μg/mL) and LPS down-regulated the expressions of p50 and p65 in nucleus and cytoplasm (Figures 6 and 7) and resulted in the down-regulation of NF-κB in the transfected RAW 264.7 macrophages as measured by firefly luciferase activity (Figure 8). According to the results, PCP-3A may down-regulate the NF-κB expression in transiently transfected RAW 264.7 macrophages and the dose-dependent expressions of p50 and p65 subunits of NF-κB in the nucleus but not in the cytoplasm. We propose that PCP-3A is able to inhibit NF-κB in the cytoplasm and block the translocation of p50 and p65 subunits of NF-κB into the nucleus.

Many researchers tried to develop potent inhibitors of NF-κB into novel anti-inflammatory agents. Hseu et al. demonstrated that the extract from *A. camphorata* inhibited iNOS and COX-2 expressions through the NF-κB pathway in RAW 264.7 macrophages.[27] We found PCP-3A to down-regulate NO formation, PGE2 formation, and NOS protein expression through the inhibition of NF-κB expression (Figures 2−8). On the basis of these results, we propose that PCP-3A from *P. citrinopileatus* is capable of down-regulating NF-κB expression in transiently transfected RAW 264.7 macrophages and inhibiting the translocation of p50 and p65 subunits of NF-κB in the nucleus. Our proposed mechanism for the anti-inflammation of PCP-3A in RAW 264.7 macrophages through the down-regulation of NF-κB expression is similar to that reported for the anti-inflammation of a peptide from soybean,[28] albaconol from the mushroom*Albatrellus confluens*,[28,29] and the extract from *Daedalea gibbosa*.[30] Our observation further suggests that PCP-3A may reduce the binding of NF-κB to its target DNA, thereby inhibiting the transcription of genes with pro-inflammatory properties.

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Author Contributions

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■ ABBREVIATIONS USED

LPS, lipopolysaccaride; MTT, filtered methylthiazole tetrazolium; SDS, dodecyl sulfate; ROS, reactive oxygen species; PGE2, prostaglandin E2; NO, nitric oxide; TNF-α, tumor necrosis factor-α); IL-6, interleukin-6; NF-κB, nuclear transcription factor κB.

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