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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

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