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ORIGINAL ARTICLE

Plantamajoside Inhibits Lipopolysaccharide-Induced MUC5AC Expression and Inflammation through Suppressing the PI3K/Akt and NF-κB Signaling Pathways in Human Airway Epithelial Cells

Chaonan Ma[1,2] and Wei Ma[1]

Abstract—It has been reported that plantamajoside (PMS), a major natural compound isolated from *Plantago asiatica*, has anti-inflammatory activities. However, the effect of PMS on respiratory inflammatory diseases has not yet been studied. The present study aimed to evaluate the effect of PMS on lipopolysaccharide (LPS)-induced airway inflammation and the underlying mechanism. The results showed that PMS did not affect the cell viability of 16-HBE cells. PMS (20 and 40 μg/ml) decreased the expression levels of MUC5AC, IL-6, and IL-1β, which were induced by LPS treatment. PMS inhibited the LPS-induced phosphorylation of Akt and p65. In addition, inhibitors of the PI3K/Akt and NF-κB pathways attenuated the effect of LPS on 16-HBE cells. In conclusion, PMS inhibits LPS-induced MUC5AC expression and inflammation through suppressing the PI3K/Akt and NF-κB signaling pathways, indicating that PMS may be a potential therapy for the treatment of respiratory inflammatory diseases.

KEY WORDS: plantamajoside; MUC5AC; PI3K/Akt; NF-κB; airway inflammation; respiratory inflammatory diseases.

INTRODUCTION

Plantamajoside (PMS; Fig. 1a) is a major natural compound isolated from *Plantago asiatica*, and it has been used in folk medicine for a long time [1]. PMS belongs to phenylpropanoid glycoside, and pharmacological studies proved that it has broad biological activities, such as anti-oxidant [2], anti-inflammatory [3], and anti-tumor effects [4]. However, the possible anti-inflammatory effects of PMS on respiratory inflammatory diseases have not yet been studied.

Inflammation is considered as an important and ubiquitous feature of respiratory airway diseases, such as asthma [5] and chronic obstructive pulmonary disease [6, 7]. Targeting inflammatory response is usually used for the treatment of these diseases [8]. Dong et al. proved that baicalin inhibited lipopolysaccharide (LPS)-induced inflammation in HBE16 airway epithelial cells [9]. The anti-inflammatory property of baicalin was resulted from the inhibition of interleukin (IL)-6, IL-8, and tumor necrosis factor α (TNF-α) expression *via* preventing the nuclear factor κB (NF-κB) signaling pathway [9]. Thus, understanding of the mechanism of inflammatory response during respiratory inflammatory diseases is helpful for the treatment of these diseases.

It has been reported that many signaling pathways are involved in the inflammation response [10]. Among the

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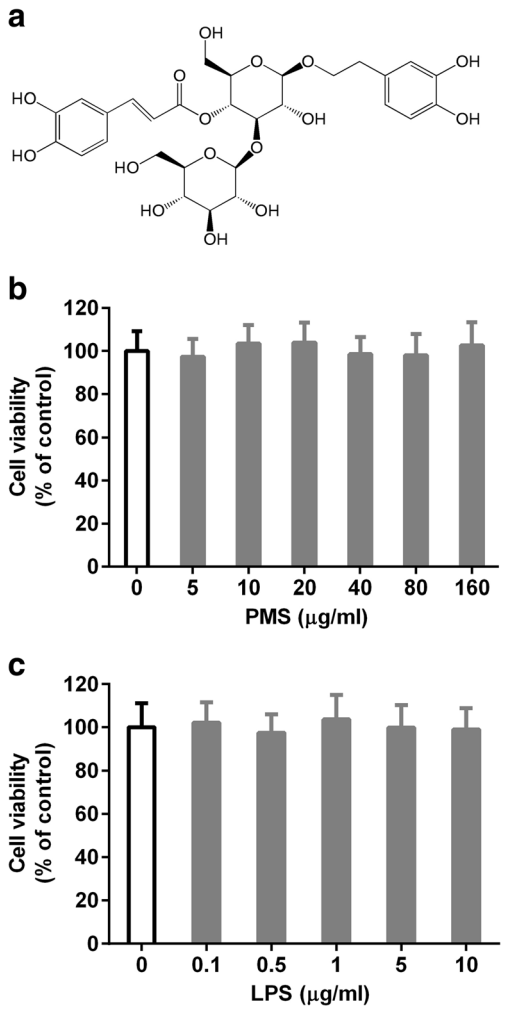

Fig. 1. Effect of PMS on viability of 16-HBE cells. To evaluate the cytotoxicity of PMS and LPS on 16-HBE cells, cells were incubated with various concentrations of PMS (0, 5, 10, 20, 40, 80, 160 μg/ml) or LPS (0, 0.1, 0.5, 1, 5, 10 μg/ml). After incubation for 24 h, the cell viability was measured by MTT assay. **a** Chemical structure of PMS. **b** Cell viability of 16-HBE cells treated with PMS. **c** Cell viability of 16-HBE cells treated with LPS.

pathways, the PI3K/Akt pathway is important in various cellular functions, including growth, proliferation, migration, and survival [11]. Previous studies showed that the inhibitor of PI3K/Akt attenuated airway inflammation and airway hyperresponsiveness in a murine asthma model [12]. Besides, NF-κB is a major regulator in inflammatory responses, and it plays a key role in mediating the expression of inflammatory proteins in airway and lung inflammation and injury [10]. Therefore, the inhibition of PI3K/Akt and NF-κB pathways may be useful for the treatment of respiratory inflammatory diseases.

In the present study, the effect of PMS on LPS-induced inflammation and the underlying mechanism in human airway epithelial cells were evaluated. The results proved that PMS inhibited LPS-induced MUC5AC expression and inflammation through suppressing the PI3K/Akt and NF-κB signaling pathways, indicating that PMS may be a potential therapy for the treatment of respiratory inflammatory diseases.

MATERIALS AND METHODS

Cell Culture and Treatment

Human bronchial epithelial cells (16-HBEs) were obtained from the American type culture collection (ATCC, Manassas, VA, USA). 16-HBE cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco) at 37 °C in 5% CO_2. For some experiments, 16-HBE cells were treated with LPS (5 μg/ml) in the absence or presence of PMS (20 or 40 μg/ml) or LY294002 (20 μM) or BAY11-7082 (10 μM) for 24 h.

MTT Assay

16-HBE cells were seeded into 96-well plates at a density of 5×10^3 cells per well and treated with a range of concentrations of PMS (0, 5, 10, 20, 40, 80, 160 μg/ml) or LPS (0, 0.1, 0.5, 1, 5, 10 μg/ml). After incubation for 24 h, MTT solution (Sigma, St. Louis, MO, USA) was added and incubated for 4 h. After that, blue formazan crystal (Sigma) dissolved in DMSO was added and incubated for 10 min. Finally, the cell viability was measured at the wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, CA, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA samples from 16-HBE cells were extracted using TRIzol (Invitrogen) and were purified using an RNeasy kit (Qiagen Inc., Valencia, CA). Reverse transcription for cDNA was performed using the PrimeScript TM RT Master Mix Kit (Takara, Shiga, Japan). Finally, qRT-PCR was carried out using SYBR premix Ex Taq II (Takara) on a real-time PCR machine (MX3000P, Stratagene, USA). The expression levels were analyzed with the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal standard. Primer sequences were listed as follows: MUC5AC

sense, 5′-CCAT AGTA CAGT GGTC GATG C-3′, antisense, 5′-GATC GATA CATG GGTA GACT T-3′; IL-6 sense, 5′-GCCT TCGG TCCA GTTG CC-3′, antisense, 5′-GCGC AGAA TGAG ATGA GTTG TCAT G-3′; IL-1β sense, 5′-GATC GTAA GGCC CGTT GCCA GACC-3′, antisense, 5′-AGTA CTGT GCAC TGCC CTFA A-3′; GAPDH sense, 5′-CAGT GGTA CGTA GATC GATA AC-3′, antisense, 5′-CGTA AGTC GATG CCGT GATC GT-3′.

Western Blot

After different treatments, 16-HBE cells were harvested and the total cellular proteins were extracted by RIPA lysis buffer (Invitrogen). Protein levels were quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins (20 μg) were separated on 12% SDS-PAGE gels, and then the gels were electro-transferred to PVDF membranes (Millipore, Billerica, MA, USA). After the transfer, the membrane was blocked with 5% skim milk at room temperature for 1 h. Then, the membranes were incubated with the specific primary antibodies against p-Akt (S473) (sc-101629; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt (sc-8312; Santa Cruz Biotechnology), p-p65 (S536) (sc-33020; Santa Cruz Biotechnology), p65 (sc-109; Santa Cruz Biotechnology), and β-actin (sc-130301; Santa Cruz Biotechnology) at 4 °C overnight. After washing for three times, the membranes were incubated with appropriate horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) with gentle agitation for 2 h at room temperature. After that, chemiluminescent detection was performed using Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad Laboratories). Finally, the protein expression levels were measured by Quantity One Image Analysis Software (Bio-Rad Laboratories) using β-actin as an internal standard.

Enzyme-Linked Immunosorbent Assay

After incubation, cell culture supernatants of 16-HBE cells were collected for ELISA analysis. The levels of IL-6 and IL-1β in the supernatants were quantified using double-antibody sandwich ELISA using a Bio-Rad ELISA reader (Bio-Rad Laboratories) at the wavelength of 450 nm. MUC5AC concentration was detected using MUC5AC ELISA kit (USCNK, Wuhan, China) following the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD). The significance of differences among groups was calculated using one-way variance analysis (ANOVA) with *post hoc* Dunnett's test. A p value less than 0.05 was considered as significantly different.

RESULTS

Effect of Plantamajoside on Viability of 16-HBE Cells

To evaluate the cytotoxicity of PMS and LPS on 16-HBE cells, cells were incubated with various concentrations of PMS (0, 5, 10, 20, 40, 80, 160 μg/ml) or LPS (0, 0.1, 0.5, 1, 5, 10 μg/ml). After incubation for 24 h, the cell viability was measured by MTT assay. The results showed that PMS and LPS did not affect the cell viability of 16-HBE cells (Fig. 1b, c). Concentrations of PMS (20 and 40 μg/ml) and LPS (5 μg/ml) were selected for further experiments.

Plantamajoside Inhibited Lipopolysaccharide-Induced MUC5AC Expression

MUC5AC is an important member of mucoprotein and has been proved to be overexpressed in airway inflammation [13]. To investigate the effect of PMS on MUC5AC expression, 16-HBE cells were induced by LPS in the presence of PMS (20 and 40 μg/ml). The messenger RNA (mRNA) and protein levels of MUC5AC were detected by qRT-PCR and ELISA, respectively. As shown in Fig. 2a, b, LPS significantly induced MUC5AC mRNA expression and increased MUC5AC concentration in the cell supernatants. PMS (20 and 40 μg/ml) decreased the MUC5AC mRNA expression and concentration induced by LPS treatment.

Plantamajoside Inhibited Lipopolysaccharide-Induced IL-6 and IL-1β Expression

IL-6 and IL-1β are two important cytokines during inflammatory responses [14]. In the present study, the mRNA levels of IL-6 and IL-1β in 16-HBE cells were detected by qRT-PCR. The secretion of IL-6 and IL-1β in the cell supernatants was detected by ELISA. As shown in Fig. 3a–d, LPS induced the mRNA levels and secretion of IL-6 and IL-1β in 16-HBE cells. However, PMS (20 and 40 μg/ml) treatment inhibited the induction of LPS. The results indicated that PMS inhibited LPS-induced expression and secretion of IL-6 and IL-1β.

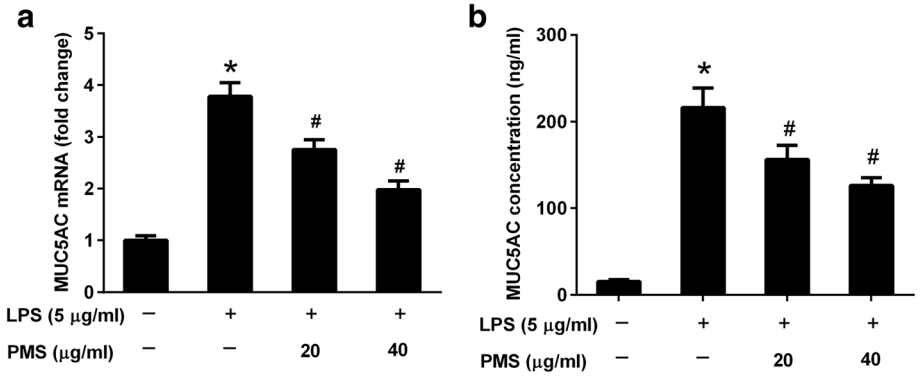

Fig. 2. PMS inhibited LPS-induced MUC5AC expression. 16-HBE cells were treated with LPS (5 μg/ml) in the presence or absence of PMS (20 or 40 μg/ml) for 24 h. The mRNA and protein levels of MUC5AC were detected by qRT-PCR and western blot, respectively. **a** The mRNA levels of MUC5AC in 16-HBE cells. **b** The protein levels of MUC5AC in 16-HBE cells. *$p < 0.05$, *vs* untreated group. #$p < 0.05$, *vs* LPS treatment group.

Plantamajoside Inhibited Lipopolysaccharide-Induced PI3K/Akt and NF-κB Pathways

To investigate whether PI3K/Akt and NF-κB pathways were involved in the effect of PMS, the expression levels of p-Akt, Akt, p-p65, and p65 were detected by western blot. The results showed that the protein levels of p-Akt and p-p65 were increased by LPS treatment, and the induction was attenuated by PMS treatment. The protein levels of Akt and p65 were not affected (Fig. 4a, b). The results indicated that LPS induced the phosphorylation of Akt and p65, while PMS inhibited the phosphorylation. The results also showed that PMS decreased the p-Akt/ Akt and p-p65/p65 ratio, which were increased by LPS treatment.

Inhibition of PI3K/Akt Pathway Attenuated the Effect of Lipopolysaccharide on 16-HBE Cells

To evaluate the role of the PI3K/Akt pathway in 16-HBE cells induced by LPS, 16-HBE cells were treated

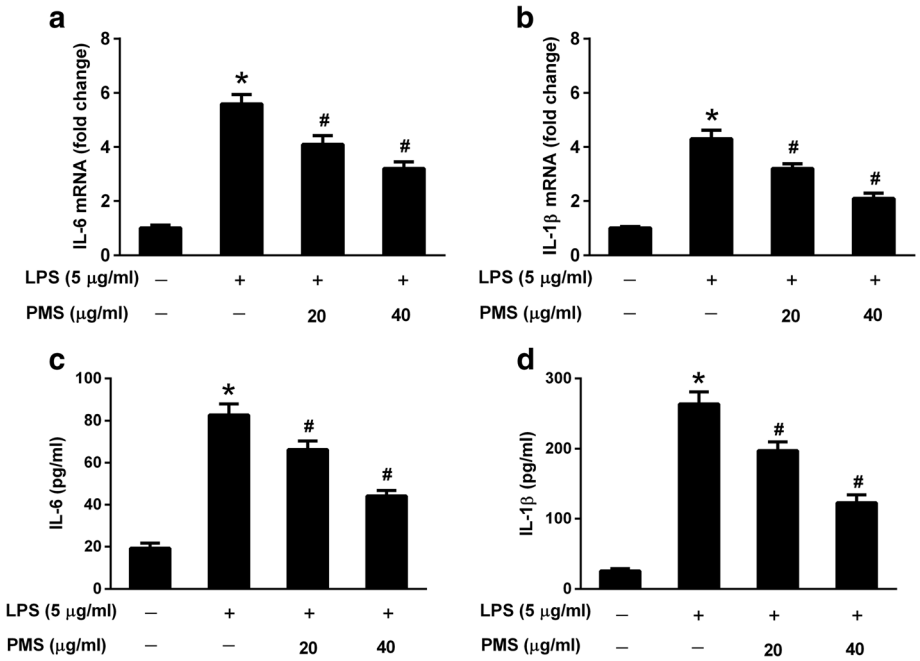

Fig. 3. PMS inhibited LPS-induced IL-6 and IL-1β expression. 16-HBE cells were treated with LPS (5 μg/ml) in the presence or absence of PMS (20 or 40 μg/ml) for 24 h. The mRNA levels of IL-6 and IL-1β were detected by qRT-PCR. The secretion levels of IL-6 and IL-1β in the cell supernatants were detected by ELISA. **a** The mRNA levels of IL-6 in 16-HBE cells. **b** The mRNA levels of IL-1β in 16-HBE cells. **c** Secretion levels of IL-6 in the cell supernatants. **d** Secretion levels of IL-1β in the cell supernatants. *$p < 0.05$, *vs* untreated group. #$p < 0.05$, *vs* LPS treatment group.

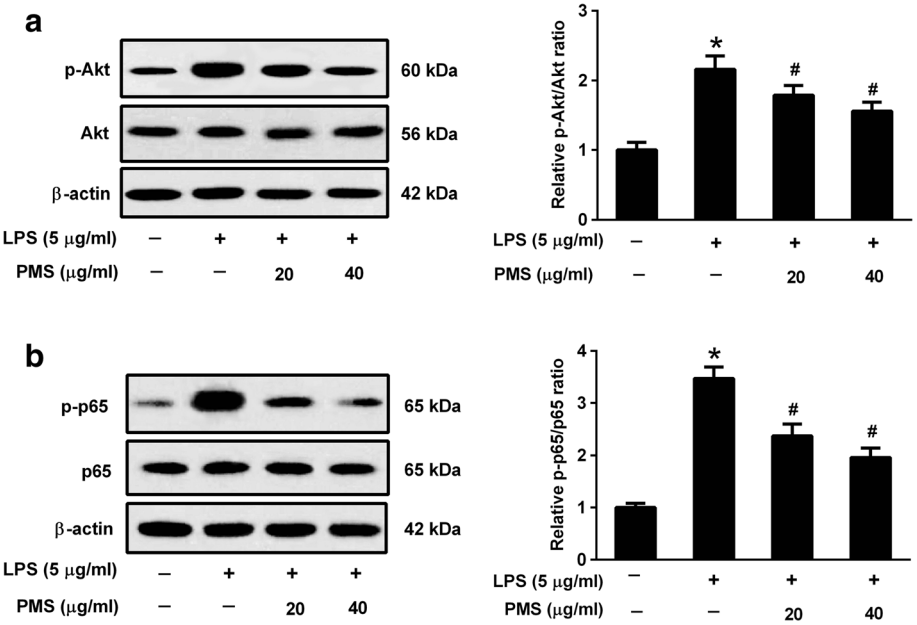

Fig. 4. PMS inhibited LPS-induced PI3K/Akt and NF-κB pathways. 16-HBE cells were treated with LPS (5 μg/ml) in the presence or absence of PMS (20 or 40 μg/ml) for 24 h. The expression levels of p-Akt, Akt, p-p65, and p65 were detected by western blot. **a** Protein levels of p-Akt and Akt in 16-HBE cells. **b** Protein levels of p-p65 and p65 in 16-HBE cells. *$p < 0.05$, *vs* untreated group. #$p < 0.05$, *vs* LPS treatment group.

with the inhibitor of PI3K/Akt (LY294002, 20 μM) for 24 h. As shown in Fig. 5a–c, LY294002 treatment significantly inhibited the phosphorylation of Akt, decreased the levels of MUC5AC mRNA and concentration, and reduced the mRNA expression of IL-6 and IL-1β. LY294002 also reduced the secretion of IL-6 and IL-1β in cell supernatants (Figs. 5d). The results indicated that inhibition of PI3K/Akt pathway attenuated the effect of LPS on 16-HBE cells.

Inhibition of NF-κB Pathway Attenuated the Effect of Lipopolysaccharide on 16-HBE Cells

To evaluate the role of NF-κB pathway in 16-HBE cells induced by LPS, 16-HBE cells were treated with the inhibitor of NF-κB (BAY11-7082, 10 μM) for 24 h. As shown in Fig. 6a, BAY11-7082 inhibited LPS-induced expression of phosphorylated p65. BAY11-7082 decreased mRNA expression and concentration of MUC5AC in the presence of LPS (Fig. 6b). BAY11-7082 inhibited the mRNA expressions of IL-6 and IL-1β, which were induced by LPS (Fig. 6c). BAY11-7082 also reduced the secretion of IL-6 and IL-1β in cell supernatants (Figs. 6d). The results indicated that inhibition of NF-κB pathway attenuated the effect of LPS on 16-HBE cells.

DISCUSSION

Mounting researches have shown that increasing kinds of herbal medicines are applied in the prevention and treatment of many diseases including respiratory diseases [15, 16]. *Plantago asiatica* is a kind of herbal medicine and is used as an expectorant in China. PMS, a major ingredient isolated from *Plantago asiatica* [17], possesses broad activities. A previous study reported that PMS had protective effect on LPS-induced acute lung injury in mice and in RAW264.7 cells. The study also proved that the anti-inflammatory effect was responsible for the protective effect [3]. Besides, PMS was reported to protect advanced glycation end-product (AGE)-induced endothelial cells against inflammatory cellular dysfunction [18]. Since many kinds of respiratory diseases are resulted from inflammation response, we speculated that PMS may have anti-inflammatory effect on respiratory inflammatory diseases, such as asthma and chronic obstructive pulmonary disease.

It is known that inflammation can be mediated by pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and IL-8 [14, 19, 20]. Reducing the production of these cytokines may be beneficial for the treatment of inflammatory diseases. PMS suppressed the production of IL-1β, IL-6, and TNF-α in a dose-dependent manner in a model of acute lung injury [3]. Besides, PMS inhibited the

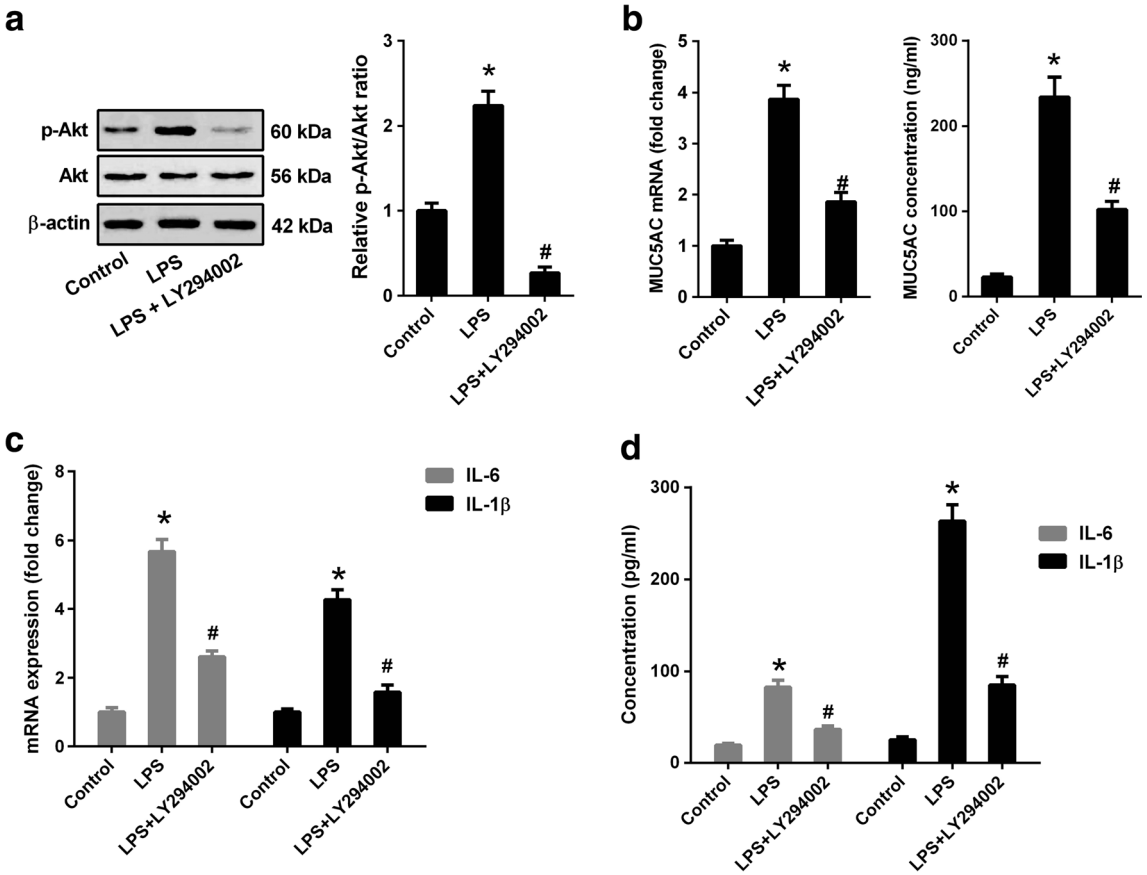

Fig. 5. Inhibition of PI3K/Akt pathway attenuated the effect of LPS on 16-HBE cells. 16-HBE cells were treated with LPS (5 μg/ml) in the presence or absence of LY294002 (20 μM) for 24 h. The mRNA levels of MUC5AC, IL-6, and IL-1β were detected by qRT-PCR. The levels of p-Akt and Akt were detected by western blot. The secretion of IL-6, IL-1β, and MUC5AC in the cell supernatants were detected by ELISA. **a** The protein levels of p-Akt and Akt in 16-HBE cells. **b** The mRNA levels of MUC5AC in 16-HBE cells and MUC5AC concentration in the cell supernatants. **c** The mRNA levels of IL-6 and IL-1β in 16-HBE cells. **d** Secretion levels of IL-6 and IL-1β in the cell supernatants. *$p < 0.05$, *vs* control group. #$p < 0.05$, *vs* LPS treatment group.

expression of pro-inflammatory cytokines including TNF-α, IL-6, and MCP-1 in glyceraldehyde-induced AGEs [18]. In the present study, we found that PMS inhibited LPS-induced expression and secretion of IL-6 and IL-1β.

Mucin hypersecretion is frequently observed in many inflammatory diseases of the human respiratory tract [21, 22]. Previous studies indicated that negative control mechanisms of mucin hypersecretion were important for developing novel therapeutic medications. MUC5AC is one of the major members of mucin, and it has been found to be overexpressed during airway inflammation [13]. Many molecules are reported to inhibit the MUC5AC expression in airway inflammation [23, 24], and the inhibitors are considered as a potential therapeutic strategy for the treatment of respiratory inflammatory diseases. In the present study, we also found that PMS inhibited LPS-induced overexpression of MUC5AC.

The PI3K family plays a prominent role in various airway and lung inflammation [10]. Choi et al. [25] proved that inhibition of protein kinase C delta attenuated allergic airway inflammation through suppression of PI3K/Akt/mTOR/HIF-1α/VEGF pathway. In another study, ovalbumin-induced allergic airway response could be promoted in part by upregulation of the PI3K/Akt pathway in pups [26]. In addition, the NF-κB pathway also plays an important role in respiratory inflammatory diseases [10]. Titanium dioxide nanoparticles augmented allergic airway inflammation *via* the NF-κB pathway in murine model of asthma [27]. In a mouse model of allergic asthma, the ethanol extract of *Citrus tachibana* leaves alleviated airway inflammation *via* inhibiting the NF-κB signaling pathway [28]. All of the results indicated that inhibition of PI3K/Akt and NF-κB pathways may be useful therapies in the treatment of respiratory inflammatory diseases. In the

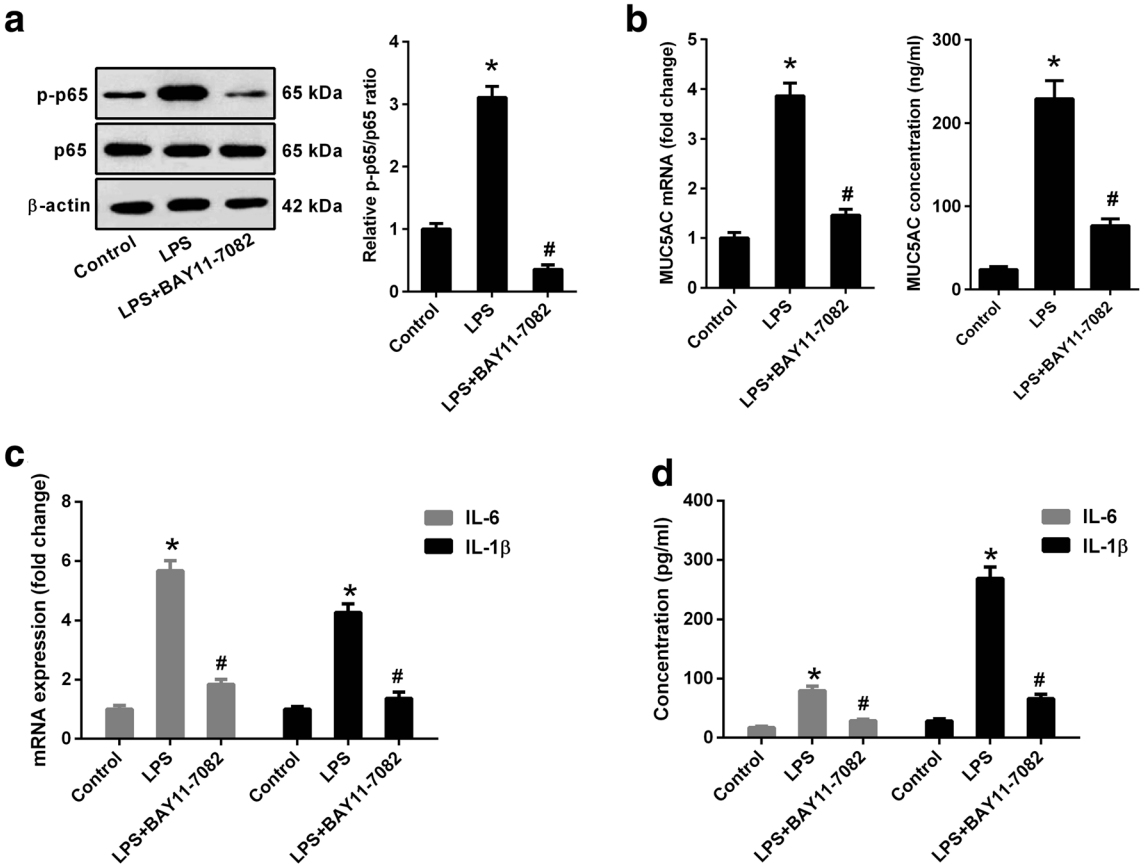

Fig. 6. Inhibition of NF-κB pathway attenuated the effect of LPS on 16-HBE cells. 16-HBE cells were treated with LPS (5 μg/ml) in the presence or absence of BAY11-7082 (10 μM) for 24 h. The mRNA levels of MUC5AC, IL-6, and IL-1β were detected by qRT-PCR. The protein levels of p-p65 and p65 were detected by western blot. The secretion of IL-6, IL-1β, and MUC5AC in the cell supernatants were detected by ELISA. **a** The protein levels of p-p65 and p65 in 16-HBE cells. **b** The mRNA levels of MUC5AC in 16-HBE cells and MUC5AC concentration in the cell supernatants. **c** The mRNA levels of IL-6 and IL-1β in 16-HBE cells. **d** Secretion levels of IL-6 and IL-1β in the cell supernatants. *$p < 0.05$, *vs* control group. #$p < 0.05$, *vs* LPS treatment group.

present study, PMS treatment attenuated LPS-induced MUC5AC expression and inflammation through suppressing the PI3K/Akt and NF-κB signaling pathways. The effect of PMS was similar with the inhibitor of PI3K/Akt and NF-κB.

In summary, the effect of PMS on LPS-induced inflammation and the underlying mechanism in human airway epithelial cells were evaluated in the present study. We found that PMS decreased the expression levels of MUC5AC, IL-6, and IL-1β, which were induced by LPS treatment. PMS inhibited the LPS-induced phosphorylation of Akt and p65. In addition, inhibition of the PI3K/Akt and NF-κB pathways attenuated the effect of LPS on 16-HBE cells. The results proved that PMS inhibited LPS-induced MUC5AC expression and inflammation through suppressing the PI3K/Akt and NF-κB signaling pathways, indicating that PMS may be a potential therapy for the treatment of asthma, chronic obstructive pulmonary disease, and other respiratory inflammatory diseases.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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