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ORIGINAL ARTICLE

(3R, 7R)-7-Acetoxyl-9-Oxo-de-O-Methyllasiodiplodin, a Secondary Metabolite of *Penicillium* Sp., Inhibits LPS-Mediated Inflammation in RAW 264.7 Macrophages through Blocking ERK/MAPKs and NF-κB Signaling Pathways

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Abstract— Twelve polyketones were isolated from the fermentation broth of *Penicillium* sp., including six new compounds (supplementary material). *Penicillium* sp. is widely used in clinic as a highly effective and low toxic antibiotic. Among these compounds, (3R, 7R)-7-acetoxyl-9-oxo-de-O-methyllasiodiplodin named PS-2 showed significant anti-inflammatory activity. So, the anti-inflammatory mechanism of PS-2 was investigated by using lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. The results showed that PS-2 can significantly inhibit the overproduction of nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6), whereas it showed no inhibition on the release of pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α). Cell-free colorimetric method demonstrated that PS-2 could obviously inhibit the enzymatic activity of cyclooxygenase-2 (COX-2).

Yanan Liu and Danna Li contributed equally to this work.

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Western blot results indicated that PS-2 could significantly inhibit high expression of iNOS and COX-2 proteins. Further investigations on the anti-inflammatory mechanism showed that PS-2 could suppress the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), but did not exhibit obvious inhibition on the phosphorylation of c-JunN-terminal kinase (JNK) and phosphorylated 38 (p38). In addition, PS-2 inhibited the degradation of inhibitor of kappa-B alpha (IκB-α) and translocation to nucleus of nuclear factor kappa-B (NF-κB) p65 in RAW 264.7 macrophages. These results suggested that PS-2 might be an effective intervention against inflammatory diseases.

KEY WORDS: *Penicillium* sp.; secondary metabolite; anti-inflammatory activity; MAPKs; NF-κB.

INTRODUCTION

Panax notoginseng is a perennial herb widely distributed in Yunnan, Guangxi, Sichuan, Hubei, Jiangxi and other provinces of China. The root of *Panax notoginseng* as a traditional Chinese medicine is used for the treatment of hemorrhages [1], inflammation [2], hyperglycemia, hyperlipidemia [3], blood stasis, remission pain [4], and the improvement of blood circulation [5]. In recent years, more and more attention has been paid to endophytic or rhizosphere fungi of *Panax notoginseng*. Many secondary metabolites from endophytic fungi of *Panax notoginseng* have been reported to have a variety of biological activities, such as antibacterial, antifungal [6], and cell cytotoxic activity [7]. In our previous study [8], *Penicillium* sp. was isolated from the root soil of *Panax notoginseng* from Wenshan, Yunnan Province, China, in December 2012. After further cultivation, extraction, and separation, 12 secondary metabolites were isolated and identified in the broth medium of *Penicillium* sp. PS-(1–5) are 12-dihydroxybenzoic acid lactone derivatives, penicimenolides. PS-6 is a new ring-opened resorcylic acid lactone derivative penicimenolide. PS-(7–12) are biogenetically related derivative. These compounds belong to polyketones and have biological activities such as antibacterial [9], anti-inflammatory [10], anti-tumor [11] activity, etc.

Nitric oxide (NO) plays an important role in the process of inflammation progress and immune response, and the ability to inhibit overproduction of NO can be considered as an indicator of anti-inflammatory activity [12, 13]. The inhibitory activity of identified 12 compounds on the production of NO were measured by Griess assay (Table 1). PS-2, (3R,7R,2′R)-7-(2′-hydroxypropionyloxy)-9-oxo-de-O-methyllasiodiplodin (PS-3), (3R,7S,2′R)-7-(2′-hydroxy-propionyloxy)-9-oxo-de-O-methyllasiodiplodin (PS-4) and cis-resorcylide (PS-7) exhibited significant inhibition on the overproduction of NO induced by LPS in RAW 264.7 macrophage cells (Fig. 1). In this study, the *in vitro* anti-inflammatory activity and molecular mechanism of PS-2 in LPS-activated RAW 264.7 macrophages are studied and reported.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS) and RPMI 1640 culture medium were purchased from Invitrogen (Thermo Fisher Scientific, Inc., USA). 3-(4, 5-dimethylthizaol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phenylmethanesulfonyl fluoride (PMSF), and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Bradford protein concentration determination kit, nitric oxide determination kit, mouse TNF-α ELISA kit, and mouse IL-6 ELISA kit were purchased from Yantai Science and Biotechnology Co., Ltd. (Yantai, China). Mouse PGE_2 ELISA kit was purchased from Shanghai Senxiong Science and Technology Co., Ltd. (Shanghai, China). COX colorimetric inhibitor screening assay kit (701050; Cayman Chemical Company, Michigan, USA). Mouse anti-rabbit iNOS polyclonal antibody (160862; Cayman Chemical Company, Michigan, USA), mouse anti-rabbit COX-2 polyclonal antibody (160106; Cayman Chemical Company, Michigan, USA), goat anti-rabbit phosphorylated extracellular signal-regulated kinase (p-ERK1/2) polyclonal antibody (AF1015; Affinity Biosciences), goat anti-rabbit phosphorylated c-Jun N-terminal kinase (p-JNK) polyclonal antibody (AF3318; Affinity Biosciences), goat anti-rabbit phosphorylated p38 (p-p38) polyclonal antibody (AF3455; Affinity Biosciences), goat anti-rabbit IκB-α polyclonal antibody (sc-371; Santa Cruz Biotechnology, Inc.), mouse monoclonal NF-κB p65 antibody (sc-8008; Santa Cruz Biotechnology, Inc.), Lamin B (AF1408; Beyotime Biotechnology, Inc.), goat anti-rabbit

Table 1. Inhibitory Activities of 12 Compounds on the Overproduction of NO in LPS-Activated RAW 264.7 Cells (IC_{50} values). Data are Presented as Mean ± S.D. of Three Independent Experiments

Compound numbers	Names of compounds	IC_{50} values(μM)
PS-1	(3R), (6E)-etheno-9-oxo-de-O-methyllasiodiplodin	49.39 ± 3.21
PS-2	(3R,7R)-7-acetoxyl-9-oxo-de-O-methyllasiodiplodin	5.53 ± 0.39
PS-3	(3R,7R,2′R)-7-(2′-hydroxypropionyloxy)-9-oxo-de-O-methyllasiodiplodin	5.97 ± 0.46
PS-4	(3R,7S,2′R)-7-(2′-hydroxy-propionyloxy)-9-oxo-de-O-methyllasiodiplodin	1.23 ± 0.10
PS-5	(3R, 9S)-(7E)-etheno-9-hydroxy-de-O-methyllasiodiplodin	68.39 ± 4.51
PS-6	(3′R)-3′-hydroxy-4′-oxo-4′-methoxy-orsellinat	8.11 ± 0.59
PS-7	Cis-resorcylide	0.73 ± 0.06
PS-8	Dihydroresorcylide	73.93 ± 7.15
PS-9	(13S, 14R)-13-hydroxydihydroresorcylide	>100
PS-10	(11S)-methoxyresorcylide	70.45 ± 6.62
PS-11	(11R)-methoxyresorcylide	>100
PS-12	7-hydroxydihydroresorcylide	55.94 ± 3.22
Hydrocortisone	Hydrocortisone sodium succinate for injection	48.66 ± 3.2

β-actin polyclonal antibody (sc-1616; Santa Cruz Biotechnology, Inc.), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit lgG (H + L) (S0001; Affinity Biosciences) were used at 1:1000 dilution. Nucleoprotein and cytoplasmic protein extraction kit (p0028; Beyotime Biotechnology, Inc.), 50 mM solution of PS-2 was prepared in 100% cell culture grade DMSO, stored at small aliquots at − 20 °C, and diluted to the required concentrations prior to use.

Fungal Material

Penicillium sp. was isolated from the rhizosphere soil of *Panax notoginseng*, which was collected from Wenshan, Yunnan province of China in November 2012. The fungus was identified according to the DNA sequence of the internal transcribed spacer (ITSI-5.8S-ITS2 region). The sequence data of the fungal strain were submitted and deposited in GenBank with the accession number KU380346. BLAST search results indicated that the fungus belongs to the genus *Penicillium* sp. and had a high sequence identity (99%) to the species *Penicillium* sp. [8]. PS-2 was extracted and isolated from fermented liquid of *Pencillium* sp.

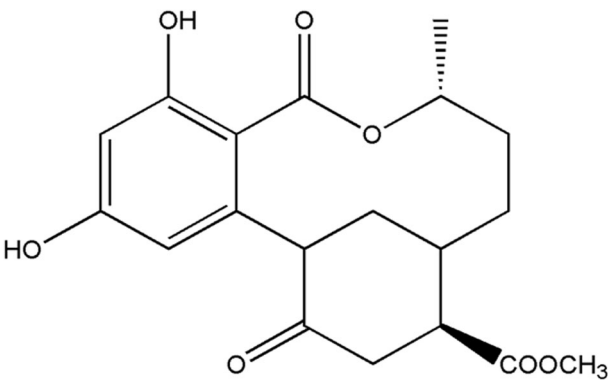

Fig. 1. Chemical structure of PS-2.

Cell Culture of RAW 264.7 Cells

Mouse monocyte-macrophage RAW 264.7 cells (ATCC TIB-71; Chinese Academy of Sciences Shanghai cell bank) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum in a humidified incubator at 37 °C with 5% CO_2 and 95% air. The medium was routinely changed every 2 days, and RAW 264.7 cells were passaged when the cell density of each dish reached 80%.

MTT Assay to Determine Cell Viability

RAW 264.7 cells were treated with PS-2 at a concentration of 3.125–100 μM for 24 h. The amount of MTT into mitochondria to form formazan was used as an indicator of cell viability. The final concentration of the MTT method was 200 μg/mL, and cells were incubated for another 4 h at 37 °C. The supernatant was removed and 150 μL DMSO was added to dissolve the formazan. The absorbance was measured by microplate reader at a wavelength of 570 nm (630 nm as a reference wavelength). Results are expressed as the percentage of viable cells compared with untreated cells.

NO Analysis

NO concentrations were measured by Griess reagent (mixture of equal amount of reagent A and reagent B, A: 1% sulfanilamide in 5% H_3PO_4, B: 0.1% naphthylethylene diamine dihydrochloride). RAW 264.7 cells (1×10^6 cells/mL) were seeded in 96-well plate and incubated for 1 h. The cells were treated with LPS (1 μg/mL) with or without PS-2 (3.125, 6.25, 12.5, 25, 50, and 100 μM) and hydrocortisone sodium succinate (100 μM) for 24 h. Cell culture supernatant (100 μL) was added to 100 μL Griess reagent and then incubated for 10 min at room temperature. The absorbance was measured at 540 nm, and nitrite concentrations were calculated using a standard calibration curve prepared from different concentrations of sodium nitrite.

Measurement of PGE_2

PGE_2 is an inflammatory mediator produced by COX-2 [14]. RAW 264.7 cells were treated by LPS (1 μg/mL) with or without PS-2 (3.125, 6.25, 12.5, 25, 50, 100 μM), or hydrocortisone sodium succinate (100 μM) for 24 h. Cell culture supernatant (100 μL) was removed to determine the level of PGE_2 using a commercial mouse PGE_2 ELISA kit according to the manufacturer's recommendations.

Measurement of Inflammatory Cytokines IL-6 and TNF-α

RAW 264.7 cells were incubated in 96-well plates at the density of 5×10^5 cells/mL. The cells were treated with LPS (1 μg/mL) with or without PS-2 (3.125, 6.25, 12.5, 25, 50, 100 μM), or hydrocortisone sodium succinate (100 μM) for 6 h. One hundred microliters of the culture supernatant was removed to determine the level of TNF-α or IL-6 by using respective ELISA kit according to the manufacturer's recommendations.

Assay of COX-2 Enzymatic Activity

The enzymatic activity of COX-2 was determined in a cell-free system by using a COX colorimetric inhibitor screening assay kit according to the manufacturer's instructions. Briefly, 160 μL of assay buffer, 10 μL of heme, and 10 μL of DMSO were added to the background wells. One hundred fifty microliters of assay buffer, 10 μL of COX-2 enzyme, 10 μL of heme, and 10 μL of DMSO were added to the 100% initial activity wells. One hundred fifty microliters of assay buffer, 10 μL of COX-2 enzyme, 10 μL of heme, and 10 μL of PS-2 or hydrocortisone sodium succinate were added to the sample wells. The plate was gently shaken for a few seconds and then incubated at 25 °C for 5 min. Twenty microliters of the colorimetric substrate solution and 20 μL of arachidonic acid were added to each well. The microplate was shaken carefully for a few seconds and incubated for 5 min at 25 °C. The absorbance was measured at 590 nm by a microplate reader, and the enzymatic activity of COX-2 was calculated in comparison with the 100% initial activity wells according to the manufacturer's instructions.

Protein Extraction for Detection of iNOS, COX-2, P-JNK, P-ERK1/2, and P-p38

RAW 264.7 cells were treated with LPS (1 μg/mL) with or without PS-2 (12.5, 25, 50, 100 μM). Cells were harvested after treating for 24 h to detect iNOS and COX-2 protein expression, for 15 min to detect p-JNK and for 60 min to detect p-ERK1/2 and p-p38 proteins. After treatment, medium was removed and the cells were washed with cold PBS and lysed in cold lysis buffer. Cell debris was removed after centrifugation (13,000 r/min, 4 °C, 6 min), and Bradford method was used to determine the total protein concentrations.

Cytoplasmic and Nuclear Protein Extraction for Detection of IκB-α and p65

After indicated treatments, according to commercial kit instructions, the medium was discarded and the RAW 264.7 cells were washed with ice-cold PBS and collected. Forty microliters of buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCL, 500 μM DTT, 0.1% (*v*/*v*) NP-40) was used to lyse the cells. After being incubated for 15 min, the cells were centrifuged at 12,000 r/min for 5 min at 4 °C. The supernatant was separated as cytoplasmic protein and used for Western blot to detect IκB-α. Suspended precipitation in 30 μL of buffer B (20 mM HEPES, 1.5 mM $MgCl_2$, 400 mM NaCl, 100 mM DTT, 20 μM PMSF) was incubated for 20 min and then centrifuged at 16,000 r/min for 10 min at 4 °C. Nuclear protein was separated and used for Western blot to detect NF-κB p65 subunit.

Western Blot Analysis

An equal amount (30 μg) of the corresponding protein was boiled in SDS-PAGE loading buffer, subjected to SDS-PAGE, and electrophoretically transferred into nitrocellulose membranes [15, 16]. Seven percent non-fat milk dissolved in Tris-buffered saline with Tween 20 (TBS-T) was used to block the membranes at room temperature for

4 h. After being washed three times for 10 min with TBS-T, the membranes were incubated in the respective primary antibody solution (iNOS, COX-2, p-ERK1/2, p-JNK, p-p38, IκB-α, p65) overnight at 4 °C. The membranes were washed three times for 10 min with TBS-T and incubated with the HRP-conjugated secondary antibody solution at room temperature for 1 h. The membranes were washed and the blots were detected by using enhanced chemiluminescence reagent (ECL) and exposed to photographic films (Kodak, USA). Images were collected and the bands corresponding to iNOS (Fig. 4a), COX-2 (Fig. 4b), p-ERK1/2 (Fig. 6a), p-JNK (Fig. 6b), p-p38 (Fig. 6c), IκB-α (Fig. 7a), and p65 (Fig. 7b) were quantitated by densitometric analysis using the DigDoc100 program (Alpha Ease FC software).

Statistical Analysis

All results are expressed as means ± standard deviation from three independent experiments. Statistical comparisons were conducted using SPSS(SPSS statistics 17.0, IBM corporation). Differences between two means were analyzed by Student's *t* test. When *P* value < 0.05, the results were considered statistically significant differences.

RESULTS

PS-2 Did Not Exhibit Cytotoxicity Against RAW 264.7

RAW 264.7 cells were treated with PS-2 for 24 h. MTT assay was used to test the cell viability. The results indicated that PS-2 did not exhibit cytotoxicity against RAW 264.7 cells in the concentration range of 3.125–100 μM (Fig. 2).

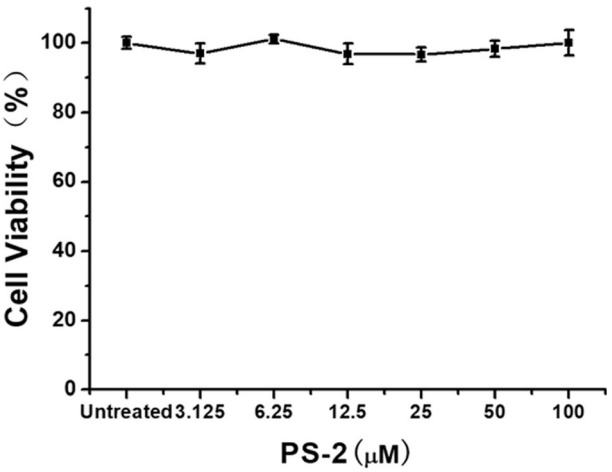

Fig. 2. Effect of PS-2 on proliferation of RAW 264.7 cells. RAW 264.7 cells were treated with serially diluted doses of PS-2 (3.125, 6.25, 12.5, 25, 50 and 100 μM) for 24 h, and MTT assay was used to detect the cell viability. The percentage of viable cells was compared to the untreated cells which were considered to have 100% viable cells. Data are presented as mean ± S.D. from three independent experiments.

Effect of PS-2 on the Release of NO and PGE_2

RAW 264.7 cells were treated by LPS with or without different doses of PS-2 for 24 h. The level of NO was determined by Griess assay and the level of PGE_2 was determined by ELISA. Hydrocortisone sodium succinate (HSS), a commonly used anti-inflammatory drug in clinic, was used as the positive control drug. HSS can strongly inhibit the production of NO and PGE_2 at the concentration of 100 μM. As shown in Fig. 3a, PS-2 significantly inhibited the overproduction of NO induced by LPS in a dose-dependent manner at the range of 12.5–100 μM. The production of PGE_2 induced by LPS was only significantly inhibited by PS-2 at the concentrations of 50 μM and 100 μM (Fig. 3b).

Effect of PS-2 on the Release of TNF-α and IL-6

RAW 264.7 cells were treated with LPS with or without different doses of PS-2 for 6 h. The levels of pro-inflammatory cytokines TNF-α and IL-6 were measured with corresponding ELISA kits. As shown in Fig. 3c, the positive control drug HSS potently inhibited the release of TNF-α, while PS-2 did not exhibit any significant inhibitory effect on the release of TNF-α. The positive control drug HSS potently inhibited the release of IL-6, and PS-2 also significantly inhibited the release of IL-6 in a dose-dependent manner (Fig. 3d).

Effect of PS-2 on the High Expression of iNOS and COX-2 Proteins

The level of inflammatory proteins iNOS and COX-2 were examined by Western blot. The untreated cells almost do not express iNOS and COX-2 proteins. After LPS stimulation, iNOS and COX-2 protein expression increased significantly. PS-2 potently inhibited the high expression of iNOS (Fig. 4a) and COX-2 (Fig. 4b) in RAW 264.7 cells and showed a good dose-dependent manner. The density of bands corresponding to iNOS and COX-2 protein was normalized by β-actin.

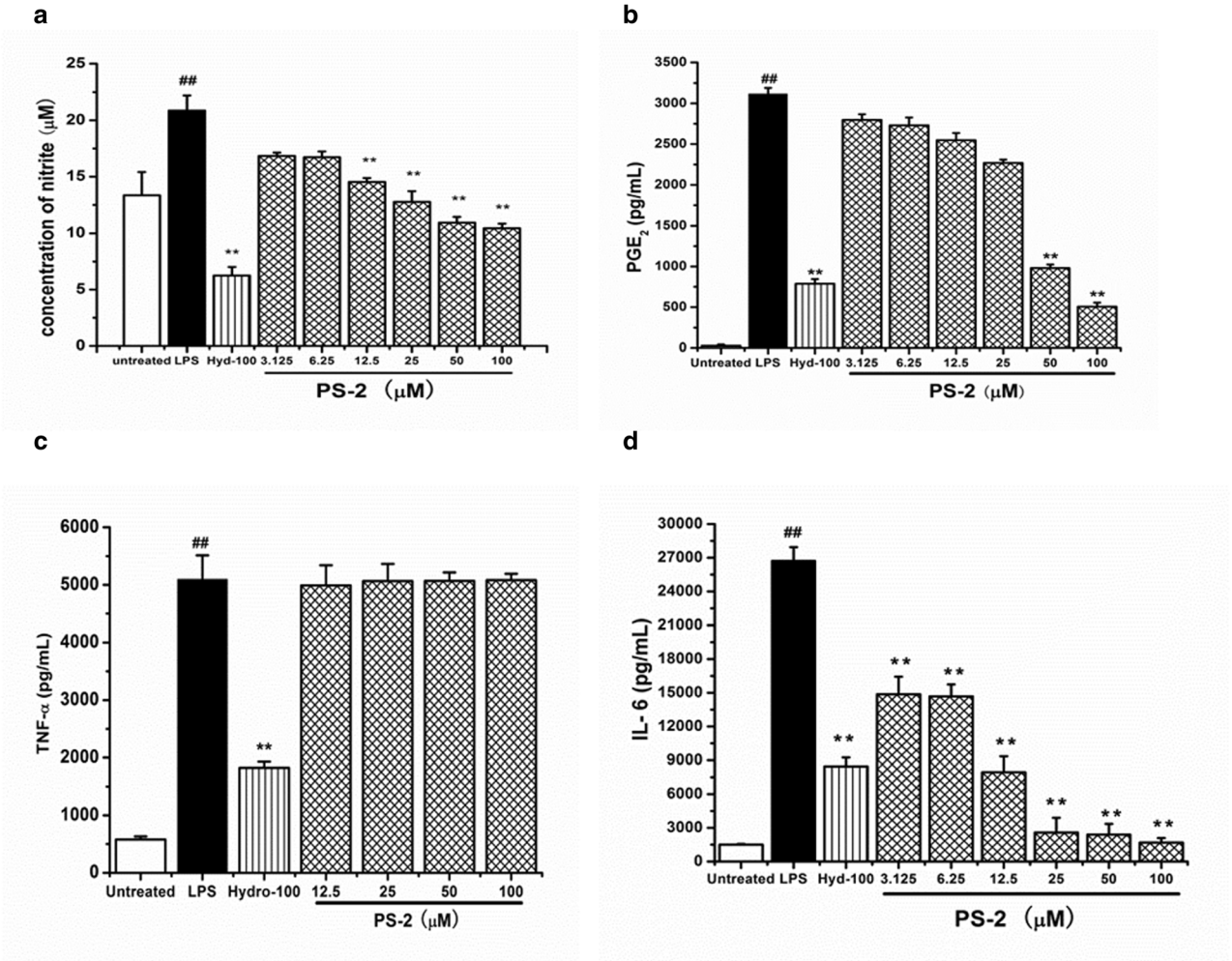

Fig. 3. Effect of PS-2 on the overproduction of NO (**a**), PGE_2 (**b**) and the release of TNF-α (**c**), IL-6 (**d**) in LPS-activated macrophages. RAW 264.7 cells were treated by 1 μg/mL LPS with or without PS-2 (3.125, 6.25, 12.5, 25, 50, and 100 μM) or hydrocortisone sodium succinate (100 μM) for 24 h. Griess assay was used to detect the concentrations of nitrite in the supernatant. Data are presented as mean ± S.D. from three separate experiments (**a**). ELISA was used to detect the levels of PGE_2 in the supernatant. Data are presented as mean ± S.D. from three separate experiments (**b**). RAW 264.7 cells were treated with 1 μg/mL LPS with or without PS-2 (3.125, 6.25, 12.5, 25, 50, and 100 μM) or hydrocortisone sodium succinate (100 μM) for 6 h. The levels of TNF-α (**c**) and IL-6 (**d**) in the supernatant were measured in triplicate by respective ELISA kit. Data are presented as mean ± S.D. from three separate experiments. (##$P < 0.01$ *vs.* untreated group, **$P < 0.01$ *vs.* LPS treatment group).

Effect of PS-2 on the COX-2 Enzymatic Activity

The inhibitory effect of PS-2 on COX-2 enzymatic activity was examined by the cell-free colorimetric method. As shown in Fig. 5, PS-2 significantly inhibited COX-2 enzymatic activity only at the concentration of 1 mM.

Effect of PS-2 on the Activation of MAPK Signaling Pathway

MAPK signaling pathway includes the protein kinases ERK1/2, JNK, and p38 kinase. Previous studies have reported that the MAPK signaling pathway is closely associated with LPS-induced inflammatory reactions. In order to investigate whether PS-2 has an effect on MAPK signaling pathway, LPS-induced phosphorylation of ERK1/2, JNK, and p38 in RAW 264.7 cells were examined by Western blot. As shown in Fig. 6a–c, the phosphorylated ERK1/2, JNK, and p38 proteins significantly increased when stimulated by LPS. Only the phosphorylation of ERK1/2 protein was inhibited by the treatment of PS-2. PS-2 treatment did not show remarkable inhibitory effect on the phosphorylation of JNK or p38 proteins.

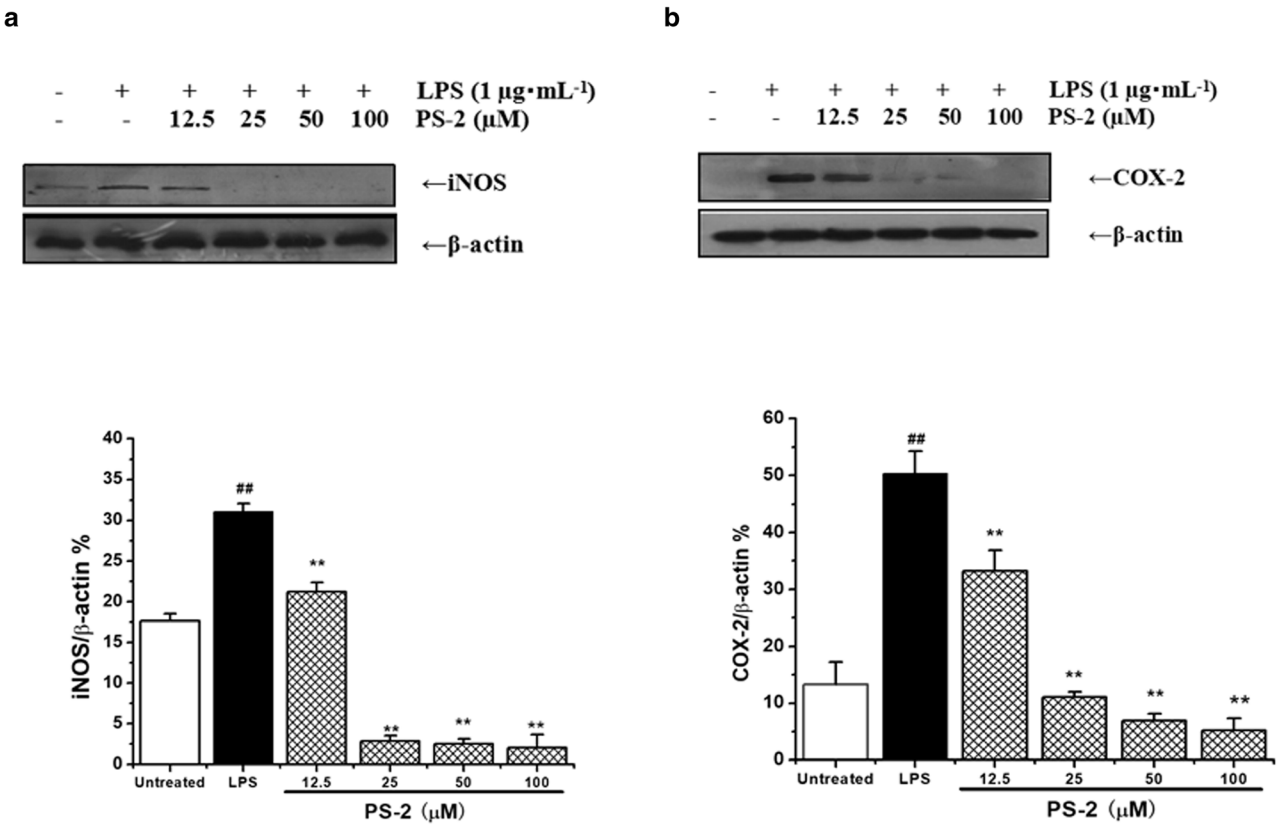

Fig. 4. Effect of PS-2 on high expression of iNOS and COX-2 proteins. RAW 264.7 cells were treated with 1 μg/mL LPS with or without PS-2 (12.5, 25, 50 and 100 μM) for 24 h, and the expression of iNOS and COX-2 proteins was detected by Western blot. The detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of iNOS (**a**) and COX-2 (**b**) was represented by mean ± S.D. from three separate experiments. Data were standardized on the basis of β-actin levels. (##$P < 0.01$ *vs.* untreated group,**$P < 0.01$ *vs.* LPS treatment group).

Effect of PS-2 on the Activation of NF-κB Signaling Pathway

Nuclear factor kappa B (NF-κB) is considered as another important signaling pathway associated with LPS-induced inflammatory reactions. The effect of PS-2 on the degradation of IκB-α and translocation of NF-κB p65 into the nucleus was detected. As shown in Fig. 7a, PS-2 remarkably inhibited the degradation of IκB-α in a dose-dependent manner. LPS-mediated translocation of NF-κB p65 to the nucleus was also suppressed by the treatment of PS-2. These results indicate that PS-2 blocks the activation process of NF-κB signaling pathway induced by LPS in RAW 264.7 cells.

DISCUSSION

Inflammation is a complex biological response when cells are stimulated by external environmental factors such as irritants, pathogens, etc. Macrophages play a critical role in the initiation and propagation of inflammatory response [17]. Pro-inflammatory cytokines or molecules including NO, IL-6, PGE_2, TNF-α, and main inflammatory signaling pathways including MAPKs, NF-κB, are involved in mediating inflammation responses.

NF-κB is one of the most important transcription factors in many cells. Activation of NF-κB pathway plays a pivotal role in inflammatory process. In untreated cells, NF-κB heterodimers are kept as inactive complexes in the cytoplasm by its inhibitory protein IκB-α. After cells are stimulated, IκB-α is phosphorylated and then degraded. Free NF-κB dimers are translocated from monomeric p65 and p50 to the nucleus and binds to the target genes of inducible nitric oxide synthase (iNOS) and cyclooxygenase [18]. Reported studies indicated that iNOS and COX-2 proteins can be inducted through NF-κB activation [19]. The high expression of iNOS and COX-2 protein directly

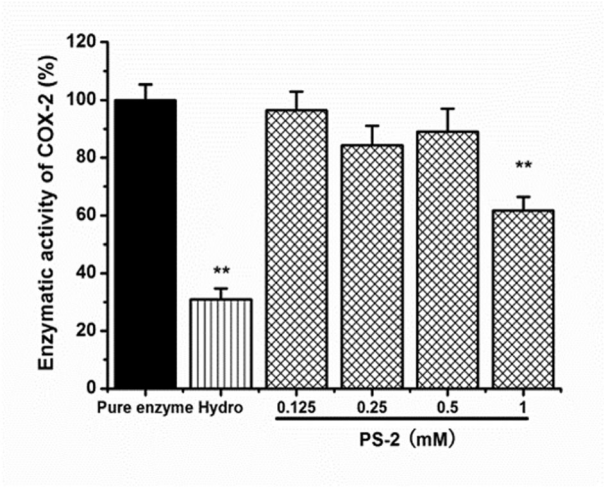

Fig. 5. Effect of PS-2 on the enzymatic activity of COX-2. The colorimetric method was used to detect the effect of PS-2 (0.125, 0.25, 0.5, and 1 mM) and hydrocortisone sodium succinate (1 mM) on COX-2 enzymatic activity. The levels of COX-2 enzymatic activity were plotted as relative units compared to the pure enzyme. The experiment was repeated three times, and data were represented by mean ± S.D. (**$P < 0.01$ *vs.* pure enzyme group).

produces a large amount of downstream inflammatory mediators NO and PGE_2 and induces the release of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, which is correlated with inflammation response [20]. Results in the present study showed that PS-2 significantly inhibited the release of NO, PGE_2, and IL-6, downregulated the expression of iNOS and COX-2 proteins, and inhibited the enzymatic activities of COX-2. Further investigation on the mechanism showed that PS-2 inhibited the degradation of IκB-α and the translocation of NF-κB p65 to the nucleus. These findings suggest that PS-2 may exert anti-inflammatory effects through blocking the activation of NF-κB signaling pathway.

Previous reports have shown that MAPK signaling pathway plays an important role in LPS-mediated inflammation [21–23]. MAPKs comprise a family of highly conserved serine/threonine protein kinases that are involved in the regulation of cellular inflammation response process [24]. There are three major classes of MAPKs, including ERK 1/2, JNK, and p38. MAPKs are also known to regulate the activation process of NF-κB through various mechanisms. Evidences indicated that ERK/MAPKs is a key element of this signaling cascade during NF-κB activation in response to external stimuli and pro-inflammatory cytokine production [25]. In the present study, PS-2 only weakly suppressed the phosphorylation of ERK/MAPKs but did not inhibit the phosphorylation of JNK/MAPKs or p38/MAPKs. These results implied

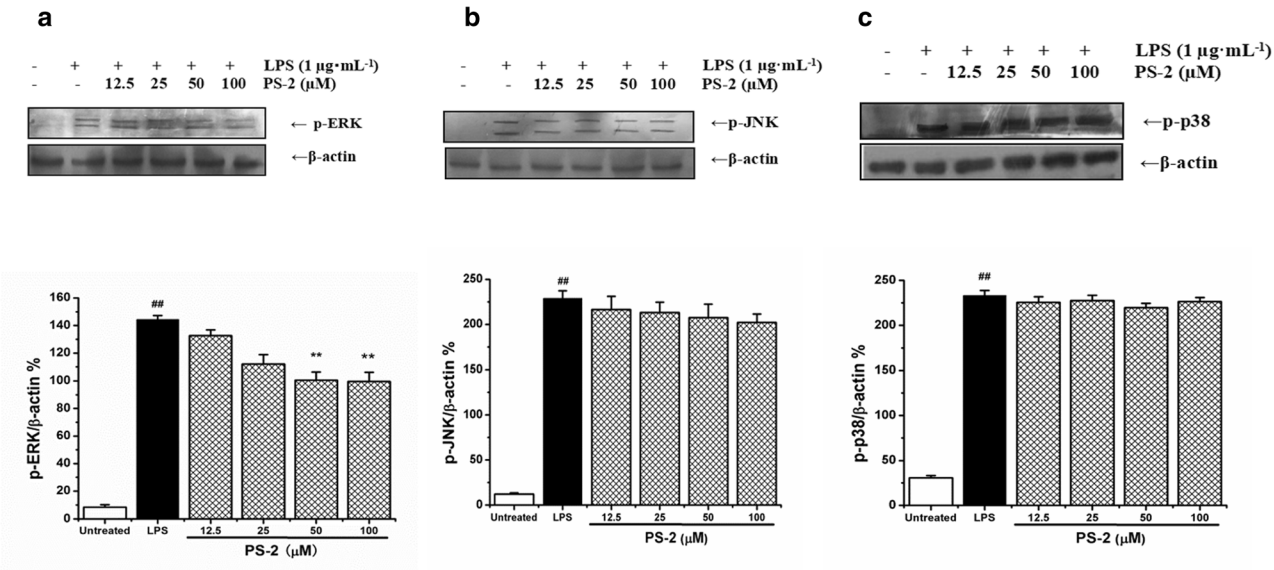

Fig. 6. Effect of PS-2 on phosphorylation of JNK, ERK1/2, and p38 proteins. RAW 264.7 cells were stimulated with 1 μg/mL LPS with or without PS-2 (12.5, 25, 50, and 100 μM) for 15 min or 60 min. Western blot was used to investigate the expression of phospho-ERK1/2, phospho-JNK, and phospho-p38 proteins activated by LPS (**a**–**c**). Detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of phospho-ERK 1/2 protein, phospho-JNK protein, and phospho-p38 protein was represented by mean ± S.D. of three separate experiments. Data were standardized on the basis of β-actin levels. (##$P < 0.01$ *vs.* untreated group, **$P < 0.01$ *vs.* LPS treatment group).

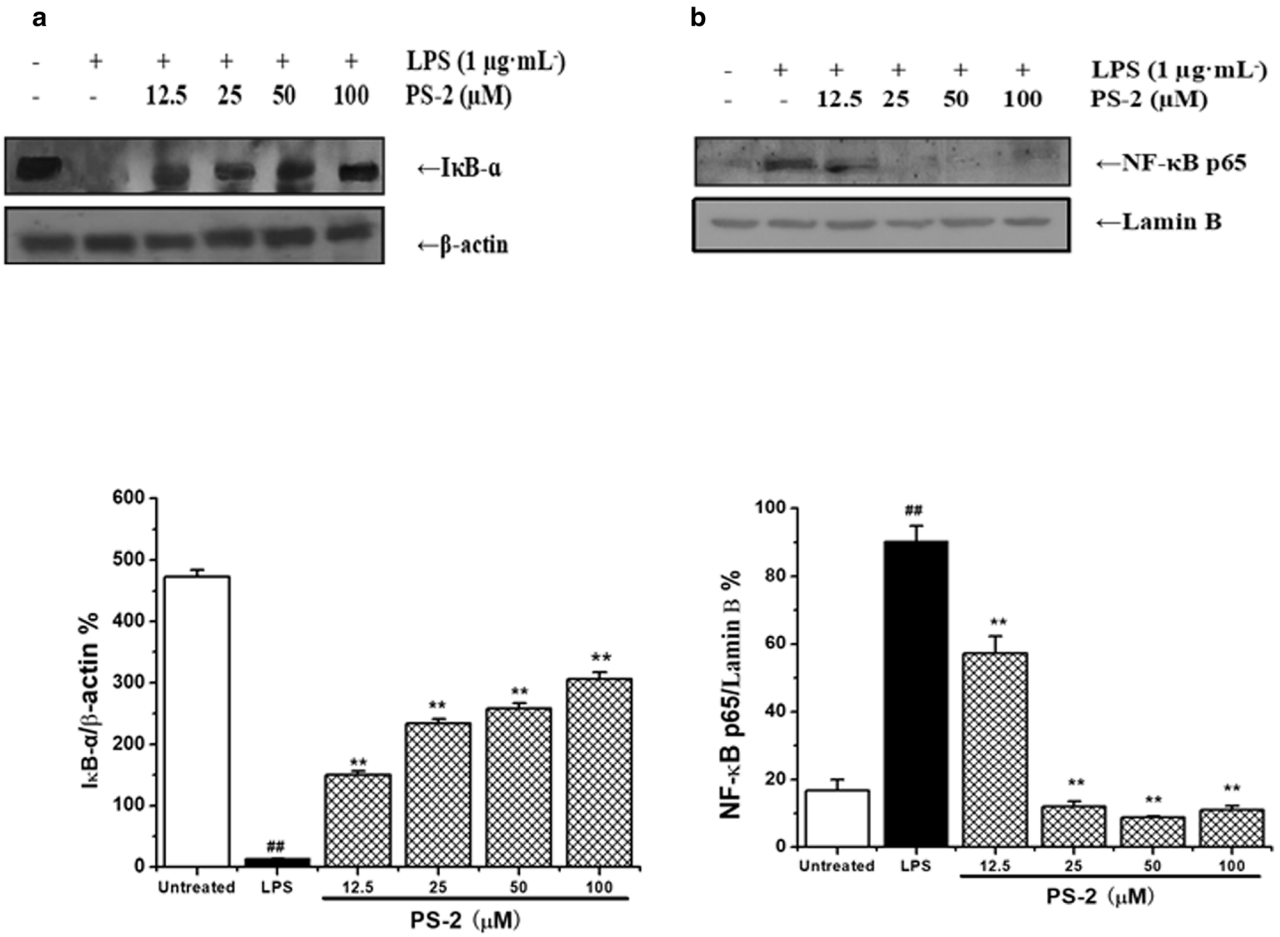

Fig. 7. Effect of PS-2 on the degradation of IκB-α (**a**) and the nuclear translocation of NF-κB p65 (**b**). RAW 264.7 cells were stimulated with1 μg/mL LPS with or without PS-2 (12.5, 25, 50 and 100 μM) for 10 min. Cytoplasmic protein was extracted and Western blot was used to investigate the level of IκB-α protein. Detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of IκB-α protein are represented by mean ± S.D. from three separate experiments. Data were standardized on the basis of β-actin levels. (##$P < 0.01$ *vs.* untreated group, **$P < 0.01$ *vs.* LPS treatment group). **a** RAW 264.7 cells were stimulated with 1 μg/mL LPS with or without PS-2 (12.5, 25, 50, and 100 μM) for 45 min. Nuclear protein was extracted and Western blot was used to investigate the expression of NF-κB p65 protein in the nucleus. Detection of Lamin B was carried out to confirm the equal loading of proteins. **b** Densitometric analysis of NF-κB p65 protein was represented by mean ± S.D. from three separate experiments. Data were standardized on the basis of Lamin B levels. (##$P < 0.01$ *vs.* untreated group, **$P < 0.01$ *vs.* LPS treatment group).

that the anti-inflammatory mechanism of PS-2 is mainly through the inactivation of NF-κB pathway rather than MAPK pathway.

Inflammation progress involves a number of inflammatory mediators including NO and many pro-inflammatory cytokines. Detection of these pro-inflammatory cytokines is considered to be essential for clinical diagnosis. In conclusion, our study results showed that PS-2 exerted significant anti-inflammatory effects by inhibiting the release of a large number of inflammatory mediators such as NO, TNF-α, and IL-6. In addition, PS-2 attenuated the high expression of iNOS and COX-2 proteins and inhibited the enzymatic activity of COX-2. PS-2 may inhibit gene expression or induce protein degradation or what other changes, but the specific molecular mechanism is still unclear. Further investigations suggested that PS-2 effectively inhibited IκB-α degradation and NF-κB p65 translocation to nucleus. All findings clearly elucidated the molecular mechanism of PS-2, which exerts anti-inflammatory effect through blocking the activation of NF-κB signaling pathway in LPS-activated mouse macrophages.

Treatment with PS-2 might be an effective treatment for inflammatory diseases including pneumonia, arthritis, cardiovascular diseases, rheumatism, glomerulonephritis, psoriasis, chronic bronchitis, rheumatoid [26], etc. Further investigations on the pharmacological activity and detailed mechanism of PS-2 using animal model are in progress.

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