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Original Article

Berberine attenuates cigarette smoke-induced airway inflammation and mucus hypersecretion in mice

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Abstract: Cigarette smoke-induced airway inflammationmucus over-production is one of the most important pathogenic features of chronic airway diseases. This study aimed to investigate the effect of berberine, a plant alkaloid with strong anti-inflammatory property, on cigarette smoke-induced airway inflammation and mucushypersecretion in mice. Mice with exposure to cigarette smoke wereintraperitonealy injected with berberin (5, 10 mg/kg·d). Inflammatory cytokines TNF-α, IL-1β and MCP-1 levels in bronchoalveolar lavage fluid were determined by ELISA. Lung tissue was examined for histopathological lesions and goblet cell hyperplasia. The expression of signaling proteins in lung tissue, ERK and P38 were detected using Western Blot. Cigarette smoke exposure significantly increased the release of inflammatory cytokines TNF-α, IL-1β, MCP-1 and inflammatory cells in bronchoalveolar lavage fluid, and it also induced goblet cell hyperplasiaand the expression of mucin-5ac in the airway of mice. Pretreatment of berberineinhibited cigarette smoke-induced airway inflammation and mucus production. Cigarette smoke exposure also increased the expression of ERK and P38, meanwhile, berberineintervention can inhibit such changes. In summary, berberine inhibits cigarette smoke exposure-induced airway inflammation and mucus hypersecretion in mice, which may partly act through inhibition of ERK and P38.

Keywords: Cigarette smoke, airway inflammation, mucus, berberine

Introduction

Cigarette smoke (CS), which contains thousands of toxic substances, is a well-known etiological factor in the development of chronic inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) [1, 2]. CS exposure damages the airway epithelium, induces consistent airway inflammatory response and high oxidative stress status, which leading to airway mucus hypersecretion, airway obstruction, and finally with an irreversible airflow limitation and progressive decline in lung function [3, 4]. To protect airway form CS-induced airway inflammation and mucus over-production will be of great importance in the prevention and management of COPD [5].

Berberine, a protoberberine alkaloid, and it exhibits a wide spectrum of pharmacological activities [6]. Berberine plays a role in the regulation of inflammation and many studies have investigate its benefit in control endotoxemia, regulation of cholesterol metabolism, and cancer treatment [7-9]. Growing studies pay attention to the effects of berberine on respiratory diseases, Lee et al reported that berberine can suppress inflammatory agents-induced cytokine production in lung cells, which may result from the inhibition of inhibitory kappaB-alpha phosphorylation and degradation, suggesting the potential role of berberine in the treatment of pulmonary inflammation [10]. However, limited study examined the effect ofberberine on CS-induced airway inflammation and mucus over-production. This study aimed to investigate the potential effect of berberine on CS-induced airway inflammation and mucus hypersecretion in an experimental mouse model.

Materials and methods

Animals

Specific pathogen-free, male BALB/c mice (6-8 weeks) were prepared (Dashuo Biological

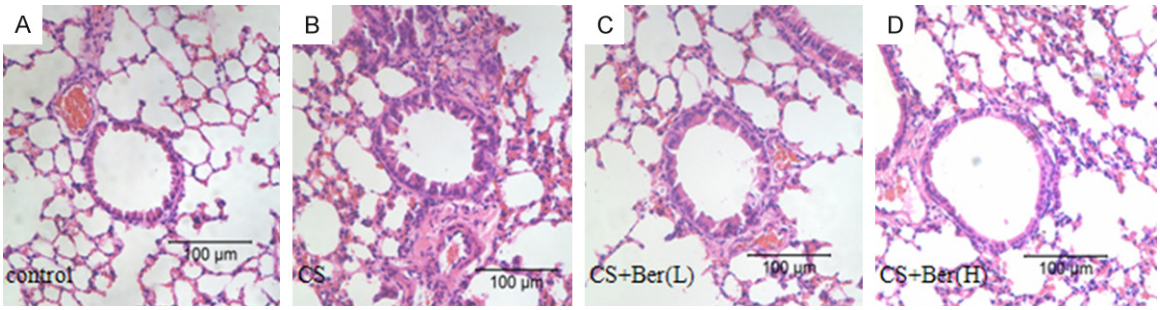

Figure 1. The effect of berberine on CS-induced lung histological changes. Lung tissues were analyzed by hematoxylin and eosin staining, A. Control, no berberine pretreatment and no exposure to cigarette smoke; B. CS, no berberine pretreatment and exposure to cigarette smoke; C. CS+Ber (L), mice pretreated with 10 mg/kgberberine and exposed to cigarette smoke; D. CS+Ber (H), mice pretreated with 20 mg/kg berberine and exposed to cigarette smoke. Scale bars=100 μm.

Technology Co, Ltd, Chengdu, China), and housed in a temperature- and humidity controlled facility and kept on a 12-h light/dark cycle, with free access to water and laboratory feed. Mice were randomly divided into the following four groups (n=8 per group):

(1) Control group (Con group), which was not exposed to CS.

(2) CS-exposed group (CS group), which received placebo and was exposed to CS.

(3) CS-exposed low-dose berberine group (CS+Ber (L)), which received 5 mg/kg berberine (q.o.d.) and was subsequently exposed to CS.

(4) CS-exposed high-dose berberine group (CS+Ber (H)), which received 10 mg/kg berberine (q.o.d.) and was subsequently exposed to CS.

CS+Ber (L) and CS+Ber (L) mice received berberine by intraperitoneal injection at the above mentioned doses 30 min before CS exposure, and then exposed to thesmoke of five commercially available cigarettes (Jiaozi, China Tobacco Chuanyu Industrial Co. Ltd; 1.1 mg nicotine and 11 mg tar per cigarette) for 30 min twice daily, 6 days per week for 4 weeks, following the methods of Yang et al [11]. After 4 weeks ofCS exposure, all the mice were sacrificed by intraperitoneal 3% sodium pentobarbital, followed by exsanguination from the right ventricle to allow tissue sample collection. All the animal preparation and treatment was handled-according to the laboratory animal care guidelines of West China School of Medicine, Sichuan University.

Bronchoalveolar lavage fluid (BALF) inflammatory cell counting

The right lung was lavaged three times with 0.5 ml of saline, with a recovery rate of 85-90%. The BALF samples were centrifuged at 1,000 g for 5 minutes, and the supernatants were removed and stored at -80°C for cytokines measurement. The pelleted cells were re-suspended in 0.2 ml phosphate-buffered saline, the total cell number was determined by a hemocytometer. Differential cell count was performed by cytocentrifugation (Cytopro7620, Wescor, Utah, USA) at 700 rpm for 10 min and stained with Wright's stain (200 cells were counted for each mouse).

Inflammatory cytokines detection

Levels of tumor necrosis factor-α (TNF-α), Interleukin-1 beta (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) in the BALF were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits for mouse cytokines (Xitang Bio-Technology Co. Ltd, Shanghai, China). The manufacturer's instructions were strictly followed during the ELISA experiments.

Lung histopathology examination and immunohistochemistry

The leftnot lavagedlung was immersed in 4% phosphate-buffered paraformaldehyde to allow complete fixation, after which it was embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin (H&E) to evaluate morphological changes in lungs.

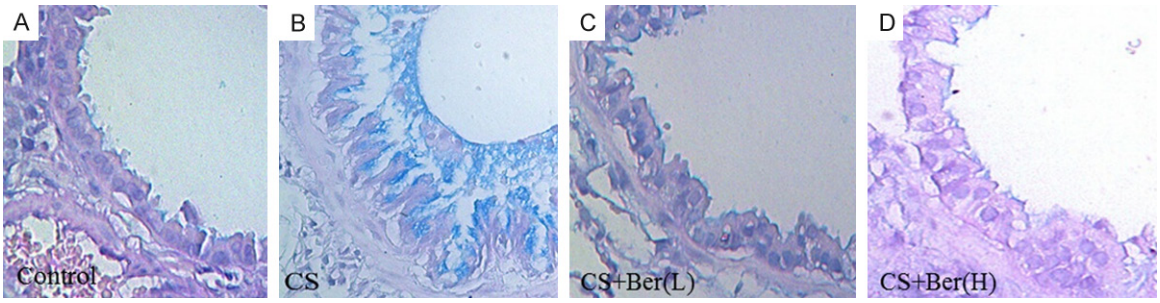

Figure 2. Changes in Alcian blue (AB)/periodic acid-Schiff (PAS) staining in mice airways.Histopathological examination of lung tissue sections stained with AB/PAS, lung tissues were treated as described in the legend to **Figure 1**. Scale bars=100 μm.

Alcian blue (AB)-periodic acid Schiff (PAS) staining was performedto assess the levels of intracellular mucous glycoconjugates. Immunohistochemical (IHC) staining for Muc5ac protein was performedusing a SPHRP kit (Santa Cruz Biotechnology, Inc. SantaCruz, CA, USA). In brief, lung sections were stained with anti-Muc5ac antibody (clone 45 M1, 1:200; Neomarkers, Fremont, CA, USA).

Western blot analysis

Lung tissues were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, and PMSF. Protein concentrations from whole lung extracts weredetermined by a BCA protein assay kit (Thermo Fisher Scientific Inc. MA, USA). To analyzeERK, P38MAPK, total protein (20 μg) was fractionated by 10% SDS polyacrylamide gel electrophoresis andtransferred to PVDF membranes. Membranes were blocked for 1 h atroom temperature with 5% BSA in TBS-Tween and incubatedovernight at 4°C with the appropriate primary antibodies. Primary antibodies were anti-ERK mAb, anti-P38MAPK, and anti-β-actin mAb (Cell SignalingTechnology, Beverly, MA, USA). After incubation with horseradish peroxidase-conjugated second antibodies (Cell Signalling Technology), the immune complexes were detected with enhanced chemiluminescence reagents (Millipore, Billerica, USA).

Statistical analysis

All values are expressed as Mean±SD. Statistical analysis was carried out using one-way ANOVA, followed by the LSD significant difference test (SPSS for Windows version 18.0, Chicago, IL, USA). A significant difference was defined at P>0.05.

Results

Berberine prevented CS-induced mouse airway histopathological changes

In this study, H&E staining was used to evaluate histopathological changes in mouse airways. Four-week's CS exposure induced significant airway inflammatory response manifested as thickening of the airway epithelium,lumen obstruction by mucus and cell debris, and inflammatory cell infiltration (**Figure 1**). Such changes were significantly attenuated by berberinepretreatment, especially with high dose berberine.

Berberine attenuated CS-induced mucus production in mice airways

Goblet cell hyperplasia was examined in the presence and absenceof chronic CS exposure using AB/PAS. CS exposure led to a prominentincrease in the numbers of goblet cells along the airway surface epithelium. This increase in AB/PAS staining was significantly inhibitedby berberine in a dose-dependent manner (**Figure 2**). Since Muc5ac isthe predominant mucin gene expressed in goblet cells, the effect ofberberine on CS-induced Muc5ac synthesis was examined by immunohistochemistry. Consistent with the AB/PAS staining results, thepercentage of bronchial epithelial surface area positively stained byanti-Muc5ac monoclonal antibody markedly increased after 24 days of CS exposure, and this increase was significantly attenuated byberberine pretreatment (**Figure 3**).

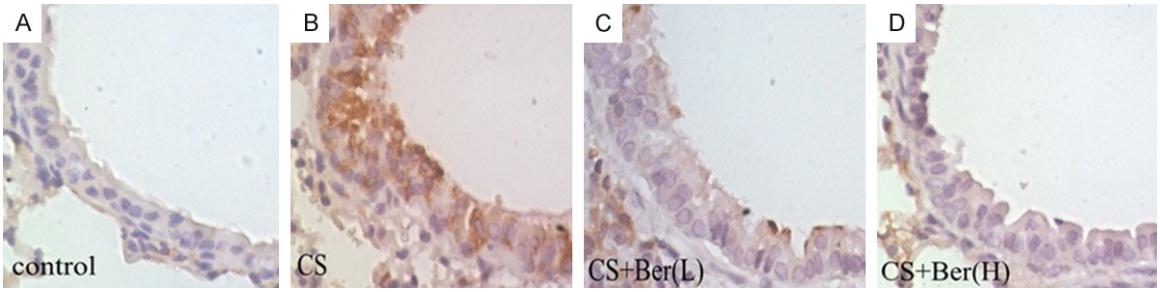

Figure 3. Changes in Muc5ac immunohistochemical staining in mice airways. Immunohistochemical staining of Muc5ac in mouse airwayepithelium. Bar ¼ 100 mm Lung tissues were treated as described in the legend to 1. Scale bars=100 μm.

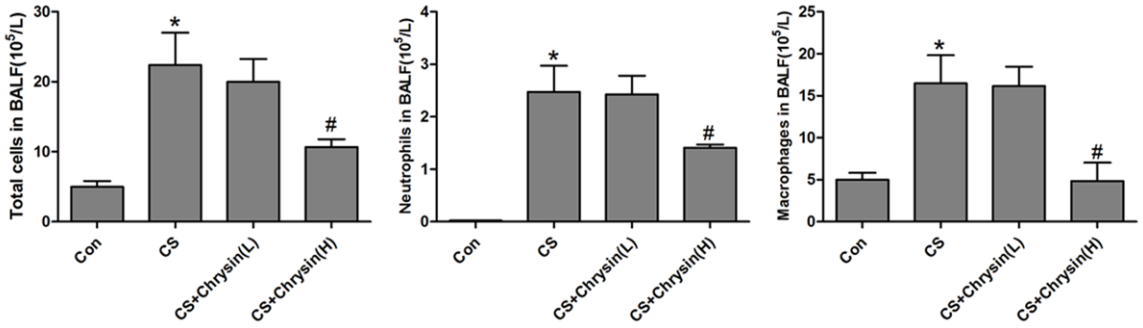

Figure 4. The effects of berberine on BALF cellularity in CS-exposed mice. Differential cell count in BALF was determined by cytocentrifugation and Wright's stain. Groups were treated as described in the legend to **Figure 1**. *P<0.05 with respect to the control group, #P<0.05 with respect to the cigarette smoke-exposed group.

Berberine attenuated CS-induced inflammatory cell influx and inflammatory cytokines release in BALF

Total BALF cell counts and differential cells counts were significantlyincreased in CS-exposed mice than in the control group. Berberinepretreatment significantly reduced the CS-induced recruitment oftotal cells and differential cells to BALF (**Figure 4**), and suppressedthe CS-induced influx of inflammatory cells into BALF.

BALF levels of TNF-α, IL-1β, and MCP-1 were significantly higher in the samples from CS-exposed mice than in the samples from control animals. Pretreatment with berberine significantly reduced the CS-induced increases in TNF-α, IL-1β, and MCP-1 in BALF (**Figure 5**).

Berberine inhibited CS-induced ERK and P38 expression

To explore the possible mechanisms involved in CS-induced airway inflammation, the expression levels of ERK and P38 were examined. Lung tissues of CS-exposed mice showed higher levels of ERK and P38 than did lung tissues of control mice, and berberine pretreatment attenuated these effectsof CS (**Figure 6**).

Discussion

In present study, CS was used to establish airway inflammation and mucus over-production model in mouse, which causes significant airwayinflammatory response, and mucus hypersecretion, and increased ERK, P38 expression in mouse lung, and pretreatment with berberineattenuatesthese effects *in vivo*.

In our study, berberine protects mouse airway from CS-induced inflammatory response through inhibiting inflammatory cell influx in BALF. Neutrophils is the main inflammatory cells in the pathogenesis of COPD [12]. Pilette C et al reported that neutrophils were increased in the small airways of COPD patients when compared with smokers, and intra-epithelial neutrophils correlated with the forced expirato-

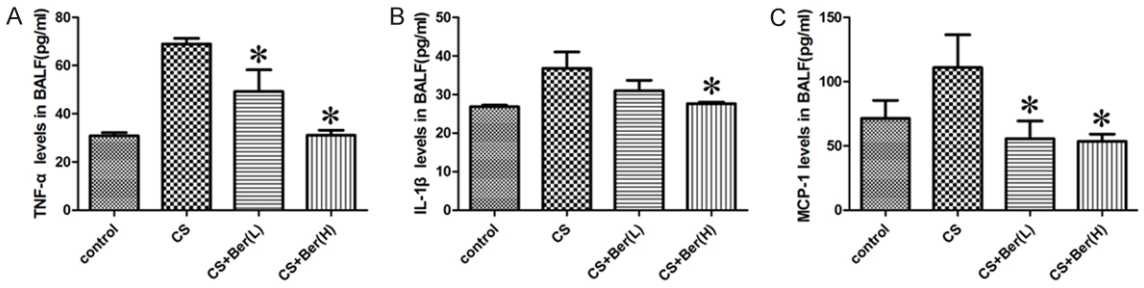

Figure 5. The effects of berberine on BALF inflammatory cytokines in CS-exposed mice. Differential inflammatory cytokines in BALF was determined by enzyme-linked immunosorbent assay. Groups were treated as described in the legend to **Figure 1**. *P<0.05 with respect to the cigarette smoke-exposed group.

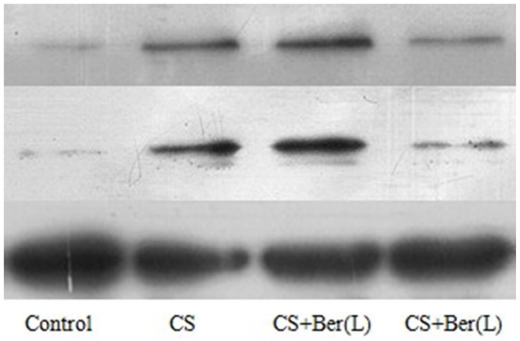

Figure 6. Expression of P38 and ERK in mice lungs. P38 (upper), ERK (Middle) and β-actinlevels were measured by Western blotting in mouse lungs. Groups were treated as described in the legend to **Figure 1**.

ry volume in one second/forced vital capacity ratio 1 [13]. Activated neutrophils recruited to the airways, and secrete tumor necrosis factor-alpha, releases reactive oxygen species, neutrophil elastase, then activates epidermal growth factor receptor, play several key roles in mucus hypersecretion in COPD [14]. Many animal studies AZD9668, a novel, oral inhibitor of neutrophil elastase, has the potential to reduce lung inflammation, while its clinical utility remainsdebatable [15, 16]. Our study finds that berberine inhibits the release of neutrophils into the BALF, and plays a role in the prevention of CS-induced airway inflammation and mucus hypersecretion.

Our study also observed that berberine significantly suppressed CS-induced airway inflammation through inhibits the release of inflammatory mediators into the BALF. TNF-α is a cytokine released primarilyfrom macrophages, and is thought to play a criticalrole in the progression of COPD. The TNF-α gene polymorphism is associated with an increased risk of COPD, serum levels of TNF-α are increased in COPD patients, and TNF-α modulates clinical severity and airflow limitation in an additive manner [17]. TNF-α also stimulates MUC5AC production in NCI-H292 cells, plays a role in mucus hypersecretion in COPD [18]. IL-1beta, a pro-inflammatory cytokine, and utilizes the NF-kappaBpathway and induce of MUC5AC mRNA and protein synthesis in human bronchial epithelial cell, and IL-1beta plays a significant role in induction of murine emphysema and small airway remodeling [19, 20]. MCP-1 wasfound to be higher in acute exacerbations of patients than in health subjects [21]. All these inflammatory mediators are involved in the pathogenesis and clinical course of COPD, and therapy targeting these mediators may supply new choice for the management of mucus overproduction and airway inflammation in COPD patients [22].

MAPK signaling pathways containing ERK and P38 play an important role in the pathogenesis of COPD [23]. Studies showed the sputum P38 MAPK activity was remarkably correlated with the CXCL8 level and neutrophils infiltration in the airway, and the decline of lung function in the COPD patients [24]. Inhibition of P38 MAPK may supply a novel therapy choice for COPD, a clinical trial has investigated the efficacy of the oral p38 inhibitor PH-797804 on COPD patients, and PH-797804 demonstrated improvements over placebo in lung function parameters and dyspnea in patients with moderate to severe COPD [25]. In addition, p38 MAPK inhibition may be beneficial in COPD by restoring CS sensitivity [26]. Studies reported that increased expression of ERK might be

involved in the pathogenesis of COPD patients, and phosphorylated ERK is involved in pro-inflammatory cytokines production and airway mucus hyepersecretion [27, 28]. Our studies confirmed that increased P38 and ERK involved in CS-induced airway inflammation, therapy or drugs targeting on these signaling pathways may help to attenuate CS-induced airway inflammation and mucus over-production. In this study, we observe that berberinetreatment significantly decreased CS-induced airway inflammation and mucus over-production, suggesting its potential role in the treatment of airway inflammatory disease. What should be pointed out is that the present study included a limited number of mice and further studies are needed to confirm the protective role of berebrine on CS-induced airway inflammation and investigate its possible mechanism, and how to translate the preclinical study findings into clinical studies.

Conclusion

Taken together, our study confirmed that CS exposure can induce significant airway inflammation, mucus hypersecretion, increased P38 MAPK and ERK expression in mice, pretreatment with berberine may attenuate these changes, thus, berberine shows the potential to treat CS-induced inflammatory airway disorders.

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Disclosure of conflict of interest

None.

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