SPANDIDOS PUBLICATIONS

Resveratrol inhibits lipopolysaccharide-induced MUC5AC expression and airway inflammation via MAPK and Nrf2 pathways in human bronchial epithelial cells and an acute inflammatory mouse model

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Abstract. Pathological mucus hypersecretion is an important clinical hallmark of chronic airway inflammatory diseases and yet there is a lack of effective therapeutic medicine. Resveratrol, a dietary polyphenol, has been shown to possess anti-aging, antioxidation, anti-inflammation and tumor prevention effects. However, the effect and underlying mechanism of resveratrol in lipopolysaccharide (LPS) induced-mucus hypersecretion remain to be elucidated. Among more than 20 mucin family members, mucin 5ac (MUC5AC) is a major glycoprotein in airway mucus. The present study investigated the therapeutic effects and mechanisms of resveratrol in LPS-induced MUC5AC expression in human bronchial epithelial (NCI-H292) cells and an acute inflammatory murine model. It found that resveratrol markedly attenuated LPS-induced MUC5AC expression and reactive oxygen species production in NCI-H292 cells. Moreover, resveratrol increased activation of nuclear factor erythroid-2-related factor 2 (Nrf2) and phosphorylation of mitogen-activated protein kinase (MAPK). Notably, compared with negative control, knockdown of Nrf2 by small interfering RNA and specific inhibitors of ERK/p38 MAPK markedly abrogated the downregulative effect of resveratrol on LPS-induced MUC5AC expression in NCI-H292 cells. Additionally, *in vivo* effects on histopathology and gene expression were assessed in lung tissues collected after intratracheal instillation of LPS with or without resveratrol treatment. Western blotting of lung tissue samples confirmed that administration of resveratrol inhibited MUC5AC expression in LPS-induced acute inflammatory mice, but increased Nrf2 expression along with phosphorylation of ERK and p38. Periodic acid-Schiff's staining also showed that resveratrol suppressed mucin production. Compared with the LPS group, administration of resveratrol effectively decreased the numbers of inflammatory cells and neutrophils in bronchoalveolar lavage fluid, as well as markedly alleviating the infiltration of exacerbated inflammatory cells in lung tissue. In conclusion, resveratrol exerted protective effects against LPS-induced MUC5AC overexpression, inflammation and oxidative stress by activating ERK/p38 MAPK and Nrf2 pathway. Furthermore, the results suggested that resveratrol might be a potential therapeutic agent to inhibit airway mucus hyperproduction.

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Introduction

Airway mucus is the host defense to protect the respiratory system against invading foreign stimuli, such as chemicals, airborne particles and pathogenic microorganisms. However, uncontrolled mucus hypersecretion impairs mucociliary clearance, increases airway resistance and decreases airflow through the lungs (1-3). Airway mucus hypersecretion is an important pathophysiological and clinical hallmark of asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis and other chronic airway inflammatory diseases. Excessive secretion of airway mucus irreversibly deteriorates lung function and notably increases pathogen colonization, the frequency of acute exacerbations, hospitalization rates and mortality (4-6).

Vestbo *et al* (4) found that in patients with COPD and chronic airway mucus hypersecretion, the risk of acute exacerbation and hospitalization increased 2.4 times in men and 2.6 times in women. A multi-center observational study on 433 patients with COPD shows that the acute exacerbation risk of subjects with chronic cough and sputum production increases by 4.15 times and the following hospitalization risk increases by 4.08 times (5). In addition, a follow-up of 14,223 patients with COPD for 10-12 years found that chronic airway mucus hypersecretion increases risk of mortality by 3.5 times compared with non-airway mucus hypersecretion (6). Despite its effect, there remains a lack of effective therapeutic medicines to suppress mucus hypersecretion (7). Among the mucin family members, mucin 5ac (MUC5AC) is a major glycoprotein in the human airway mucus. A number of studies have indicated that abnormal expression of MUC5AC plays an essential role in the pathogenesis of respiratory diseases (1-3). High levels of MUC5AC expression are often induced by various stimuli, including lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, epidermal growth factor (EGF), phorbol myristate acetate (PMA) and interleukin-13. As a major component of the outer membrane of gram-negative bacteria, LPS can cause excessive inflammation and oxidative injury to lung epithelium and endothelium, accompanied by airway mucus hypersecretion (8). Accumulating evidence demonstrates that LPS is a critical factor in developing COPD, bronchiectasis and cystic fibrosis pulmonary disease (9-12). Therefore, it is essential to explore effective therapeutic drugs to regulate LPS-induced MUC5AC overexpression.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenol found in red grape skin, *Polygonum cuspidatum*, peanuts, blueberries and other berries. Several studies have reported that resveratrol exhibits various biological effects, including anti-inflammatory, anti-oxidative, anti-aging, immunomodulatory and tumor-preventive effects. Resveratrol has been reported to ameliorates LPS-induced acute lung injury (13-16). In addition, resveratrol-loaded lipid-core nanocapsules can modulate acute lung inflammation and oxidative imbalance in an LPS-induced lung injury model (17,18). Resveratrol prevents inflammation and oxidative stress response in LPS-induced human gingival fibroblasts by targeting the PI3K/AKT and Wnt/β-catenin signaling pathways (19). Moreover, resveratrol exerts protective effects on LPS and cigarette smoke-induced COPD in a murine model (20). However, only a few studies have focused on the role of resveratrol in mucus regulation. Studies have demonstrated that resveratrol inhibits MUC5AC synthesis induced by EGF, PMA and TNF-α in the human airway epithelium and decreases MUC5AC expression in murine asthma models (21-23). Nonetheless, to the best of the authors' knowledge there is no comprehensive study on the effects and mechanisms of resveratrol on MUC5AC expression induced by LPS *in vitro* and *in vivo*.

Nuclear factor erythroid 2-related factor 2 (Nrf2), an important transcription factor involved in oxidative stress and plays a critical role in regulating cellular redox status. Resveratrol, a potent antioxidant and Nrf2 modulator, is known to have antioxidant and anti-inflammatory effects by activating the Nrf2 and mitogen-activated protein kinase (MAPK) signaling pathways (23-26). The MAPK signaling pathway has been found to be involved in regulating LPS-induced MUC5AC overexpression (27-31). One study demonstrates that resveratrol decreases MUC5AC expression by inhibiting ERK and AKT signaling in mucus-producing A549 human lung carcinoma cells (23). Orally administered resveratrol-loaded lipid-core nanocapsules ameliorate LPS-induced acute lung injury via ERK and PI3K/Akt pathways (17). A previous study from our laboratory demonstrates that Nrf2 ameliorates cigarette smoking-induced MUC5AC overproduction in A549 cells and mouse lung (32). Another study reveals that Nrf2 is involved in the regulatory effect of curcumin on LPS-induced MUC5AC hypersecretion and airway inflammation (33). However, it remains uncertain whether resveratrol inhibits LPS-induced MUC5AC production via MAPK and Nrf2 pathways.

Therefore, the present study aimed to determine whether resveratrol could alleviate LPS-induced MUC5AC overexpression and inflammatory response in NCI-H292 cells and an acute airway inflammatory murine model. Furthermore, it examined the role of MAPK and Nrf2 signaling in the protective effects of resveratrol against LPS-induced MUC5AC expression.

Materials and methods

Reagents and kits. Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific, Inc.). Lipopolysaccharide, resveratrol, PD98059/SP600125/SB203580 (inhibitors of ERK/JNK/p38, respectively) and methyl-thiazolyl-tetrazolium (MTT) were from MilliporeSigma. Resveratrol, PD98059, SP600125 and SB203580 were dissolved in DMSO and lipopolysaccharide and MTT were diluted in a filtered PBS. Other reagents and kits included reactive oxygen species (ROS) Assay Kit (Enzo Life Sciences, Inc.), Nrf2 small interfering (si)RNA (Invitrogen; Thermo Fisher Scientific, Inc.), TRIzol® reagent (Thermo Fisher Scientific, Inc.), total RNA isolation and reverse transcription system (Promega Corporation), mouse MUC5AC antibody (Thermo Fisher Scientific, Inc.), human heme oxygenase-1 (HO-1) antibody (Enzo Life Sciences, Inc.) and human GCLC antibody (Abnova, Inc.). Antibodies against human MUC5AC, Nrf2, β-actin, and GAPDH were purchased from Abcam. Antibodies against NAD(P)H: quinine oxidoreductase 1 (NQO1), phosphorylated (p-)ERK, ERK, p-P38, P38, p-JNK and JNK were purchased from Cell Signaling Technology, Inc.

Cell culture. NCI-H292 cells, a human airway epithelial cell line, was obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in RPMI 1640 supplemented with 10% FBS +1% penicillin/streptomycin in a 5% CO_2 incubator at 37˚C. When cells were ~80% confluent, varying doses of resveratrol dissolved in DMSO were added to the culture media for 30 min and then co-incubated with or without LPS (10 μg/ml) for 24 h.

Cell viability analysis. The MTT assay was used to evaluate resveratrol cytotoxicity. Cells (6x10^3 cells/well) were seeded in 96-well plates and treated with resveratrol (10-100 μM), with or without LPS (10 μg/ml) for 24 h. The cells were then incubated with 5 mg/ml MTT solution for 4 h and the absorbance was measured at 490 nm to estimate viable cells. The relative

Table I. Primers for quantitative real-time PCR analysis.

Species	Genes	Primer sequence (5'-3')
Human	GAPDH	Forward: CTCCTGCACCACCAACTGCTTAG Reverse: GACGCCTGCTTCACCACCTTC
Human	MUC5AC	Forward: CAGCACAACCCCTGTTTCAAA Reverse: GCGCACAGAGGATGACAGT
Human	Nrf2	Forward: AAACCAGTGGATCTGCCAAC Reverse: ACGTAGCCGAAGAAACCTCA
Human	HO-1	Forward: ATGGCCTCCCTGTACCACATC Reverse: TGTTGCGCTCAATCTCCTCCT
Human	NQO1	Forward: GTTGCCTGAAAAATGGGAG Reverse: AAAAACCACCAGTGCCAGT
Human	GCLC	Forward: TCTCTAATAAAGAGATGAGCAACATGC Reverse: TTGACGATAGATAAAGAGATCTACGAA
Mouse	β-actin	Forward: GGCTGTATTCCCCTCCATCG Reverse: CCAGTTGGTAACAATGCCATGT
Mouse	MUC5AC	Forward: CTGTGACATTATCCCATAAGCCC Reverse: AAGGGGTATAGCTGGCCTGA
Mouse	Nrf2	Forward: GGACATGGAGCAAGTTTGGC Reverse: GCTGGGAACAGCGGTAGTATC

cell viability (%) was calculated as the ratio of the absorbance of the administered cells to that of the untreated cells.

ROS generation assay. An ROS detection kit (Enzo Life Sciences, Inc.) was used to measure the total ROS levels following the manufacturer's protocol. Briefly, cells were collected, stained with a ROS Detection Reagent and incubated in the dark at 37˚C for 30 min. Finally, the fluorescence intensity of each sample was measured using flow cytometry.

siRNA. *NFE2L2* siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) or scrambled RNA (as a negative control) was transfected into NCI‑H292 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, $4x10^5$ cells were seeded and cultured in 6‑well plates until they reached 50‑70% confluence and then treated with 50, 75, or 100 nM siRNA for 48 h at room temperature. Transfection efficiency was assessed by RT‑qPCR and western blotting. The sequences were: siRNA‑Nrf2 forward, 5'‑AAUGAGUUCACUGUCAACUGGUUGG‑3'; siRNA‑Nrf2 reverse, 3'‑CCAACCAGUUGACAGUGAACU CAUU‑5' and negative control (NC)‑siRNA forward, 5'‑UUC UCCGAACGUGUCACGUdTdT‑3' and NC‑siRNA reverse, 3'‑ACGUGACACGUUCGGAGAAdTdT‑5'.

NCI‑H292 cells were divided into six groups: siRNA‑NC, LPS + siRNA‑NC, LPS+ resveratrol + siRNA‑NC, Nrf2‑siRNA control, LPS + Nrf2‑siRNA and LPS + resveratrol + Nrf2‑siRNA. LPS (10 µg/ml) was added to the NCI‑H292 cell culture of the LPS group for 24 h. The cells of the LPS + resveratrol group were pretreated with resveratrol (50 µM) for 30 min and then incubated with LPS (10 µg/ml) for 24 h. At 6 h after small interfering RNA transfection, the cells were incubated with LPS (10 µg/ml) or resveratrol (50 µM) + LPS (10 µg/ml) for 24 h.

Reverse transcription‑quantitative (RT‑q) PCR. Total RNA was isolated from cells ($1x10^7$ cells) and lung tissues using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse‑transcribed using a Reverse Transcription Reagent Kit (Takara Bio, Inc.) according to the manufacturer's instructions. SYBR Green Premix Ex Taq II (Takara Bio, Inc.) was used to perform real‑time qPCR on an Applied Biosystems StepOnePlus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR primers used in this study are listed in Table I. The relative mRNA expression levels of the target genes were normalized to the CT value of GAPDH using the $2^{-\Delta\Delta Cq}$ formula (34). All the experiments were replicated three times.

Protein extraction and western blotting. Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) with a protease inhibitor cocktail (Roche Diagnostics) and quantified using a BCA protein assay kit. Equal weights of proteins (20 µg/lane) were separated by SDS‑PAGE on 8‑10% gel and electrotransferred onto polyvinylidene difluoride membranes (MilliporeSigma). Membranes were blocked with 5% skimmed milk in tris‑buffered saline containing 0.1% Tween 20 (TBST) for 1 h at 37˚C and incubated with various primary antibodies overnight at 4˚C. The following primary antibodies were used: Human MUC5AC (1:100, ab24070, Abcam), human Nrf2 (1:1,000, ab62352; Abcam), GAPDH (1:5,000, ab9485, Abcam), β‑actin (1:5,000, ab8227, Abcam), HO‑1 (1:1,000, ADI‑SPA‑896, Enzo Life Sciences), NQO1 (1:1,000, ma1‑16672, cell signaling Technology), GCLC (1:1,000, H00002729, Cell Signaling Technology), MAPK p38 (1:1,000, #9212, Cell Signaling Technology), pp38 (1:1,000, #9215S, Cell Signaling Technology), ERK (1:1,000, #4695, Cell Signaling Technology), p‑ERK (1:1,000, #4377S, Cell

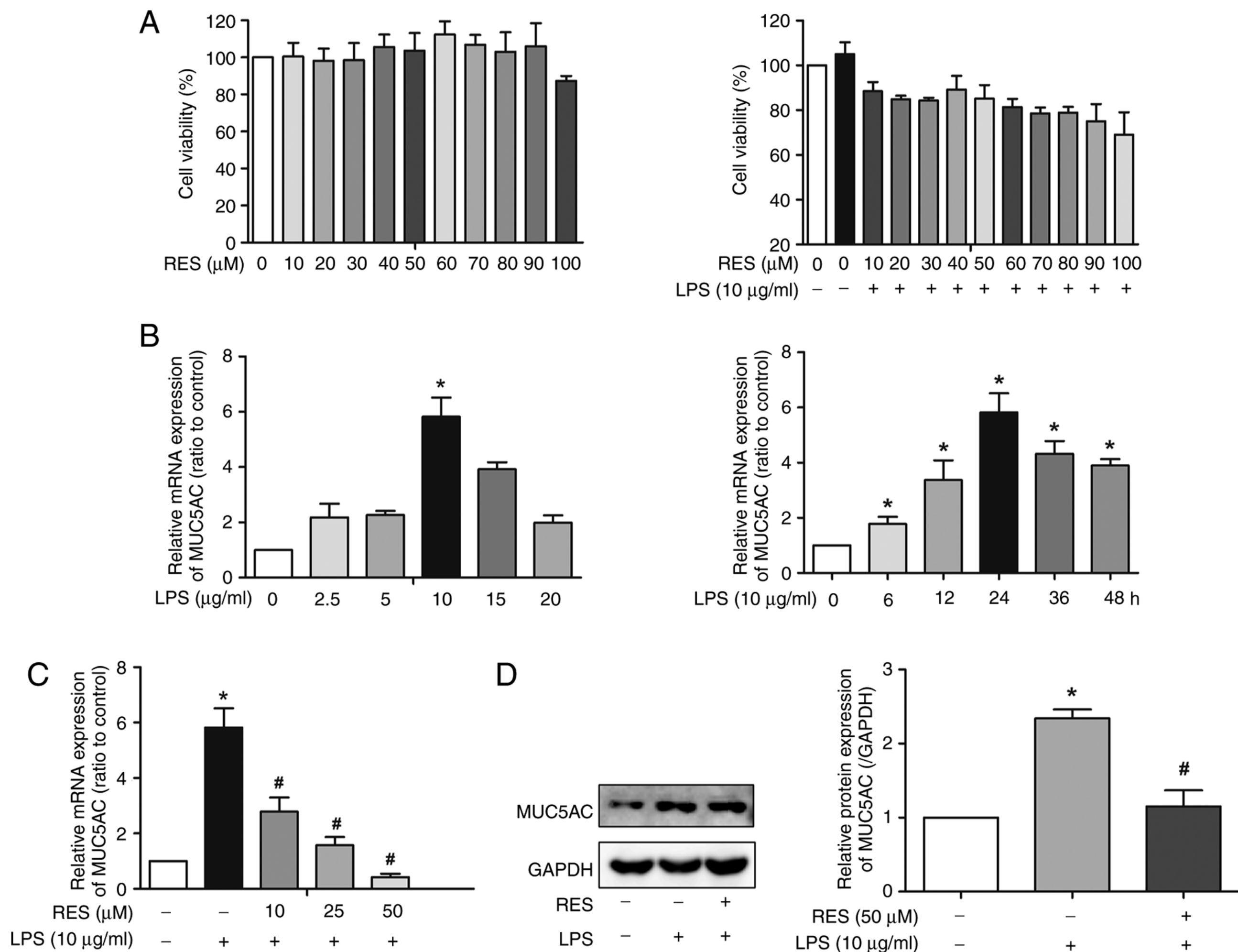

Figure 1. Effects of resveratrol on MUC5AC expression induced by LPS in NCI-H292 cells. (A) MTT assay demonstrated that treatment with resveratrol for 24 h had little toxic effect on NCI-H292 cells. (B) The mRNA expression of MUC5AC induced by LPS at different concentrations and different times. (C) Resveratrol inhibited MUC5AC mRNA expression in a dose-dependent manner. (D) Resveratrol (50 μM) inhibited MUC5AC protein expression induced by LPS (10 μg/ml). All data are expressed as the mean ± standard error of the mean, n=3/group. *P<0.05 vs. control group; #P<0.05 vs. LPS group. MUC5AC, mucin 5ac; LPS, lipopolysaccharide; RES, resveratrol.

Signaling Technology), mouse MUC5AC (1:100, MA5-12178, Thermo Fisher Scientific). The next day, the membranes were washed in TBST and hybridized with HRP-conjugated secondary antibodies (1:10,000, BA1050 and BA1054, Wuhan Boster Biological Technology) at room temperature for 1 h. Immunoreactive protein bands were visualized using enhanced chemiluminescence (cat. no. #32109; Thermo Fisher Scientific, Inc.) on a GE ImageQuant LAS4000mini System (Cytiva). The intensities of the relative bands were quantified using Quantity One 1-D 4.62 software (Bio-Rad).

Animal experiments. All procedures were approved by the Ethics Committee of the second affiliated hospital of Fujian Medical University (approval no. 2022-FYFE-559) and all experiments were performed in accordance with the ARRIVE guidelines for animal care (35).

A total 21 of male C57BL/6 mice (8-10 weeks old and body weight of 18-22 g) were purchased from the Experimental Animal Center of the Fujian Medical University (Fujian, China). Mice were housed under standard conditions and had unlimited access to sterilized food and distilled water. Room temperature was maintained at 25±2˚C and relative humidity at 60±10% and a 12-h light/dark cycle was used. After 7 days of acclimation, the mice were randomly divided into three experimental groups (n=7 per group): i) Control group, ii) LPS exposure group and iii) LPS + resveratrol group. Group i) mice served as the normal control group and were challenged with an equal volume of PBS. Group ii) was the LPS-exposed group and the mice were treated with 100 μg LPS (in 50 μl saline) by intratracheal instillation. Group iii) mice were pretreated with an intraperitoneal injection of resveratrol (50 mg/kg) 2 h prior to intratracheal LPS instillation (100 μg/50 μl saline). The total duration of the animal study was 7 days. The mice were monitored daily for food and water intake, weight, body posture, behavior, distress and response to external stimuli. Animals were sacrificed when they reached the humane endpoints, such as a loss of >20% body weight, severe dehydration, refusal of food, severe pain or distress, or a moribund state. However, none were humanely sacrificed or found dead during the present study. All mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium and 0.5-1 ml blood collected by cardiac

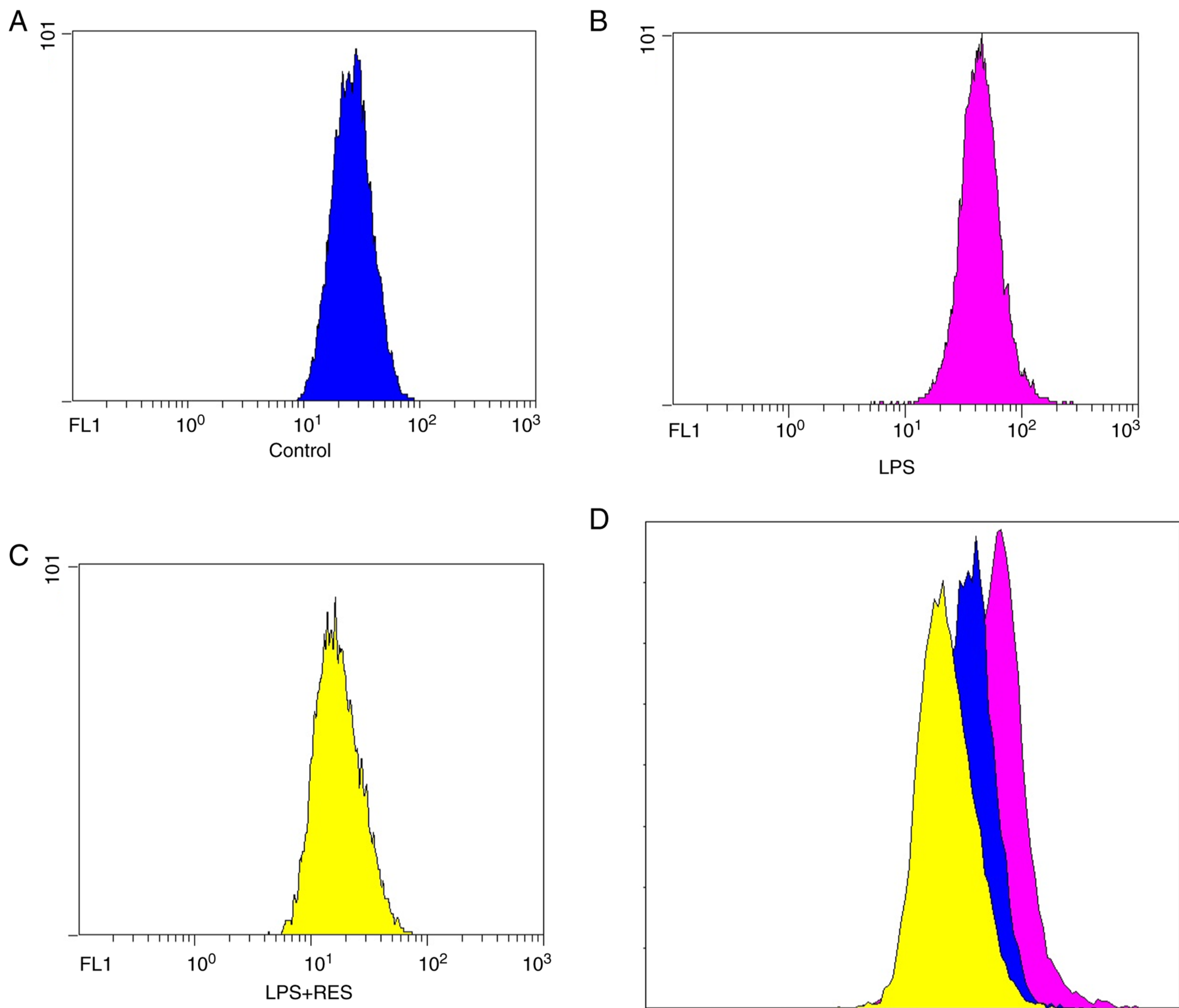

Figure 2. Resveratrol significantly attenuated LPS-induced ROS generation in NCI-H292 cells. The fluorescence intensity was detected using a flow cytometry. ROS content in (A) the control group, (B) the LPS group and (C) the LPS + resveratrol group. (D) Comparison of three groups. n=3/group. LPS, lipopolysaccharide; RES, resveratrol; ROS, reactive oxygen species.

puncture, then sacrificed by exsanguination. Indexes such as breathing, heartbeat, pupils and nerve reflexes were assessed to confirm death.

Bronchoalveolar lavage fluid (BALF) and airway inflammation analysis. The left bronchus of the mouse was ligated and ice-cold PBS (0.4 ml) was lavaged twice into the right lung. Then, the collected BALF was centrifuged at 400 x g for 10 min at 4˚C and the cell pellets were resuspended in cold PBS. The total count of inflammatory cells was assessed using a chemocytometer and neutrophil counts were determined using Wright and Giemsa staining (BaSO Diagnostics Inc.).

Histological analysis. After BALF sample collection, left lung tissue was fixed in 4% (v/v) paraformaldehyde at room temperature for 24 h, dehydrated with graded ethanol, embedded in paraffin and sliced into 4-μm sections. Hematoxylin staining was conducted at room temperature for 10 min and eosin staining for 2 min, followed by clearing in xylene for 5 min. Additionally, the slides were subjected to periodic acid Schiff (PAS) stain to assess mucus production. At room temperature, lung tissues were incubated in 0.1% periodic acid for 10 min, immersed in Schiff's reagent for 10 min, counterstained with Mayer's hematoxylin for 2 min, dehydrated in two changes of 96% alcohol, and finally cleared in xylene.

Statistical analysis. Statistical analyses were performed using the SPSS software (version 20.0; IBM Corp.) and GraphPad Prism 8.0 statistical software (Dotmatics). All data were presented as the mean ± SD. An independent-sample t-test was used to compare two groups and one-way ANOVA with Tukey's post hoc test was used to compare three or more groups of data. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of resveratrol on cell viability in NCI-H292 cells. The cytotoxicity of resveratrol on NCI-H292 cell viability was analyzed using the MTT assay at various times. As shown in

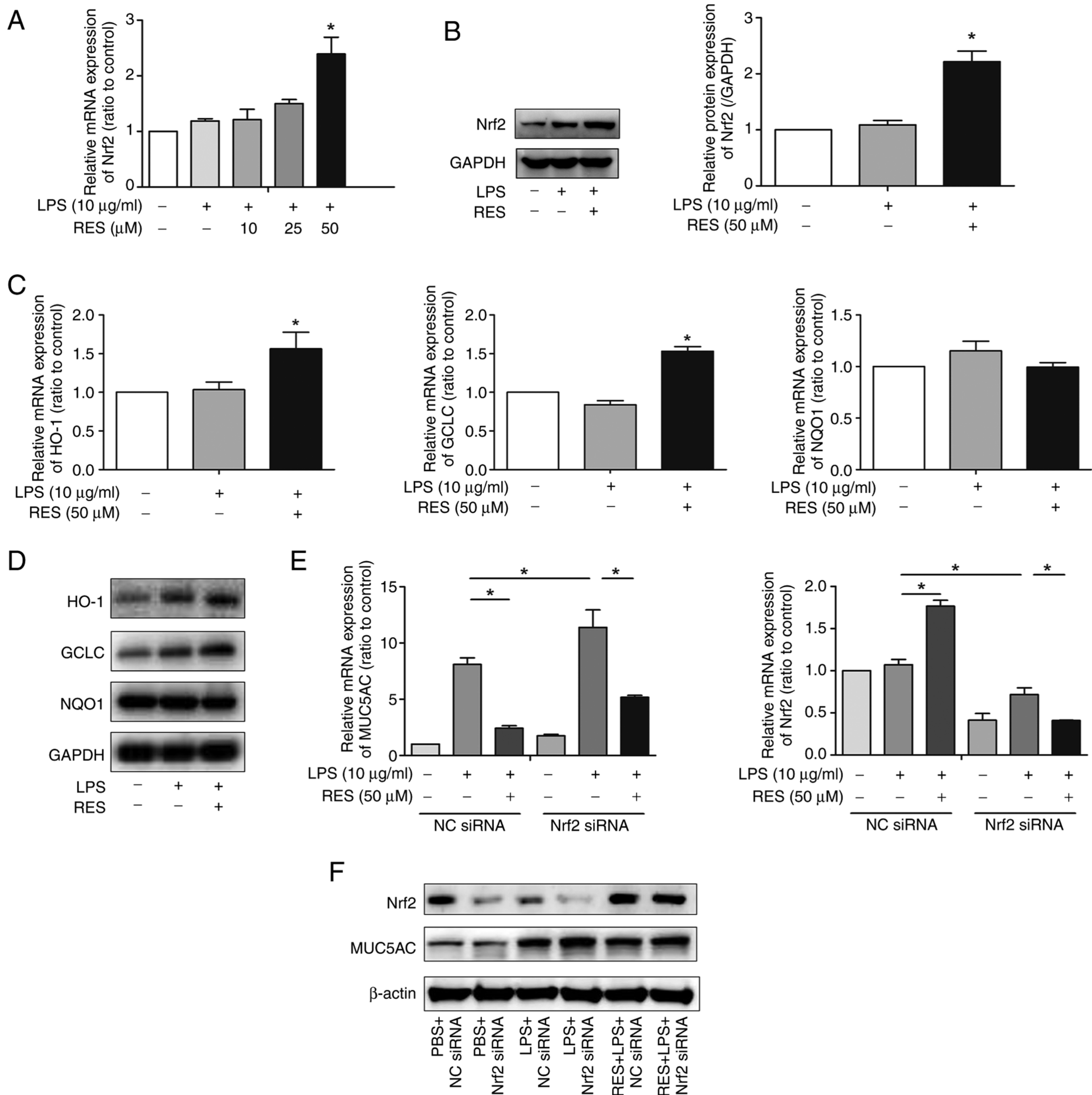

Figure 3. Resveratrol inhibited LPS-induced MUC5AC expression via Nrf2 in NCI-H292 cells. (A) Resveratrol induced Nrf2 mRNA expression in a dose-dependent manner in NCI-H292 cells. (B) Resveratrol (50 μM) induced Nrf2 protein expression. The effect of resveratrol on the (C) mRNA and (D) protein expression of downstream antioxidant gene HO-1, GCLC and NQO1. Knockdown of Nrf2 by Nrf2 siRNA, the downregulative effect of resveratrol on LPS-induced MUC5AC (E) mRNA and (F) protein expression was significantly suppressed. All data are expressed as mean ± standard error of the mean, n=3/group. *P<0.05. LPS, lipopolysaccharide; MUC5AC, mucin 5ac; Nrf2, nuclear factor erythroid 2-related factor 2; RES, resveratrol; si, small interfering.

Fig. 1A, ≤90 μM resveratrol and ≤70 μM resveratrol +LPS (10 μg/ml) showed no obvious toxicity to NCI-H292 cells at 24 h. Thus, concentrations of 10, 25 and 50 μM were selected for further *in vitro* experiments.

Resveratrol inhibits LPS-induced MUC5AC overexpression in NCI-H292 cells. Previous studies, and this study, show that LPS induced MUC5AC expression in a time-dependent manner in NCI-H292 cells and that this effect was most apparent with 10 μg/ml LPS for 24 h (Fig. 1B). Therefore, for the subsequent experiments, the time point of 24 h was used. However, treatment with resveratrol significantly decreased LPS-induced MU5AC mRNA expression in a dose-dependent manner in NCI-H292 cells (Fig. 1C). Consistent with the RT-qPCR results, the expression of MUC5AC protein was markedly increased in LPS-stimulated NCI-H292 cells but was obviously suppressed in resveratrol-treated NCI-H292 cells (Fig. 1D).

Resveratrol attenuates LPS-induced ROS production in NCI-H292 cells. To investigate the anti-oxidative effects of resveratrol, intracellular ROS production in NCI-H292 cells was measured. As shown in Fig. 2, LPS greatly induced ROS production (Fig. 2B), whereas resveratrol significantly attenuated LPS-induced ROS production (Fig. 2C).

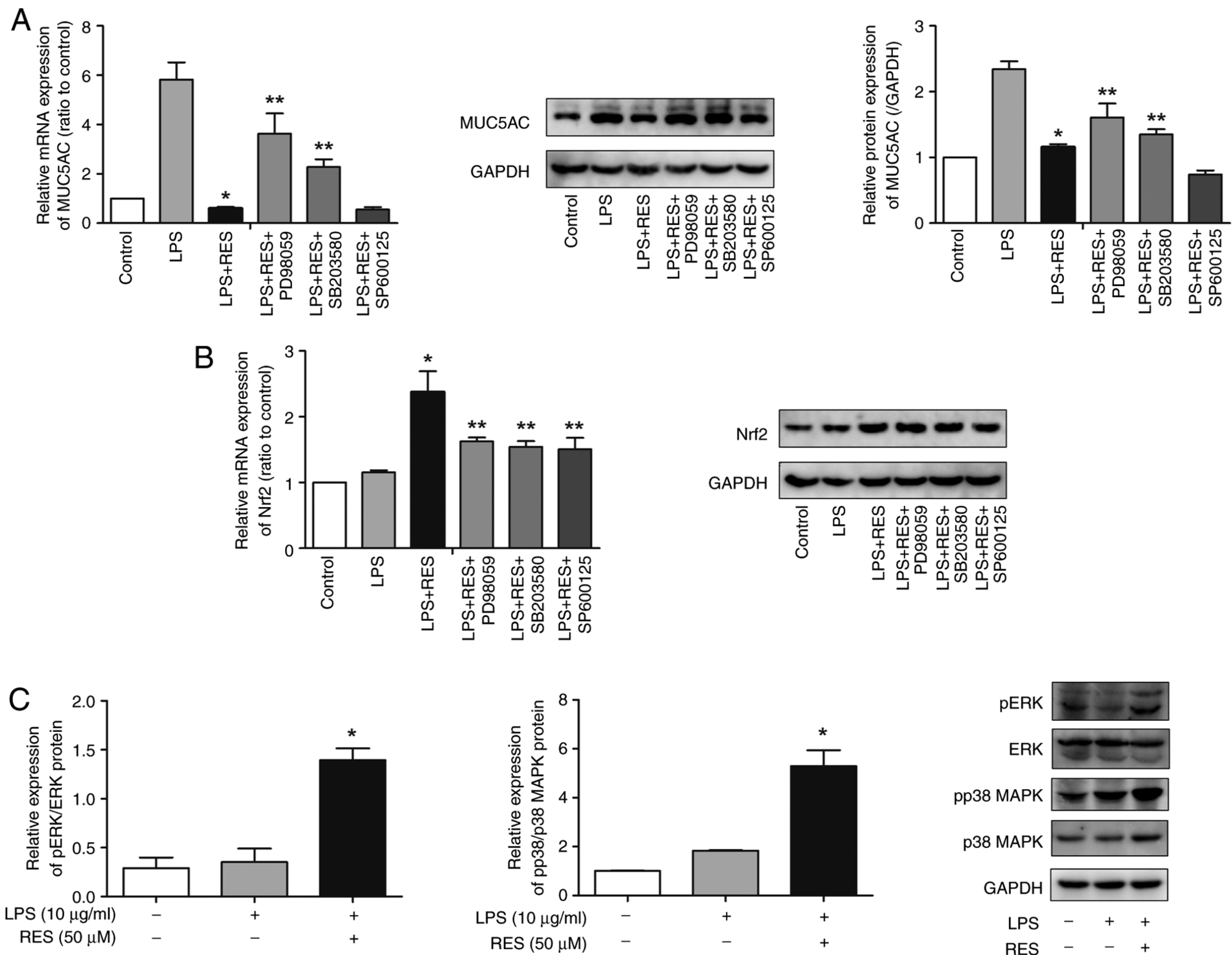

Figure 4. Involvement of MAPK pathway in the regulatory effect of resveratrol on LPS-induced MUC5AC expression in NCI-H292 cells. (A) Resveratrol (50 μM) failed to suppress LPS-induced MUC5AC expression in NCI-H292 cells when treated with PD098059 and SB203580, but not SP600125. (B) The inductive effects of resveratrol on Nrf2 was suppressed by MAPK inhibitors. (C) Resveratrol induced the phosphorylation of ERK and p38 MAPK. All data are expressed as mean ± standard error of the mean, n=3/group. *P<0.05 vs. LPS group; **P<0.05 vs. LPS+RES group. MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; MUC5AC, mucin 5ac; Nrf2, nuclear factor erythroid 2-related factor 2; p, phosphorylated; RES, resveratrol.

Resveratrol inhibits LPS-induced MUC5AC expression in NCI-H292 cells via Nrf2 pathway. To determine whether resveratrol inhibited LPS-induced MUC5AC expression via the Nrf2 signaling pathway, the mRNA and protein expression of Nrf2 and its downstream antioxidant genes, such as HO-1, glutamate-cysteine ligase catalytic subunit (GCLC) and NQO1 were analyzed by RT-qPCR and western blotting. First, it was found that resveratrol induced the mRNA expression of Nrf2 in a dose-dependent manner and this effect was most apparent at 50 μM (Fig. 3A). Compared with the LPS group, resveratrol significantly induced mRNA and protein expression of Nrf2 (P<0.05; Fig. 3B), as well as the downstream antioxidant genes HO-1 and GCLC (P<0.05; Fig. 3C and D). However, there was no effect on the expression of NQO1 (Fig. 3C and D).

Furthermore, the expression of Nrf2 was knocked down through siRNA transfection in NCI-H292 cells. Nrf2-siRNA could effectively decrease Nrf2 expression with 100 nM for 24 h and the mRNA and protein expression were decreased by 71.7 and 58.2% (Fig. S1). When Nrf2 expression was knocked down by Nrf2 siRNA, the downregulating effect of resveratrol on LPS-induced MUC5AC expression was significantly suppressed (P<0.05) (Fig. 3E and F). These results indicate that resveratrol inhibited LPS-induced MUC5AC expression via Nrf2 activation.

Involvement of MAPK pathway in the regulatory effect of resveratrol on LPS-induced MUC5AC expression. The MAPK pathway plays an important role in the regulation of inflammation, oxidative stress and mucin expression. To assess the role of the MAPK pathway in the regulatory effect of resveratrol on LPS-induced MUC5AC expression, MAPK inhibitors, including PD098059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor), were used. Resveratrol failed to suppress LPS-induced MUC5AC expression in NCI-H292 cells when ERK and p38 MAPK activation were blocked by their specific inhibitors, PD098059 and SB203580 (P<0.05), respectively (Fig. 4A). However, SP600125 did not inhibit this effect. Resveratrol induced the phosphorylation of ERK and p38 MAPK (P<0.05; Fig. 4C). Additionally, the inductive effects of resveratrol on Nrf2 were

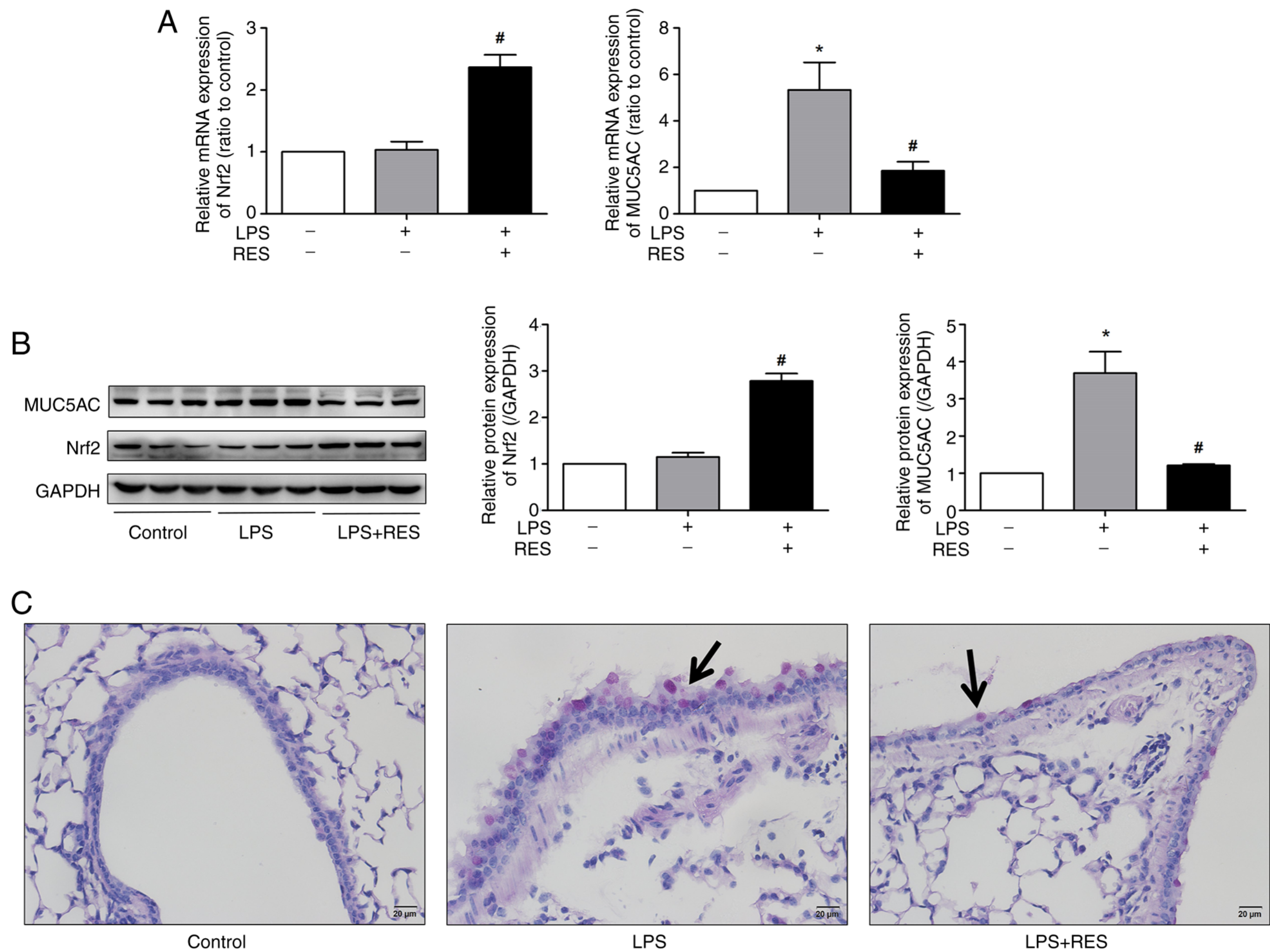

Figure 5. Effect of resveratrol on MUC5AC and Nrf2 expression in the lung of mice induced by LPS. Administration of resveratrol (50 mg/kg) induced the (A) mRNA and (B) protein level of Nrf2 in mouse lung tissue. Resveratrol significantly suppressed MUC5AC (A) mRNA and (B) protein expression in mouse lung tissue induced by LPS (100 μg/50 μl). (C) Periodic acid-Schiff staining showed that resveratrol decreased mucin production (arrows; magnification, x200). All data are expressed as mean ± standard error of the mean, n=3/group. *P<0.05 vs. control group; #P<0.05 vs. LPS group. MUC5AC, mucin 5ac; Nrf2, nuclear factor erythroid 2-related factor 2; LPS, lipopolysaccharide; RES, resveratrol.

suppressed by the MAPK inhibitors (Fig. 4B). These results suggested that resveratrol inhibited LPS-induced MUC5AC expression via the activation of the ERK/p38 MAPK pathway in NCI-H292 cells.

Resveratrol attenuates mucus hypersecretion in the lung of mice induced by LPS. To confirm the mitigating effect of resveratrol on LPS-induced mucus hypersecretion in the lungs, the mucus expression was assessed using PAS staining, RT-qPCR and western blotting. Consistent with the *in vitro* experimental results, resveratrol significantly suppressed MUC5AC mRNA and protein expression in mouse lung tissues (Fig. 5A and B) and LPS-induced mucus hypersecretion (Fig. 5C).

Resveratrol alleviates airway inflammation of mice exacerbated by LPS exposure. Compared with the control group, the LPS treatment group showed a significant increase in the total number of cells and neutrophils in BALF (Fig. 6A and B). However, administration of resveratrol effectively decreased the number of inflammatory cells and neutrophils exacerbated by LPS in BALF (Fig. 6A and B). In addition, H&E staining of the lungs collected from mice in each group was performed to analyze histological changes in the lungs. Compared with LPS-challenged mice, administration of resveratrol markedly alleviated the infiltration of exacerbated inflammatory cells in the lung tissue (Fig. 6C).

Resveratrol induces the phosphorylation of ERK and Nrf2 activation in the lung of mice. The present study investigated the protein expression levels of the MAPK signaling pathway in a mouse model using western blotting. As shown in Fig. 6D, the phosphorylation levels of ERK and p38 in the resveratrol + LPS group were significantly higher than those in the LPS group. Meanwhile, the mRNA and protein level of Nrf2 were increased in the resveratrol + LPS group compared with the LPS group (P<0.05; Fig. 5A and 5).

Discussion

Mucus overproduction is a major pathophysiological factor in chronic airway inflammatory diseases, contributing to airway obstruction, atelectasis and impaired gas exchange (1,3). However, there remains a lack of effective therapeutic

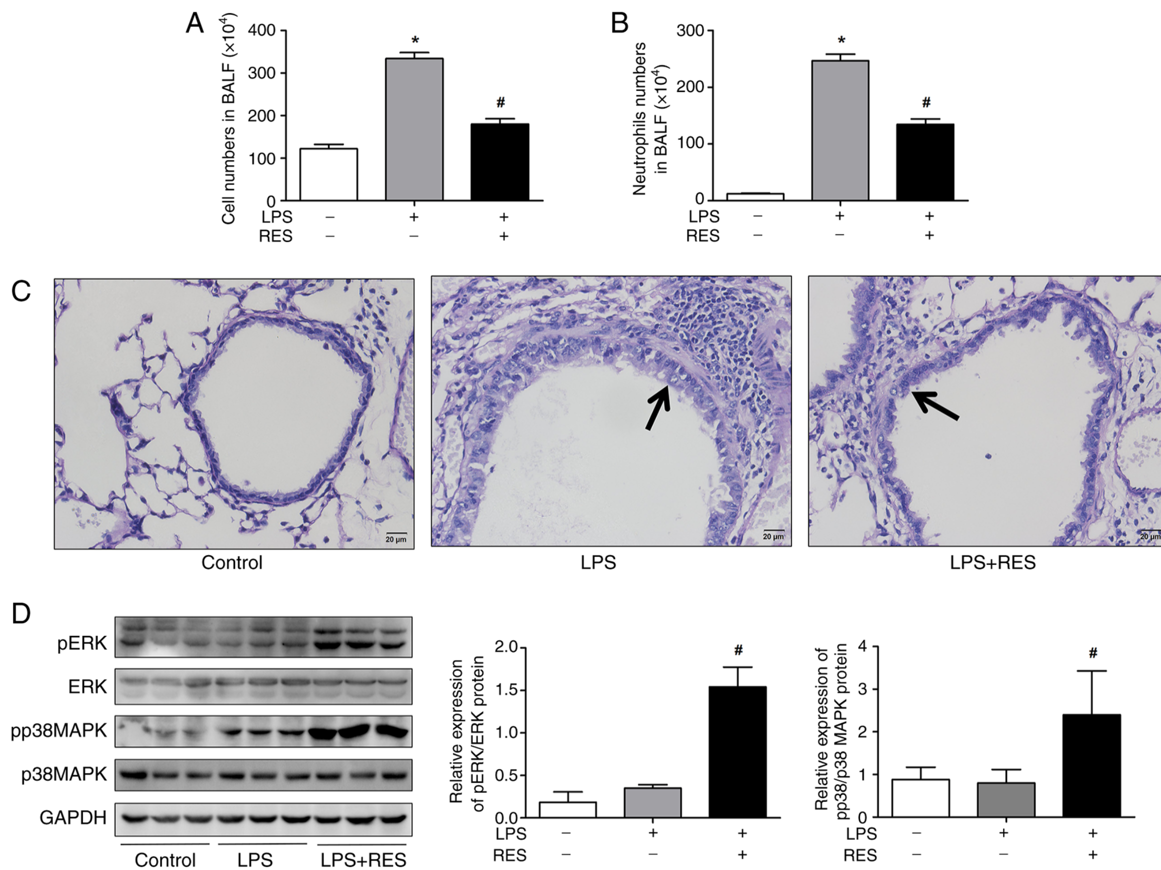

Figure 6. Resveratrol alleviated airway inflammation of mice exacerbated by LPS exposure. Resveratrol (50 mg/kg) effectively decreased the number of (A) total inflammatory cells and (B) neutrophils exacerbated by LPS (100 μg/50 μl) in BALF. (C) Resveratrol markedly alleviated the infiltration of exacerbated inflammatory cell in lung tissue hematoxylin and eosin staining (arrows; magnification, x200). (D) Resveratrol induced the phosphorylation of ERK and p38 MAPK. All values are expressed as mean ± standard error; *P<0.05 vs. control group; #P<0.05 vs. LPS group. LPS, lipopolysaccharide; BALF, Bronchoalveolar lavage fluid; p, phosphorylated; RES, resveratrol; MAPK, mitogen-activated protein kinase.

drugs to regulate the expression of mucus secretion. The present study found that resveratrol inhibited LPS-induced MUC5AC expression via MAPK and Nrf2 signaling cascades in airway epithelia and mice (Fig. 7), providing new insight into the molecular mechanisms underlying the regulation of LPS-induced mucin overproduction. Therefore, it suggested that resveratrol may be a potential therapeutic agent for excessive mucus secretion.

Resveratrol, a natural phenol, has been reported to possess various biological effects including anti-oxidative, anti-aging, anti-inflammatory, anti-cancer and immunomodulatory effects. A recent study indicates that resveratrol suppresses MUC5AC expression induced by EGF, PMA and TNF-α in the human airway epithelium (21), but the underlying mechanism remains to be elucidated. Resveratrol treatment inhibits benzo(a) pyrene-induced MUC5AC overexpression in NCI-H292 cells (36). Several studies have demonstrated that LPS is a potent inducer of MUC5AC expression and hence it is widely used (8-12). However, it is unclear whether resveratrol has a similar effect on LPS-induced MUC5AC overexpression. The present study found that resveratrol alleviated LPS-induced MUC5AC expression in NCI-H292 cells and reduced LPS-induced lung inflammation in mice, using RT-qPCR, western blotting and immunohistochemistry, respectively.

The detailed mechanisms underlying resveratrol-regulated LPS-induced mucus production remain to be elucidated and necessitate further investigation. Oxidative stress plays an important role in mucin production (37,38). ROS have been found to mediate LPS-induced MUC5AC expression in A549 cells (39-41). ROS also play a role in MUC5AC expression induced by neutrophil elastase and the aryl hydrocarbon receptor in human biliary and airway epithelial cells (38,42). Resveratrol is a polyphenol nonflavonoid compound with strong antioxidative activity. Several studies have reported that resveratrol alleviates hypoxic pulmonary hypertension in rats via anti-inflammatory and antioxidative pathways (43), alleviates pulmonary inflammation and lung injury in LPS-induced acute lung injury mice (13,15) and prevents inflammation and oxidative stress response in LPS-induced human gingival fibroblasts (19). The present study found that resveratrol decreased LPS-induced ROS production in NCI-H292 cells and confirmed the role of the antioxidative pathway in resveratrol-mediated MUC5AC regulation. It also observed that resveratrol alleviated mucus overexpression and airway inflammation exacerbated by LPS exposure in mice.

It is well known that the transcription factor Nrf2 effectively reduces ROS production and helps restore redox balance (44-46). Previous reports revealed that Nrf2 plays an important role in MUC5AC expression induced by cigarette smoking or neutrophil elastase (32,42). As a potent activator of Nrf2, resveratrol has been demonstrated to exert antioxidative

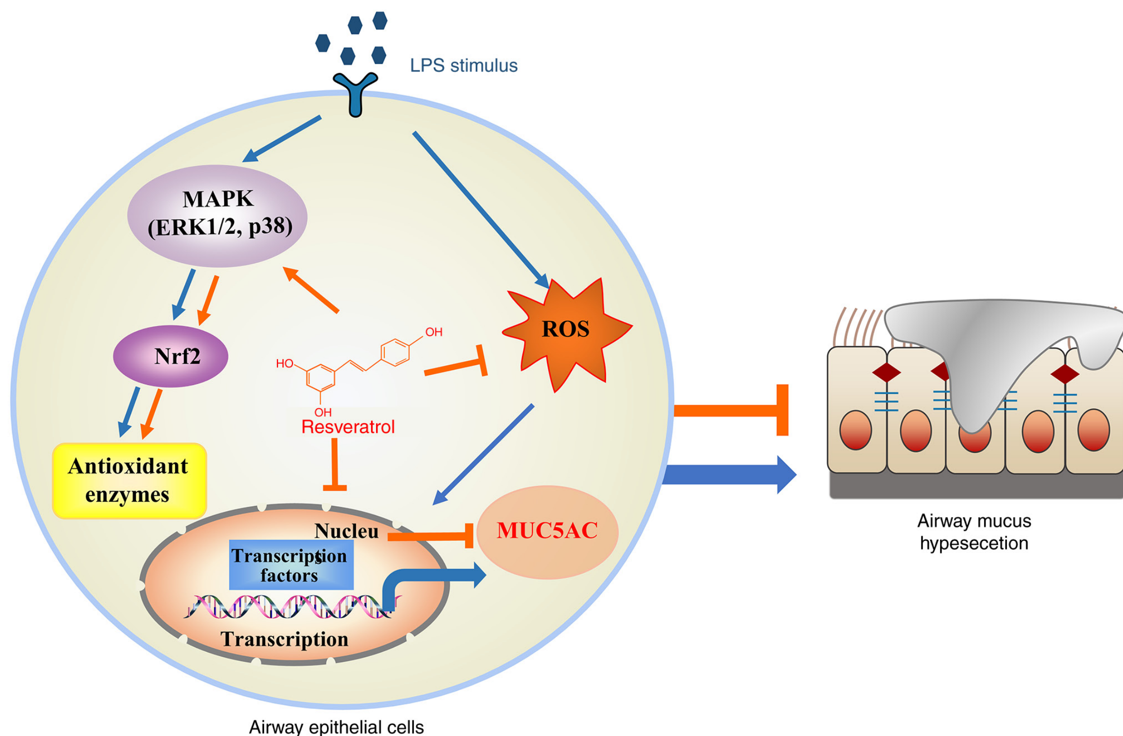

Figure 7. Illustration of the proposed working model. Resveratrol inhibited LPS-induced airway mucus hypersecretion by MAPK and Nrf2 pathway in human airway epithelial cells and acute inflammatory mouse model. LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Nrf2, nuclear factor erythroid 2-related factor 2; MUC5AC, mucin 5ac; ROS, reactive oxygen species.

and anti-inflammatory effects by inducing Nrf2-mediated protective pathways. It is reported that resveratrol alleviates rotenone-induced inflammation and oxidative stress through Nrf2/Keap1/SLC7A11 pathway in a microglia cell line (26). A study found that resveratrol protects intestinal integrity and attenuates intestinal inflammation and oxidative stress via aryl hydrocarbon receptor/Nrf2 pathways in weaned piglets exposed to diquat (25). Moreover, dietary resveratrol alleviates the LPS-induced inflammatory response in ducks through Nrf2 signaling pathways (47,48). However, the role of Nrf2 in resveratrol-mediated regulation of MUC5AC expression is not well characterized. The present study revealed that resveratrol increased the expression levels of Nrf2 in NCI-H292 cells and mice, along with its downstream antioxidant genes, such as HO-1 and GCLC. Therefore, the biological function of resveratrol may be associated with ROS clearance through Nrf2 signaling. To further investigate the mechanism by which resveratrol regulates MUC5AC, Nrf2 knockdown was achieved through transfection with Nrf2-siRNA in NCI-H292 cells, which abrogated the downregulation of LPS-induced MUC5AC expression in these cells. Based on previous reports and the present study, it is hypothesized that resveratrol attenuates LPS-induced MUC5AC expression via the Nrf2 signaling pathway.

Aside from Nrf2, it is widely recognized that the MAPK signaling pathway plays a pivotal role in regulating Nrf2-dependent transcription. Studies have indicated that various compounds downregulate LPS-induced MUC5AC expression via the MAPK signaling pathway. Ghrelin suppresses LPS/leptin-induced MUC5AC expression in human nasal epithelial cells by modulating the ERK1/2 and p38 MAPK pathways (30). Peroxiredoxin 6 decreases MUC5AC expression in LPS-induced airway inflammation through the H_2O_2-EGFR-MAPK signaling pathway (49). L-Glutamine alleviates MUC5AC expression and inflammation in mice with LPS-induced acute lung injury via the TLR4/MAPK signal pathway (50). In addition, resveratrol decreases MUC5AC expression by inhibiting ERK and AKT signaling in mucus-producing A549 human lung carcinoma cells (23). However, there is no study demonstrating the role of the MAPK pathway in LPS-induced MUC5AC expression. Consequently, the present study investigated whether the MAPK pathway is involved in the cytoprotective effects of resveratrol against LPS exposure. In the *in vitro* inflammation model, MAPK inhibitors were used to investigate the role of the MAPK pathway in the effect of resveratrol on LPS-induced MUC5AC expression in NCI-H292 cells. The findings revealed that resveratrol significantly induced the phosphorylation of ERK and p38, as well as the activation of Nrf2 in NCI-H292 cells and C57BL mice. However, inhibitors of ERK (PD98059) and p38 (SB203580) all suppressed Nrf2 expression in NCI-H292 cells. Additionally, the inhibition of ERK and p38 MAPK abolished the resveratrol-induced downregulation of MUC5AC expression. Therefore, these results suggested that resveratrol inhibited LPS-induced MUC5AC expression via activating ERK/p38 MAPK and Nrf2 pathways. The present study did not involve pharmacokinetics and pharmacodynamics of resveratrol and clinical studies, which should be performed in the future to guide clinical practice.

In conclusion, the present study indicated that resveratrol alleviated LPS-induced MUC5AC expression, inflammation and oxidative stress response through the ERK/p38 MAPK

and Nrf2 pathways in bronchial epithelial cells and mice. These findings provided new perspectives on the therapeutic approaches for airway mucus hypersecretion.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

QC, LX and JW contributed to the literature search and study design. XL conceived and designed the study. QC, LX, JW, XY, XS and XL drafted the manuscript. QC, LX and JW performed the experiments. QC and LX contributed to data collection and analysis. XS and XY provided overall guidance and helped with the experiments. QC, LX and XL revised the manuscript. QC, LX, JW and XL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study protocol was reviewed and approved by the Experimental Animal Ethics Committee of the second affiliated hospital of Fujian Medical University (approval no. 2022-FYFE-559). All methods were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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