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Phloretin attenuates mucus hypersecretion and airway inflammation induced by cigarette smoke

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ABSTRACT

Backgrounds: Cigarette smoke (CS)-induced airway mucus hypersecretion and inflammation are the prominent features of chronic obstructive pulmonary disease (COPD). As an anti-inflammatory flavonoid, phloretin was found to be involved in various inflammatory disorders such as sepsis. In this study, the effects of phloretin on CS-induced airway mucin secretion and inflammation were investigated *in vivo* and *in vitro*.

Methods: Phloretin dissolved in 1% DMSO was daily injected intraperitoneally to mice, which were then exposed to CS for four weeks. Mouse lung histologic changes were evaluated, the expression of mucin 5 ac (MUC5AC) was measured, bronchoalveolar lavage fluid (BALF) total cells, neutrophils, and macrophages were counted. BALF and lung levels of tumor necrosis factor-alpha and interleukin-1 beta (IL-1β) were quantified. Moreover, the effects of phloretin on cigarette smoke extract (CSE)-induced expression of MUC5AC and IL-1β were investigated in NCI-H292 cells. Then, to explore the potential mechanisms, the signaling molecules including epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK) and P38 were evaluated.

Results: Phloretin pretreatment dramatically suppressed the mucins secretion, inflammatory cell infiltration and inflammatory cytokine release in mouse lungs induced by CS, and it also suppressed CSE-induced expression of MUC5AC and IL-1β in NCI-H292 bronchial epithelial cells. Furthermore, western blot showed that phloretin attenuated the activation of EGFR, ERK and P38 both *in vivo* and *in vitro*.

Conclusions: This study highlights the protective effect of phloretin on CS-related airway mucus hypersecretion and inflammation, where EGFR, ERK and P38 might be involved. These findings suggest that phloretin could be a potential therapeutic drug for COPD.

1. Introduction

As a worldwide health problem, Chronic obstructive pulmonary disease (COPD) was ranked eighth in the causes of disease burden which was evaluated by disability-adjusted life years (DALYs) in 2015 [1]. As a well-known risk factor of pulmonary disorders, cigarette smoke (CS) has been found to induce chronic airway inflammation and mucus hypersecretion, leading to the mechanical obstruction of small airways, which contributes to the irreversible airflow limitation in COPD [2,3]. Therefore, attenuating CS-induced airway inflammatory response and mucin secretion has been identified as an effective approach to the therapy of COPD.

Phloretin, which is widely distributed in the leaves, bark and fruit of apple trees, belongs to the chalcone class of flavonoids, and exhibits diverse biologic properties such as antioxidase activities, regulation of glucose transportation, and anti-tumor abilities in various diseases [4–6]. Recently, the anti-inflammatory potentials of phloretin inspired plenty of studies. It was found to alleviate rat sepsis induced by cecal ligation and puncture [7], and inhibit LPS-induced release of inflammatory cytokines through down-regulating the activation of nuclear transcription factor kappa-B (NF-κB) and Mitogen Activated Protein Kinase (MAPK) pathways in macrophages and dendritic cells [8–10]. Furthermore, phloretin also exhibited protective roles in various pulmonary disorders. Animal studies showed that phloretin decreased ovalbumin-induced mouse airway allergic inflammation [11], *in vitro* studies found that phloretin suppressed interleukin-1β (IL-1β)-

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induced inflammatory response *via* inhibiting MAPK pathways in human alveolar epithelial cells [12]. However, the role of phloretin in CS exposure-related airway inflammation and mucus hypersecretion is still not clear.

Thus, in the current study, we explored the role of phloretin in CS exposure-induced mucus hypersecretion and airway inflammation both *in vivo* and *in vitro*, and we found that phloretin dramatically suppressed CS-induced inflammatory response and mucus hypersecretion possibly through downregulating the activation of epidermal growth factor receptor (EGFR)/MAPK signaling pathways.

2. Materials and methods

2.1. Mouse groups and treatments

Animals were handled-according to the ARRIVE guidelines developed by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs), the study protocol was reviewed and approved by the animal ethics committee of West China Hospital, Sichuan University.

Specific pathogen-free male BALB/c mice (6–8 weeks, 22–24 g) were purchased from Dashuo Biological Technology Co, Ltd. (Chengdu, China). They were housed on a 12-h light/12-h dark cycle (lights on from 6:00 AM to 6:00 PM) at a room temperature of 23 °C ± 2 °C with 50% ± 10% humidity. Mice were allowed free access to food and water. Experimental procedures were conducted under aseptic conditions. Chambers and cages were cleaned every 3 days.

Mice were randomly assigned into four groups (n = 6 per group): control group (C), which received vehicle and was housed in room air; CS-exposed group (CS), which received vehicle and was exposed to CS; CS-exposed low-dose phloretin-pretreated group (CS + PhL), which received 10 mg/kg phloretin (q.d.) and was subsequently exposed to CS; and the CS-exposed high-dose phloretin-pretreated group (CS + PhH),which received 20 mg/kg phloretin (q.d.) and was subsequently exposed to CS [13,14].

Mice were allowed to adjust to the animal housing facilities for one week before any interventions were carried out. Phloretin (Selleckchem, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) just before use, and diluted with sterile saline to the final concentration of DMSO at 1% v/v. Mice in group CS + PhL and CS + PhH were pretreated daily with phloretin through intraperitoneal injection 1 h before CS exposure. In parallel, mice in group C and CS received an equal volume of 1% v/v DMSO in sterile saline intraperitoneally as the vehicle.

2.2. Cigarette smoke exposure

For cigarette smoke exposure experiment, a commercially available cigarette was used (Marlboro, Philips Morris, USA; 1.0 mg nicotine and 11 mg tar per cigarette). Mice in group CS, CS + PhL, and CS + PhH were exposed to cigarette smoke for 2 h twice daily, 6 days per week for 4 weeks according to a modified procedure based on the methods described by our previous studies [15]. Briefly, a ventilated plastic chamber containing the mice was connected to a smoke generator (CH Technologies, Westwood, NJ, USA) and filled with fixed concentration of smoke (250 mg total particulate matter (TPM)/m^3) by pumping mainstream cigarette smoke from burning cigarettes at a constant rate (each cigarette took 3.5 min to burn out) while using another pump to deliver fresh air from outside simultaneously at a fixed rate. The rate of airflow passing through the box was constant at 1.22 L/min. In parallel, group C were exposed to room air following the same schedule. After 4 weeks of CS or room air exposure, mice were sacrificed with intraperitoneal phenobarbital (Sigma-Aldrich).

2.3. Bronchoalveolar lavage fluid (BALF) collection and cell counting

Mouse right lungs were lavaged with 0.5 mL of sterile phosphate-buffered saline (PBS) for three times, and more than one 1.3 mL of fluid was recovered for each mouse. The lavage fluid was centrifuged at 1000*g* for 5 min, and the supernatant was stored at − 80 °C for analysis of cytokines using enzyme-linked immunosorbent assay (ELISA). The cell pellet was suspended in 0.2 mL PBS, and the total cell number was evaluated with a hemocytometer. Then the differential cell count was performed by cytocentrifugation (Cytopro7620, Wescor, Utah, USA) at 800 rpm for 10 min followed by staining with Wright's stain (200 cells were counted for each mouse).

2.4. BALF inflammatory cytokine detection

BALF levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) were measured with the ELISA kit for mice (ExCell Bio, Shanghai, China) following the manufacturer's instructions. The absorbance data were measured with a Bio-Rad 680 microplate reader, and analyzed with accompanying software Microplate Manager 5.2 (Bio-Rad, Hercules, CA, USA).

2.5. Mouse histology

Mouse left lungs without lavage were fixed with 4% phosphate-buffered paraformaldehyde under a constant pressure of 25 cm H_2O, embedded in paraffin, and sliced at 4-mm thickness. Paraffin sections were stained with hematoxylin and eosin solution (H&E) or Alcian blue-periodic acid Schiff (AB-PAS) to evaluate morphological changes and mucus secretion in lungs. An experienced pathologist who was blinded to the treatments graded a lung inflammation score for each H&E staining slice, which was based on the severity of lung lesions including peribronchiolar infiltrates, alveolar septal infiltrates, perivascular infiltrates, and combined bronchus-associated lymphoid tissue hyperplasia [16]. For each possible lesion, the score ranged from 1 to 4 (1: minimal, 2: mild, 3: moderate, and 4: marked), and the group histopathology scores were obtained by averaging the scores of individual mouse in each group. Percentages of positively stained areas by AB/PAS to the total airway epithelial areas were quantified by Image-Pro plus 4.5 software (Media Cybernetics, Bethesda, MD, USA) [17].

2.6. Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was freshly prepared as previously described with a few modifications [18]. Briefly, mainstream smoke from 3 cigarettes (Marlboro, Philips Morris) was drawn slowly into a 50 mL syringe and bubbled through 10 mL of RPMI-1640 medium prewarmed at 37 °C. Then this solution, considered to be 100% CSE, was adjusted to pH 7.4 and sterilized with a 0.22 μm filter (Millipore, Bedford, MA, USA). Before use, this 100% CSE was diluted with serum-free RPMI-1640 medium to the required CSE concentrations.

2.7. Cell culture and treatments

NCI-H292 cells were purchased from American Type Culture Collection (CRL-1848™), and were cultured in RPMI 1640 Medium (Invitrogen, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen), 50 U/mL penicillin G sodium and 50 μg/mL streptomycin sulfate (penicillin–streptomycin, Invitrogen). After 24-hour incubation with serum-free medium, cells were treated with different concentration of CSE, and/or assigned with 1-hour pretreatment of different doses of phloretin.

2.8. CCK-8

Cell viability was measured using the tetrazolium salt WST-8-based

cell counting kit (CCK8; Dojindo Molecular Technologies, Rockville, MD, USA) according to the protocol of manufacturers. Briefly, after 24-hour starvation with serum-free medium, NCI-H292 cells seeded in 96-well plates were treated with various concentrations of CSE (2%, 4%, 8%, and 12%) or different dose of phloretin (1 μM, 2.5 μM, 5 μM, and 10 μM) in 100 microliter (μL) serum-free medium. Twenty-four hours after the treatment, 10 μL of CCK8 solution was added to each well followed by another 1-hour incubation. Then the absorbance at 450 nm was measured. Three replicates were performed for each condition, and the results were expressed as the percentage of viable cells in treated groups to those in control groups.

2.9. Real-time PCR

RNA was isolated from mouse right lungs or NCI-H292 cells with E.Z.N.A.® Total RNA kit I (Omega Bio-tek, Norcross, GA, USA) following the manufacture's protocol. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). Real-time RT-PCR was conducted in triplicates with the LightCycler® 96 PCR system (Roche Molecular Systems, Indianapolis, IN, USA) according to the manufacturer's specifications, and FastStart Essential DNA Green Master (Roche) which was based on SYBR® Green I was used to prepare the PCR reaction substrate. The primer sequences were listed in Table 1, and the amplification was performed following these three steps: step 1, pre-incubation: 95 °C for 10 min for 1 cycle; step 2, 3-step amplification: 95 °C for 10 s, Tm (shown in Table 1) for 15 s, and 72 °C for 15 s for 35 cycles; step 3, melting: 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s for 1 cycle. All data were normalized to GAPDH gene expression [19,20], and relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

2.10. Western blotting

Protein samples were isolated from mouse right lungs or cells with RIPA lysis buffer supplied with 1 mM PMSF (Cell Signaling Technology, Danvers, MA, USA). Total protein (10 μg) was fractionated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. After 1-hour blocking with 5% bovine serum albumin (BSA) in tris buffered saline tween at room temperature, these membranes were incubated overnight at 4 °C with anti-MUC5AC (Abcam, Cambridge, MA, USA), anti-epidermal growth factor receptor (EGFR), anti-p-EGFR, anti-P38, anti-p-P38, anti-extracellular signal-regulated kinase (ERK), anti-p-ERK, and anti-β-actin antibodies (Cell Signaling Technology) respectively. Then they were incubated with horseradish peroxidase-conjugated second antibodies, and the immune complexes were detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Band intensities were quantified with QuantityOne software (Bio-Rad).

2.11. Statistical analysis

All the original data were described as mean and standard deviation, their difference between various groups was evaluated with one-way ANOVA, Fisher's LSD test was performed for multiple comparisons, and two-side *P* value lower than 5% was regarded as statistical significant. Data were analyzed and figures were drawn with GraphPad Prism 6.01 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Phloretin alleviates mouse lung histologic changes induced by CS

To evaluate the histologic changes following CS exposure and phloretin pretreatment, H&E staining was performed based on the fixed mouse left lungs. The slices showed that four-week CS exposure dramatically increased peribronchial inflammatory cell infiltration, airway epithelial cell hyperplasia, airway epithelium thickening, and lumen obstruction by mucus and cell debris. However, all these changes were alleviated by both low-dose and high-dose phloretin pretreatment (Fig. 1).

3.2. Phloretin suppresses the mucus hypersecretion induced by CS in mouse lungs

AB-PAS staining showed that CS exposure highly increased the secretion of airway mucus proteins which were stained as blue, and both low-dose and high-dose phloretin pretreatment suppressed the expression of mucins in mouse airways (Fig. 2).

As the major source of airway mucus, mouse MUC5AC expression levels were detected with RT-PCR and western-blot. After 4-week CS exposure, both the mRNA and protein levels of MUC5AC in mouse lungs were dramatically increased (Fig. 3), and the elevated MUC5AC proteins were attenuated by both low-dose and high-dose phloretin (Fig. 3a–b). However, only high-dose phloretin decreased the elevated MUC5AC mRNA levels (Fig. 3c).

3.3. Phloretin decreases CS-induced inflammatory cell release in mouse BALF

To evaluate the infiltration of inflammatory cells in mouse lungs, the amounts of total cells, neutrophils and macrophages in mouse BALF were counted. They were all elevated in CS-exposed mice, and neutrophils were dominated. However, phloretin pretreatment significantly decreased CS-induced release of BALF total cells, neutrophils, and macrophages with a dose-dependent manner, as the high-dose phloretin exhibited stronger effects to suppress inflammatory cell release. (Fig. 4).

3.4. Phloretin down-regulates CS-induced increase of IL-1β and TNF-α in mouse lungs

Aside from inflammatory cells, inflammatory cytokines in mouse BALF were also detected using ELISA. Four-week CS exposure sharply increased the release of IL-1β and TNF-α, and these elevated cytokines were attenuated by different dose of phloretin (Fig. 5a–b). Similarly, the increased mRNA levels of IL-1β and TNF-α in the lungs of CS-exposed mice were also diminished by the pretreatment of phloretin (Fig. 5c–d).

Table 1
The detailed information about the primers used in this experiment.

Gene name	Forward 5′-3′	Reverse 5′-3′	Product length	Tm (°C)
Human IL-1β	CACGATGCACCTGTACGATCA	GTTGCTCCATATCCTGTCCCT	121 bps	60
Human MUC5AC	CAGCCACGTCCCCTTCAATA	ACCGCATTTGGGCATCC	64 bps	60
Human GAPDH	AACGGATTTGGTCGTATTGG	CTCCTGGAAGATGGTGATGG	213 bps	60
Mouse IL-1β	GTTGACGGACCCCAAAAG	GTGCTGCTGCGAGATTTG	93 bps	60
Mouse MUC5AC	AACGGCAGTCCAAAATCC	GAAGGTTCCCAAACTCAAGG	73 bps	60
Mouse TNF-α	GTCGCTACATCACTGAACCTCT	ATGACCCGTAGGGCGATTA	64 bps	60
Mouse GAPDH	AACTTTGGCATTGTGGAAGG	GTCTTCTGGGTGGCAGTGAT	62 bps	60

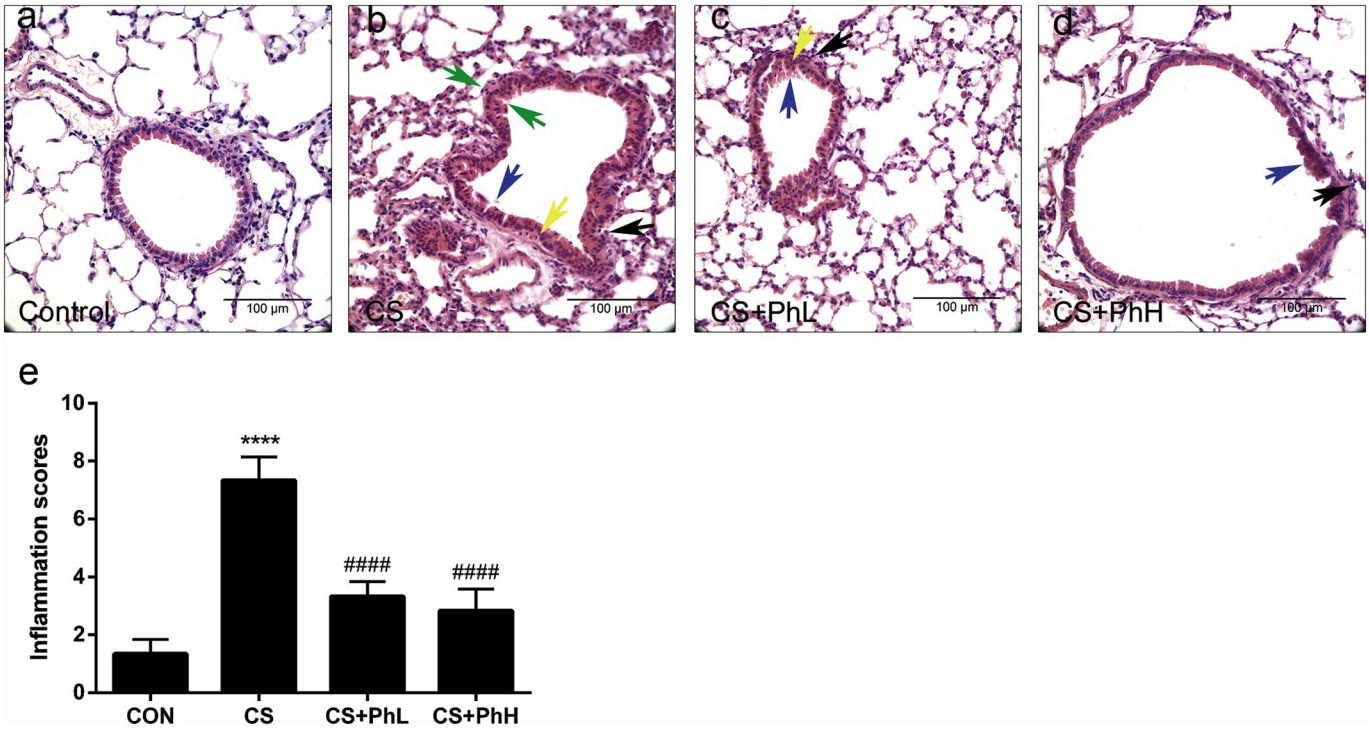

Fig. 1. Phloretin attenuates CS exposure-induced mouse lung histologic changes. Notes: mouse lungs from control group (a), CS group (b), CS + PhL group (c) and CS + PhH (d) group were stained with Hematoxylin and Eeosin, and inflammation scores of mouse lungs were evaluated (e); n = 5 or 6 for each group; black arrow: peribronchial inflammatory cells, yellow arrow: airway epithelial cell hyperplasia, green arrow: airway epithelium thickening, blue arrow: cell debris; ****: $P < 0.0001$ compared with control group, ####: $P < 0.0001$ compared with CS-exposed group. Abbreviations: CS, cigarette smoke; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Phloretin attenuates CSE-induced expression of MUC5AC and IL-1β in NCI-H292 cells

To confirm the *in-vivo* findings that phloretin pretreatment attenuated CS-induced mucus hypersecretion and inflammation in mouse airways, the *in-vitro* properties of phloretin were explored in NCI-H292 cells. Firstly, CCK-8 test was performed to evaluate the toxicity of CSE and phloretin. The cell viabilities in 2%, 4% and 8%

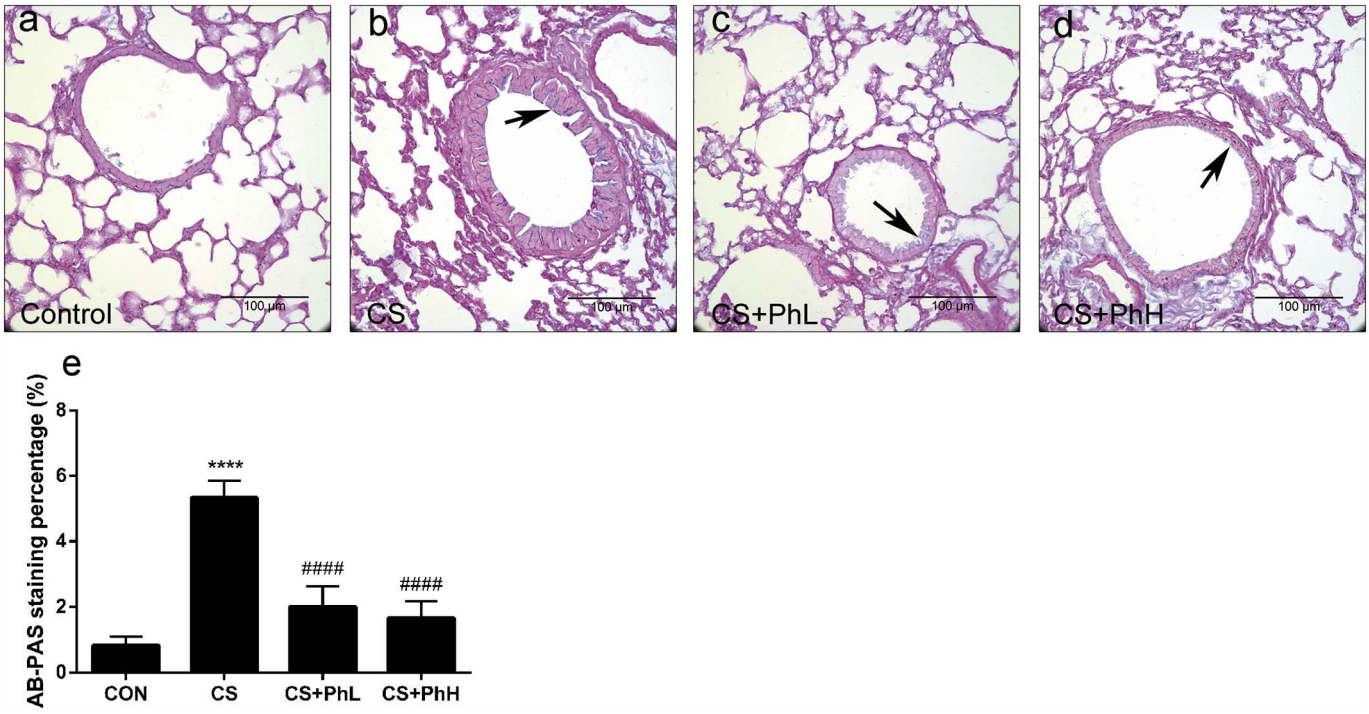

Fig. 2. Phloretin attenuates CS exposure-induced mouse airway mucus hypersecretion. Notes: Mouse lungs from control group (a), CS group (b), CS + PhL group and CS + PhH group were stained with AB-PAS; n = 5 or 6 for each group; black arrow: mucus stained by AB-PAS; ****: $P < 0.0001$ compared with control group, ####: $P < 0.0001$ compared with CS-exposed group. Abbreviations: CS, cigarette smoke; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg); AB-PAS: alcian blue-periodic acid schiff.

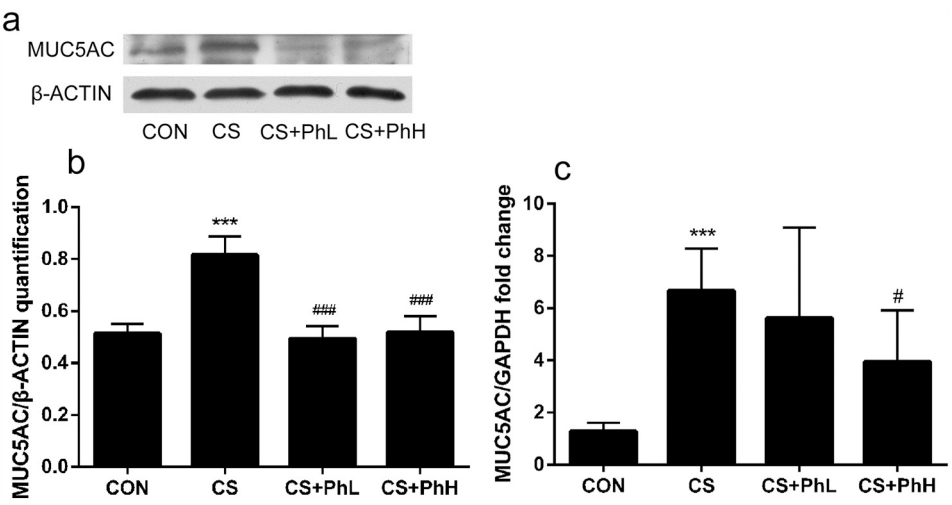

Fig. 3. Phloretin alleviates CS exposure-induced MUC5AC hypersecretion in mouse lungs. Notes: The protein and mRNA levels of MUC5AC in mouse lungs were evaluated with western blot (a) and PCR (c), and the quantification of protein blots were performed with Quantity-one software (b), β-actin or GAPDH was used as internal controls, n = 5 or 6 for each group, ***: $P < 0.001$ compared with control group, #: $P < 0.05$ compared with CS-exposed group, ###: $P < 0.001$ compared with CS-exposed group. Abbreviations: CS, cigarette smoke; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg); MUC5AC, mucin 5 ac; GAPDH, glyceraldehyde-phosphate dehydrogenase.

CSE-treated groups were all above 80% (Fig. 6a) when comparing with control group, but 12% CSE significantly decreased the ratio of living cells (lower than 80%) compared to controls. However, various doses of phloretin tested in this study showed no toxicity to NCI-H292 cells (Fig. 6b).

Then, the MUC5AC mRNA was detected to evaluate the optimal concentration of CSE. With the stimuli of graded concentrations of CSE, the expression of MUC5AC mRNA dramatically increased with a dose-dependent manner, and peaked at 8%CSE (Fig. 7a). Thus, to mimic CS-induced airway inflammation with bronchial epithelial cell models, 8%CSE was selected to treat NCI-H292 cells in the following experiments.

With One-hour pretreatment of phloretin, the elevated MUC5AC mRNA, which was induced by CSE, sharply decreased with a dose-dependent manner, and dropped to the bottom at the 5 μM of phloretin (Fig. 7b). Moreover, 8% CSE highly increased the expression of IL-1β mRNA, this was not affected by low-dose of phloretin (1 μM), but was largely diminished by high-dose of phloretin and was decreased to the lowest by the treatment of 5 μM phloretin (Fig. 7c).

3.6. Phloretin inhibits CS-induced phosphorylation of EGFR, ERK and P38 both in vivo and in vitro

Based on the protein samples isolated from mouse tissues or NCI-H292 cells, western blot was performed to explore the potential pathways involved in the effects of phloretin both *in vivo* and *in vitro*. Firstly, four-week CS exposure highly promoted the phosphorylation of EGFR, ERK and P38 in mouse lungs. Both low-dose and high-dose of phloretin alleviated the activation of these molecules (Fig. 8).

In NCI-H292 cells, CSE activated EGFR, ERK and P38 with a time-dependent manner. Briefly, following the treatment of CSE, the phosphorylation of EGFR began to increase at 10 min and peaked at 2 h, the phosphorylation of ERK began to increase and peaked at 30-minute treatment. For P38, the phosphorylation began to elevate at 30 min and peaked at 2 h following the CSE treatment (Fig. 9a–b). Thus in the next cell experiment for western blot, the time duration of CSE stimuli was set at 2 h. With the pretreatment of phloretin, CSE-induced activation of EGFR, ERK and P38 were all dramatically diminished (Fig. 9c–d).

4. Discussion

Phloretin, as a bioactive flavonoid primarily extracted from apple trees, has been found to be protective in inflammatory disorders such as sepsis and allergic airway inflammation [11,14]. In the current study, we found that phloretin attenuated CS exposure-induced airway inflammation and mucus secretion both in CS-exposed mice and CSE-treated NCI-H292 cells, and these effects were related to the down-regulation of activated EGFR and MAPK signaling pathways.

CS-induced chronic airway inflammation has been identified as one of the main features of COPD. Previous studies showed that BALF and circulating inflammatory cytokines and chemokines in COPD smokers were much higher than those in non-smoker COPD patients or non-smoker healthy subjects [21], and CS exposure dramatically activated airway inflammatory responses both *in vivo* and *in vitro* [22,23]. During the last decade, the anti-inflammatory properties of phloretin has been identified in LPS-treated human peripheral blood mononuclear cells and ultraviolet B-exposed skin [24,25]. Moreover, phloretin was also found to be protective in pulmonary inflammatory disorders, Huang et al. found that phloretin suppressed the release of inflammatory cytokines in the lungs of allergic mice induced by ovalbumin [11]. *In-vitro* studies showed that phloretin alleviated interferon γ-induced

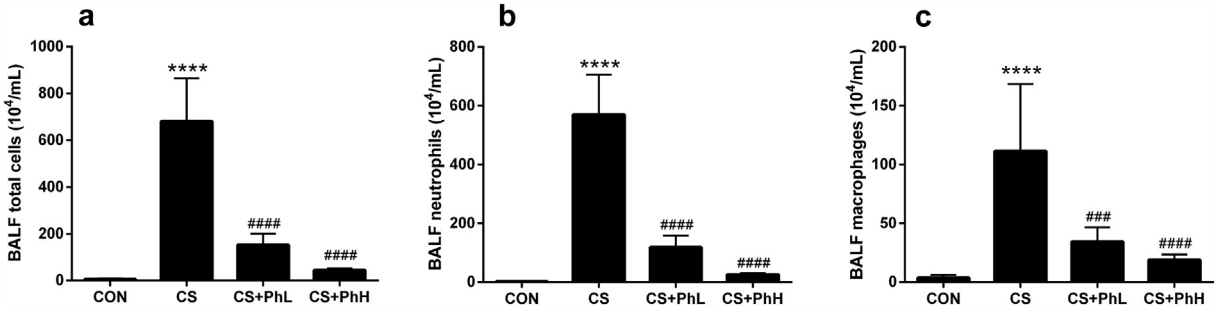

Fig. 4. Phloretin alleviates CS exposure-induced inflammatory cell infiltration in mouse lungs. Notes: The total cells (a), neutrophils (b) and macrophages (c) in mouse BALF were counted, n = 5 or 6 for each group, ****: $P < 0.0001$ compared with control group, ###: $P < 0.001$ compared with CS-exposed group, ####: $P < 0.0001$ compared with CS-exposed group. Abbreviations: CS, cigarette smoke; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg); BALF, bronchoalveolar lavage fluid.

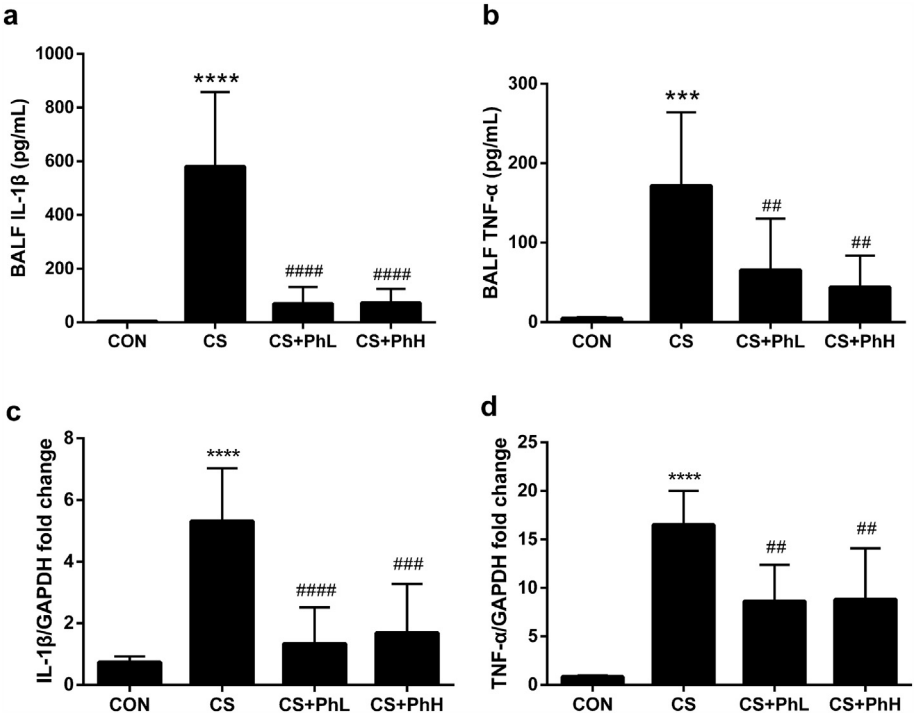

Fig. 5. Phloretin attenuates CS exposure-induced inflammatory cytokine release in mouse lungs. Notes: The release of IL-1β (a) and TNF-α (b) in mouse BALF was evaluated with ELISA, and the mRNA expression of IL-1β (c) and TNF-α (d) in mouse lungs were assessed with PCR, GAPDH was used as an internal control, n = 5 or 6 for each group, ***: $P < 0.001$ compared with control group, ****: $P < 0.0001$ compared with control group, ##: $P < 0.01$ compared with CS-exposed group, ###: $P < 0.001$ compared with CS-exposed group, ####: $P < 0.0001$ compared with CS-exposed group. Abbreviations: CS, cigarette smoke; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg); BALF, bronchoalveolar lavage fluid; IL-1β: interleukin-1 beta; TNF-α, tumor necrosis factor-alpha; GAPDH, glyceraldehyde-phosphate dehydrogenase.

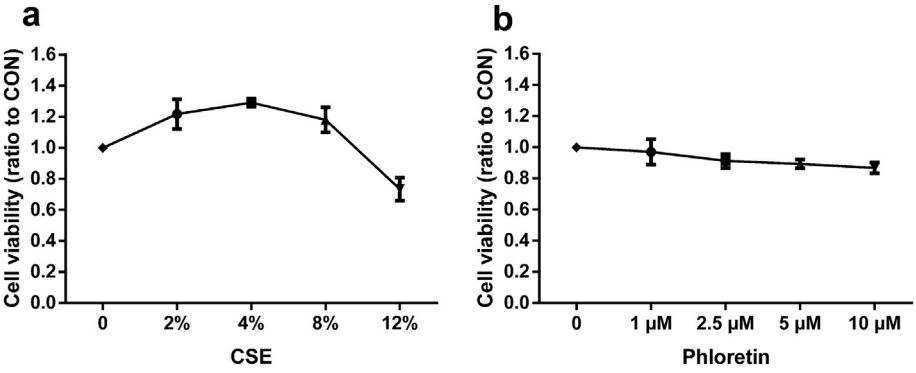

Fig. 6. Phloretin showed no toxicity to NCI-H292 cells. Notes: Cell viability was measured with CCK-8 following the treatment of gradient concentration of CSE (a) or phloretin (b), data were from three independent experiments. Abbreviations: CSE, cigarette smoke extract; CCK-8, WST-8-based cell counting kit.

inflammation in human lung fibroblasts [26]. In the current study, we provided the novel evidence that phloretin exhibited protective roles in CS-induced airway inflammation, implying the therapy potentials of phloretin in CS-related pulmonary inflammatory disorders such as COPD.

Aside from chronic inflammation, airway mucus hypersecretion also contributed to the onset and progression of COPD, and chronic CS exposure has been found to be the most usual promotor of mucin secretion. As a detectable mucin, MUC5AC has been widely investigated in mucus hypersecretion animal and cell models [17,20]. This study found

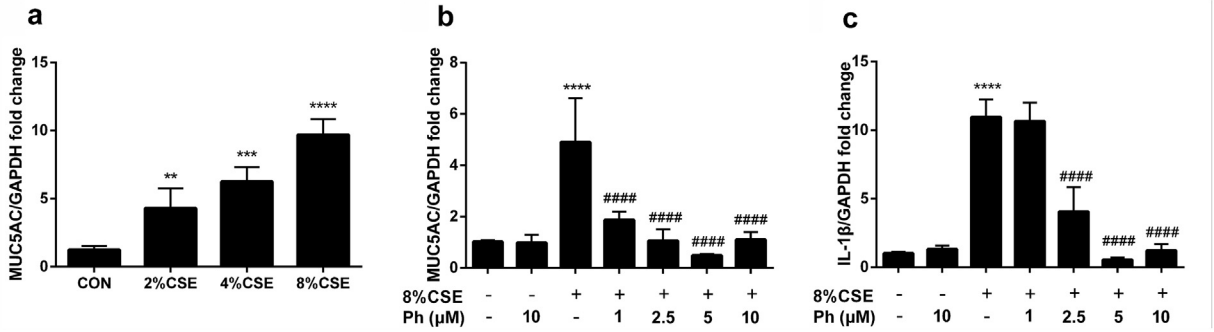

Fig. 7. Phloretin attenuates CSE-induced expression of mucin and inflammatory cytokine in NCI-H292 cells. Notes: The mRNA levels of MUC5AC were detected to assess the optimal concentration of CSE (a), the effects of phloretin pretreatment on the expression of MUC5AC and IL-1β was evaluated with PCR (b, c), GAPDH was used as an internal control, data were from three independent experiments, **: $P < 0.01$ compared with control group, ***: $P < 0.001$ compared with control group, ****: $P < 0.0001$ compared with control group, ####: $P < 0.0001$ compared with CSE-exposed group. Abbreviations: CSE, cigarette smoke extract; Ph, phloretin; MUC5AC, mucin 5ac; IL-1β: interleukin-1 beta; GAPDH, glyceraldehyde-phosphate dehydrogenase.

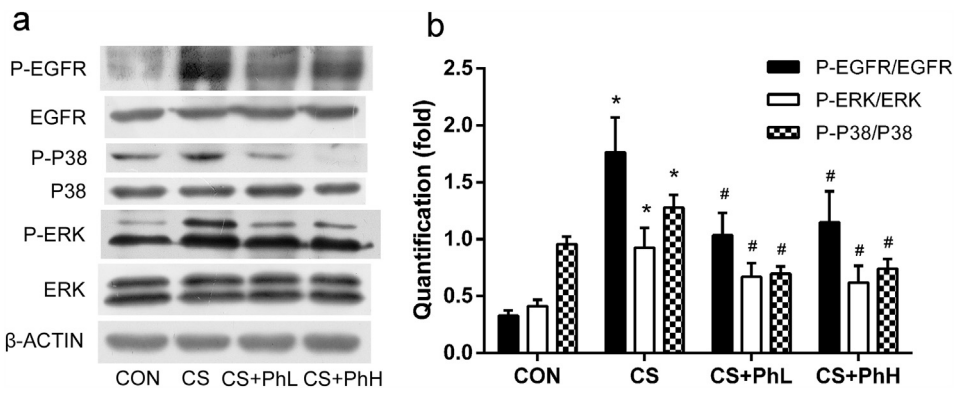

Fig. 8. Phloretin alleviates CS exposure-induced phosphorylation of EGFR, ERK and P38 in mouse lungs. Notes: The protein levels of p-EGFR, p-ERK, p-P38, EGFR, ERK and P38 were detected with western blot (a), and the activation of these signaling molecules was evaluated with the density ratio of phosphorylated proteins to the accompanying total proteins (b), β-actin was used as an internal control, n = 5 or 6 for each group, *: $P < 0.05$ compared with control group, #: $P < 0.05$ compared with CS-exposed group. Abbreviations: CSE, cigarette smoke extract; PhL, low-dose phloretin (10 mg/kg); PhH, high-dose phloretin (20 mg/kg); p-EGFR, phosphorylated epidermal growth factor receptor; P-ERK, phosphorylated extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase.

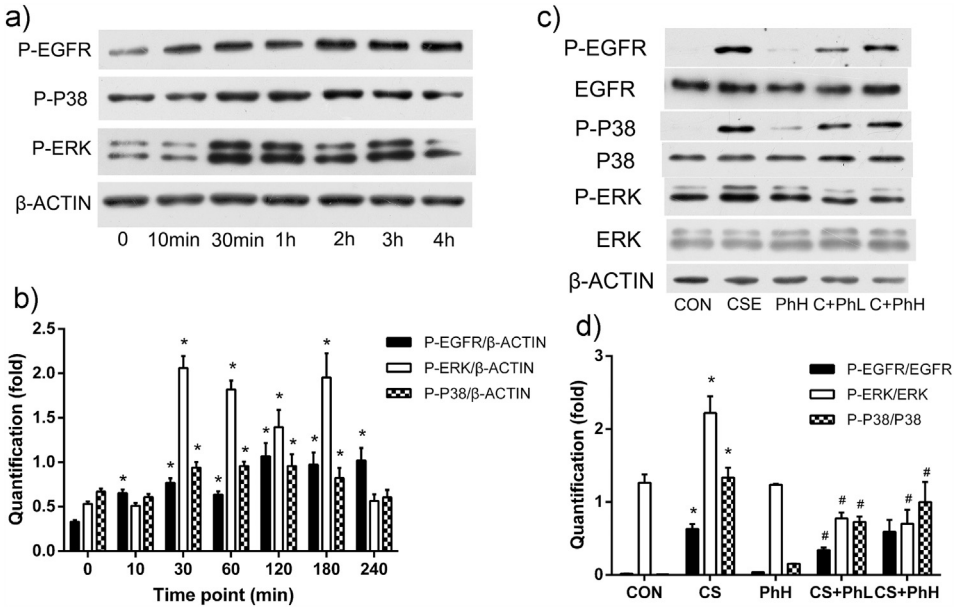

Fig. 9. Phloretin alleviates CSE exposure-induced phosphorylation of EGFR, ERK and P38 in NCI-H292 cells. Notes: The protein levels of p-EGFR, p-ERK and p-P38 were detected with western blot (a), the densities were measured to explore the optimal time duration of CSE treatment (b), the effects of phloretin on the activation of these signaling pathways were investigated (c, d), β-actin was used as an internal control, data were from three independent experiments, *: $P < 0.05$ compared with control group, #: $P < 0.05$ compared with CSE-treated group. Abbreviations: CSE, cigarette smoke extract; PhL, low-dose phloretin (2.5 μM); PhH, high-dose phloretin (10 μM); p-EGFR, phosphorylated epidermal growth factor receptor; P-ERK, phosphorylated extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase.

that four-week exposure of CS dramatically increased the expression of MUC5AC in mouse lungs, and CSE treatment largely up-regulated MUC5AC mRNA levels in human airway epithelial cells, suggesting that CS exposure indeed promoted mucus hypersecretion. Moreover, in the current study, we found that phloretin effectively attenuated CS exposure-induced MUC5AC overexpression both *in vitro* and *in vivo*, suggesting phloretin as a potential therapy drug to reduce mucus in CS exposure-related pulmonary disease especially COPD.

As a canonical inflammatory signaling pathway, the phosphorylation of MAPK pathways, including P38 kinase, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK), was found to promote the release of inflammatory cytokines and chemokines, and the inhibition of MAPK cascade attenuated inflammatory response [27]. On the other hand, MAPK pathways have been identified to be involved in regulating mucus production. Previous studies showed that EGFR inhibitor suppressed the phosphorylation of MAPK, and down-regulated the expression of MUC5AC [28], identifying EGFR as the upstream singling molecule of MAPK pathways in the regulation of mucus secretion. These were observed in the current study, which showed that CS exposure sharply increased the phosphorylation of EGFR, ERK and P38, promoting the release of inflammatory mediators and expression of MUC5AC both in mouse lungs and human airway epithelial cells. These imply that EGFR/MAPK pathways are involved in CS-induced airway inflammation and mucus hypersecretion.

As an anti-inflammatory flavonoid, phloretin has been found to be involved in the regulation of MAPK pathways. Chung and colleagues found that phloretin attenuated the release of allergic inflammatory cytokines such as IL-4 and IL-13 through down-regulating the phosphorylation of ERK, P38 and JNK in human basophils [19]. Phloretin was also found to inhibit the activation of MAPK signaling molecules, and decreased downstream inflammatory cytokine release in interferon-γ-stimulated lung fibroblast, TNF-α-stimulated macrophages, LPS-stimulated dendritic cells, and LPS-stimulated mice [14,29]. The current study provided a novel idea that phloretin attenuated the activation of MAPK pathways induced by CS exposure, and down-regulated downstream inflammatory response. On the other hand, phloretin was found to suppress the expression of EGFR in astrocytes [30], and our data for the first time, to the best of our knowledge, reported the effects of phloretin on EGFR and connected it with mucus hypersecretion.

Although this study provided several new ideas that phloretin would be a potential therapy drug for COPD, some limitations still need to be issue. Firstly, for purpose of minimizing the use of animals, the phloretin control group which received phloretin pretreatment in room air was not performed in mouse experiments. In addition, CCK-8 test performed in NCI-H292 cells showed that phloretin was not toxic to cells. Secondly, the MUC5AC protein levels in mouse BALF and cell supernatant were not detected, as it was extremely difficult to measure MUC5AC with ELISA, and we detected the mRNA of MUC5AC to evaluate the changes of mucus secretion.

In summary, phloretin exhibited anti-inflammatory and anti-mucus proprieties in CS exposure-stimulated mice and airway epithelial cells,

and the down-regulating of EGFR/MAPK pathways would be involved. These data suggest phloretin as a potential therapy approach to CS-induced lung diseases especially COPD.

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Disclosure

The authors declare that they have no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2017.12.009.

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