Inhalation of ambroxol inhibits cigarette smoke-induced acute lung injury in a mouse model by inhibiting the Erk pathway

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Abstract

Oral and injection administration of ambroxol has been clinically used to treat airway disease. However, little is known about its potentials in inhalation therapy. In present studies, we tested the effects of ambroxol by inhalation with intravenous administration, and explored the underlying working mechanism. The mice received 10 cigarettes exposure every day for 4 days. Inhaled solution of ambroxol was aerosolized 20 min before the exposure of cigarette smoke (CS). The effect of ambroxol on the expression of mucoprotein 5AC (MUC5AC) and proinflammatory cytokines in NCI-H292 cells stimulated with cigarette smoke extract (CSE). Four days of daily inhalation of ambroxol at 3.75 or 7.5 mg/ml for 20 min suppressed the accumulation of neutrophils and macrophages in the bronchoalveolar lavage fluid (BALF) and lung tissues, and inhibited increases in the mRNA and protein levels of tumor necrosis factor (TNF)- α , CCL-2 and KC, but not interleukin (IL)-1 β in the CS-exposed mice. Moreover, ambroxol at 3.75 or 7.5 mg/ml facilitated airway mucosa cilia clearance, reduced glycosaminoglycans level in BALF and MUC5AC mRNA levels in lung tissues. The effects of ambroxol by inhalation at 7.5 mg/ml was comparable to that of ambroxol at 20 mg/kg i.v. and dexamethasone at 0.5 mg/kg i.p. Using cultured lung epithelial cells, we demonstrated that pretreatment with ambroxol at 2 or 20 μ M inhibited the CSE-induced up-regulation of MUC5AC, TNF- α , IL-1 β mRNA levels, which was through inhibiting Erk signaling pathway. Our results demonstrate the beneficial effects of ambroxol as an inhalation replace systemic administration for COPD therapy.

Keywords: Ambroxol; Drug delivery; Mucokinetic activity; Airway inflammation

1. Introduction

Chronic obstructive pulmonary disease (COPD) remains a major public health problem. The World Bank/World Health Organization reported that COPD will rank fifth worldwide based on the disease burden in 2020 [1]. According to the global initiative for COPD guidelines, COPD is recognized as an inflammatory disease state that includes increases in the levels of a complex cascade of inflammatory mediators, such as tumor necrosis factor- α (TNF- α), monocyte chemotactic protein (MCP-1 or CCL-2), interleukin-1 β (IL-1 β) and IL-8 [2]. Cigarette smoke (CS) is a complex mixture of oxidant radicals and different chemical compounds, including reactive aldehydes and semiquinones that are known to cause oxidative stress in the lungs. CS exerts major effects on human health and is widely recognized as a primary risk factor associated with the progression of COPD.

Smoking and its associated tissue damage give rise to inflammation with an increase in the sequestration of neutrophils and inflammatory cells, resulting in the activation of redox-sensitive transcription factors, which are critical for the transcription of pro-inflammatory genes, including CCL-2, keratinocyte chemo-attractant (KC or mouse IL-8), IL-1 β and TNF- α [3-5]. Indeed, CS leads to oxidative stress, high sputum secretion, small airway fibrosis, emphysema, and progressive airflow limitation [6,7]. It is believed that steroid treatment is primarily resisted by cigarette smoking patients with COPD, which indicates the urgent needs for efficient treatments [8].

Ambroxol (Amb, 2-amino-3,5-dibromo-N-[*trans*-4-hydroxycyclohexyl]benzylamine) is a metabolite of bromhexine. Both bromhexine (Bisolvon) and Amb are semisynthetic derivatives of vasicine, used in the treatment of respiratory disorders with productive cough. Its major pharmacodynamic actions are surfactant stimulation, mucokinetic and secretagogue activity [9]. In addition to a mucolytic action, Amb has antioxidant and anti-inflammatory properties *in vitro* [10-14] and *in vivo* [15-17]. Amb has been proposed to treat chronic pulmonary disorders, such as COPD [18-20], acute lung injury/acute respiratory distress syndrome [21-23],

idiopathic pulmonary fibrosis [24-26] and upper respiratory disease [27]. Amb significantly reduced the lung hemorrhage, edema, exudation, neutrophil infiltration, the histological score of lung injury and the cytokine levels in a murine model of lipopolysaccharide-induced lung injury [17]. In another study, Amb enhanced LPS-induced secretion of IL-12 and the ratio of IL-12/IL-10, which suggests that Amb appears to strengthen innate immune response and cell-mediated immunity, and facilitate the development of Th-1 cells [28]. The effects of Amb on the release of histamine, leukotrienes, cytokines and superoxide anions from a variety of cells involved in the pathogenesis of allergic reaction and inflammation [14]. Amb can inhibit the release of mediators of allergic reaction from mast cells and leukocytes, which make it potent in the treatment of allergic and inflammatory respiratory diseases.

In clinical drug delivery, in general, oral or injection administration of Amb is the most commonly used method for airway disease. However, little is known about its potentials as an inhalation (i.h) therapy to treat CS-induced mucous hypersecretion and inflammatory responses. In this study, we hypothesize that Amb is a potential anti-inflammatory drug exerting biological and pharmacokinetic properties suitable for delivery by inhalation. Inhalation of Amb may overcome low clinical efficacy, and weaken the side effects from oral administration or injection. We aimed to investigate whether the inhalation of Amb could inhibit pathological changes in a rodent model of CS-induced acute lung injury, as well as in cigarette smoke extract (CSE)-exposed lung epithelial cells. We intended to explicit the beneficial effects of Amb in inhalation therapy for COPD.

2. Materials and methods

2.1. Animals

Female ICR mice (weighing 22 ± 2.5 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (No. SCXK 2012-0002). The animals were housed in isolated ventilated cages (4–5 mice/cage) under a 12-h light/12-h dark cycle and received food and water *ad libitum* in the Laboratory Animal Center of Zhejiang

University. All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine.

2.2. Drug administration

The inhaled solution of ambroxol hydrochloride (Amb, 15 mg/2 ml, Tianjin Institute of Pharmaceutical Research., Tianjin, China) for inhalation was prepared at concentrations of 1.875, 3.75, and 7.5 mg/ml. Then, Inhaled solution of Amb was aerosolized for 20 min with a jet nebulizer (BARI Co. Ltd., Germany) 20 min before the exposure of the animals to cigarettes. The plasma concentrations of 1.875, 3.75, and 7.5 mg/ml by i.h for 20 min are the dose equivalent of intravenous injection (i.v.) of 0.7, 1.34, 2.78 mg/kg, which are measured by HPLC. As a reference drug, Amb hydrochloride injection (15 mg/2 ml, Tianjin Institute of Pharmaceutical Research., Tianjin, China) 20 mg/kg was injected by i.v. 10 min before their exposure to cigarettes. As a positive control drug, dexamethasone sodium phosphate (Tianjin Jin Yao Group Hubei Tianyao Pharmaceutical Co., LTD) at 0.5 mg/kg was intraperitoneally (i.p.) injected into mice 1 h before their exposure to cigarettes. The control and model mice received solvent inhalation.

2.3. Cigarette exposure

The mice were exposed to whole-body CS generated from research grade cigarettes (3R4F; University of Kentucky, Lexington, KY, USA) in a square plastic box (45 × 45 × 20 cm) once a day for 4 days as described previously [29–31]. The mice were exposed to seven cigarettes on the first day, nine cigarettes on the second day, and 11 cigarettes on both the third and fourth days. Lung tissues and bronchoalveolar lavage fluid (BALF) were collected 18 h after the last CS exposure. The control animals were exposed to room air.

2.4. Preparation of bronchoalveolar lavage fluids

Eighteen hours after the last CS exposure, the mice were euthanized through an i.p. pentobarbital injection of 6 g/kg urethane. The BALF was obtained by cannulating the trachea and lavaging with PBS containing 1% BSA and 5000 IU/l heparin. The BALF cells were centrifuged once at 500 g and 4 °C for 10 min with PBS containing 2%

FCS. The pelleted BALF cells were resuspended in PBS, and the total number of leukocytes was counted using a Neubauer chamber. A total of 200 cells in a cytocentrifuged preparation of BALF stained with Wright–Giemsa were differentiated under a light microscope according to the classical cell morphology. The total number of each cell type was determined by multiplying the percentage by the total number of cells. The results were expressed as the number of each cell population in 1 ml of BALF.

2.5. Tissue processing and histological analysis

With the mice under terminal anesthesia, the left lungs were removed, infused with 10% formalin, and immersed in the same solution, and the tissue was then processed in paraffin-embedded blocks. The sections were stained with H&E to evaluate the general morphology. To determine cell counts in the alveolar spaces and severity of the infiltration of the inflammatory cells were performed based on the 5-point scoring system described in our previous papers [32, 33]. The analyses were performed in a blind fashion, and the slides were presented in a random order for each examination.

2.6. Mucociliary clearance and glycosaminoglycans level in CS-exposed mice

Mucociliary clearance in CS-exposed mice were performed according to the report of Hosoe and colleagues [34]. Under pentobarbital anesthesia (45 mg/kg, i.p.), the carbon solution was instilled to evaluate the mucociliary clearance 30 min after CS exposed mice. BALF was performed 2 h after carbon instillation and the OD value of BALF was determined. Drugs pretreatment were administered to see the section 2.2.drug administration. The glycosaminoglycans concentration in BALF was estimated according to the report of Goldberg and Kolbas [35].

2.7. RNA isolation and quantitative PCR

To investigate the effects of Amb on IL-1 β , TNF- α , CCL-2, and KC as well as **mucoprotein 5AC (MUC5AC)** mRNA expression in the lung tissues and pulmonary epithelial cells, the mice inhaled Amb 7.5mg/ml 20 min before CS exposure or Amb pretreated epithelial cells, and the lung tissues were obtained 18 h after the final CS or

epithelial cells were obtained 24 h after cigarette smoke extract (CSE) exposed cells. The total RNA of lung tissue homogenates and epithelial cells were extracted with the TRIzol reagent (Takara Bio, Dalian, China) according to the manufacturer's instructions. The PCR primers were purchased from Shanghai Bioengineering (Shanghai, China). All of the primers were checked using a basic local alignment search tool to determine their selectivity. Real-time PCR cycling was conducted (7500 Real-Time PCR System; Applied Biosystems, Carlsbad, CA, USA) under the following conditions: the PCR mixture consisted of 10.4 μ l of SYBR GreenMasterMix, 0.4 μ l of both the sense and antisense primers, 2.0 μ l of the sample cDNA solution, and distilled water to obtain a final volume of 20 μ l. The program for chymase was conducted as follows: a denaturation step at 95 °C for 40 s and 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 34 s. The primer sequences are described in [Table 1](#), and β -actin was used as an internal control.

2.8. Enzyme-linked immunosorbent assay (ELISA)

To investigate the effects of Amb on IL-1 β , TNF- α , CCL-2, and KC cytokines protein expression as well as MUC5AC protein expression in the BALF, the BALF were analyzed using ELISA kits (Boster, Wuhan, China and Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China) according to the manufacturer's

2.9. Cell culture

NCI-H292 cells, a human pulmonary epithelial cell line, were obtained from the Cell Bank, Chinese Academy of Sciences. The cells were maintained in RPMI 1640 (HyClone, Logan, UT) containing 10% FBS (HyClone) at 37°C in the presence of 5% CO₂.

2.10. Preparation of cigarette smoke extract (CSE)

Research-grade cigarettes (3R4F) were obtained from the Kentucky Tobacco Research Council (University of Kentucky). The composition of 3R4F research-grade cigarettes was as follows: total particulate matter, 10.9 mg per cigarette; tar, 9.4 mg per cigarette; and nicotine, 0.726 mg per cigarette. CSE was prepared by bubbling smoke from three cigarettes into 30 ml PBS, modifying the method used in previous

research. CSE was standardized by measuring the absorbance at a wavelength of 320 nm. After filtering through a 0.45- μ M filter, CSE was frozen in aliquots and stored at -80°C . An aliquot of CSE was thawed immediately before use [29].

2.11. Immunoblotting analysis

NCI-H292 cells were seeded into a six-well plate. After reaching the confluent, the cells were incubated in serum-free medium (RPMI 1640) overnight and then exposed to CSE in the presence or absence of PHPS1 for 15 min. After treatment, the cells were washed three times with ice-cold PBS and lysed in 100 μ l radioimmunoprecipitation assay buffer with 10 mM PMSF (Beyotime, Haimen, China). The protein concentration was measured by the BCA Protein Assay Kit (cwbiotech, Beijing, China). A sample of protein (20–50 μ g) from the cell lysates was separated by SDS-PAGE in 12% polyacrylamide gel and transferred to nitrocellulose membranes (Pall, Port Washington, NY), which were blocked with 5% fat-free milk (1 h at room temperature). The membranes were then incubated with p-Erk and p-P38, Erk and P38 (Cell Signaling Technology) and actin primary Abs (Bioworld, St. Louis Park, MN). Afterward, the membranes were rinsed with TBST and then probed with secondary Abs (Invitrogen) for 1 h at room temperature. Immunoreactive bands were visualized by a two-color infrared imaging system (Odyssey; LI-COR, Lincoln, NE).

2.12. Statistical analysis

The data are expressed as the means $\pm$ S.E.M. The statistical tests were performed using the SPSS software (version 16.0; SPSS, Chicago, IL, USA). One-way ANOVA followed by the Student–Newman–Keuls test was used for the multiple comparisons. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Ambroxol alleviates CS-induced lung inflammation in mice

To evaluate the effects of Amb on CS-induced pulmonary inflammation, the infiltrated inflammatory cells were counted following four days of CS exposure. In the BALF, CS exposure resulted in increased numbers of total inflammatory cells, neutrophils, macrophages, and lymphocytes compared with the control ($P < 0.001$)

(Fig. 1A). Twenty min inhalation of Amb at 1.875, 3.75, and 7.5 mg/ml 20 min before mice was exposed to CS concentration-dependently inhibited the CS-induced inflammatory cell accumulation, including total leukocytes, neutrophils and macrophages in the BALF. The lung sections were further histologically analyzed. As illustrated in Fig. 1B, C, H&E staining revealed that Amb inhalation, compared with CS exposure alone, alleviated the infiltration of neutrophils and macrophages into the alveolar spaces. Amb at 7.5 mg/ml by aerosol seems to be more potent than that of Amb at 20 mg/kg by i.v. administration in Fig. 1C ($p < 0.05$). Dex at a dose of 0.5 mg/kg, as a positive reference drug, also showed significant inhibitory effects on the accumulation of inflammatory cell. These observations reveal a potent role of Amb inhalation in reducing the recruitment of inflammatory cells in acute mouse models of CS.

3.2. Ambroxol reduces mRNA levels of IL-1 β , TNF- α , CCL-2 and KC in the lung tissues and their protein levels in the BALF

To measure the IL-1 β , TNF- α , CCL-2 and KC mRNA expression and protein level, the lung tissues and the BALF were harvested and examined 18 h after the final CS exposure. As shown in Fig. 2A and B, the CS-exposed mice showed a significant up-regulation in the IL-1 β , TNF- α , CCL-2 and KC mRNA and protein levels compared to the control mice, which were all significantly reduced by the aerosol pretreatment with Amb at 1.875, 3.75, or 7.5 mg/ml in a dose-dependent manner, except the IL-1 β mRNA expression. The inhibitory effects of Amb at 7.5 mg/ml i.h. were comparable to that of Dex at 0.5 mg/kg i.p. Moreover, the inhibitory effect of Amb at 3.75 mg/ml i.h. on TNF- α and KC protein expression are more potent than that of Amb at 20 mg/kg by i.v. administration in Fig. 2B ($p < 0.05$).

3.3. Ambroxol increases the clearance function of airway mucosa cilia and decreases mRNA expression of MUC5AC in the lung tissues and glycosaminoglycans in the BALF.

To measure the clearance function of airway mucosa cilia, glycosaminoglycans level, MUC5AC mRNA and protein expression and in vivo, the BALF and the lung tissues were harvested and examined after the final CS exposure. As shown in Fig. 3A,

B, C and D, the CS-exposed mice significantly decreased the clearance function of airway mucosa cilia, up-regulated glycosaminoglycans levels and MUC5AC mRNA and protein expression. Amb dose-dependently enhanced the clearance function of airway mucosa cilia, normalized the glycosaminoglycans levels in BALF as well as the MUC5AC mRNA and protein levels in lung tissues in the CS-exposed mice. Importantly, the effects of Amb at 3.75 mg/ml i.h. on inhibiting glycosaminoglycans levels in the BALF were comparable to that of Amb at 20 mg/ml i.v. and Dex at 0.5 mg/kg i.p.

3.4. Ambroxol suppresses the CSE-induced IL-1 β , TNF- α and MUC5AC mRNA expression in the pulmonary epithelial cells

To examine the effects of CSE on IL-1 β , TNF- α and MUC5AC mRNA expression in lung epithelia, pulmonary epithelial cells were treated with CSE at different concentration with various duration, then followed by measures of IL-1 β , TNF- α and MUC5AC mRNA expression. We found remarkable elevations of IL-1 β , TNF- α and MUC5AC in lung epithelial cells with CSE exposure that were in a concentration- and time-dependent manner (Fig. 4 A and B). Amb 20 μ M significantly decreased the CSE-mediated IL-1 β , TNF- α and MUC5AC mRNA expression (Fig. 4 C).

3.5. Ambroxol inhibits the CSE-induced Erk1/2 activity

We then further explored the molecular mechanisms of Amb in the expression of CS-induced inflammatory factors and MUC5AC. We found that CSE-induced Erk and P38 activation. Further, Erk activation, but not P38 activation was inhibited by Amb at 20 μ M (Fig. 5 A and B). Erk inhibitor - U0126 10 μ M (Fig. 5C), but not p38 inhibitor - SB203580 (data not shown) significantly decreased the CSE-mediated IL-1 β , TNF- α and MUC5AC mRNA expression. Our data suggest that Amb decreased the CSE-induced Erk1/2 activation, which may explain part of the molecular mechanisms.

4. Discussion

In this article, we report that the inhalation of Amb prevents the CS-induced

inflammatory responses in pulmonary epithelia *via* Erk signaling pathway. These findings extended our view regarding the improved potency of administering drug through a novel route. Furthermore, the biological roles of Amb in the pathogenesis of pulmonary inflammation and mucolytic action were also extensively explicated.

Although several articles have described the *in vivo* anti-inflammatory activity of ambroxol [9, 17], conclusion is still controversial. As Amb has activity to facilitate airway mucosa cilia clearance, its anti-inflammatory activity is possibly due to the reduction of the exposure of cigarette smoke extract or other inflammatory stimulants. Despite of the CS-induced lung injury model and our *in vitro* results, we suggest that the anti-inflammatory effects of Amb involve both the facilitation of airway mucosa cilia clearance and its direct anti-inflammatory effects on other inflammatory stimulants, such as LPS [17], N-formyl-methionyl-leucyl-phenylalanine (FMLP) [12], concanavalin A and compound 48/80 [10], hydrogen peroxide [15] and doxorubicin [16].

Cigarette smoking is a leading cause of COPD, which is associated with persistent inflammatory responses and increased viscous sputum secretion [36, 37]. The lung inflammatory responses to CS exposure are more complex than the neutrophil accumulation, including high sputum secretion, small airway fibrosis and emphysema. We studied a novel delivery that is inhalation of Amb - a conventional drug for treating COPD. Amb is frequently used as a mucolytic agent in respiratory diseases associated with increased mucus production like acute or chronic bronchitis [18-20]. In addition to mucus regulatory effects, a wide range of pharmacological anti-inflammatory properties of ambroxol have been described *in vitro* and *in vivo* [9], including the inhibition or scavenging of oxidative and nitrosative stress, the increases in local defense molecules involved in respiratory virus replication, the reduction of pro-inflammatory cytokines and arachidonic acid metabolites, inflammatory cell chemotaxis, and lipid peroxidation of tissues [38, 39].

Ambroxol is marketed in various pharmaceutical formulations, including intravenous and intramuscular solutions, liquids, granules, tablets, capsules, suppositories and oral slow release formulations. Systemic administration of Amb

requires substantially high dose and may be associated with a greater incidence of systemic side effects. On the other hand, the absorption of i.v. administration is faster than oral administration. But it also requires a substantially higher dose; thereby it may also increase systemic side effects and lead to poor patient compliance. Logically, the inhalation administration of Amb could overcome some of the drawbacks from the above delivery routes. Recently, Ren et al [40,41] reported that Amb DPI (dry powder inhalation) at 20 mg/kg, given *via* tracheal administration (TA), achieved a high local concentration in lung epithelial lining fluid (ELF) and reached a maximum concentration (C_{max}) at 1.5 h in plasma. After the same dose Amb was given by i.v., Amb reached a C_{max} in ELF at 1.25 h. The AUC (0-t) (ELF)/(AUC(0-t)) (plasma) ratio (1.05-2.25) after TA differed significantly from the ratio (0.029-0.039) observed after i.v. Amb ($p < 0.05$). Their results indicate that Amb DPI can be locally delivered to achieve high concentration in ELF. Therefore, the inhalation of Amb could be a useful drug delivery system for treating pulmonary diseases. In addition, a clinical literature described that the volume of distribution is 17 folds higher for dose deposition within the lung than within the plasma if Amb was administrated intratracheally [42]. These results suggest that the lung tissue and plasma concentration following i.v. administration of Amb was only 3% to 4% compared to that following i.h. or i.t. delivery administration. However, in rats, these values were 8% to 10%, and a healthy volunteer study showed 55% to 60% increases in the pattern of deposition within the lung following a treatment with HFA-beclomethasone metered-dose inhaler [43]. As discussed above, these results suggest that Amb is a potential anti-inflammatory drug that has pharmacological and pharmacodynamic properties suitable for delivery *via* inhalation.

Although intragastric administration (i.g) and intraperitoneal administration (i.p) of Amb have shown good efficacy in preclinical models [15-17], it is not clear if i.h. administration of Amb influences the pro-inflammatory factors and mucolytic action. In the present study, i.h. administration of Amb significantly inhibited the CS-induced increases in the mRNA and protein levels of TNF- α , IL-1 β , CCL-2 and KC in lung tissues and in the BALF. Moreover, Amb at a dose of 1.875, 3.75 and 7.5 mg/ml

dose-dependently increased the clearance function of airway mucosa cilia, decreased glycosaminoglycans level in the BALF and MUC5AC mRNA levels in lung tissues in the CS-exposed mice. The inhalation of Amb 1.875, 3.75, and 7.5 mg/ml concentration-dependently inhibited CS-induced accumulation of inflammatory cells, including total leukocytes, neutrophils and macrophages in the BALF and the infiltration of neutrophils and macrophages within the alveolar spaces in lung tissues. Amb 7.5 mg/ml by i.h. administration was more potent than that of Amb 20 mg/kg by i.v. administration. In *in vitro* studies, we found that pretreatment with Amb 0.2, 2 and 20 μ M inhibited Erk signaling. Subsequently, Amb concentration-dependently inhibited the CSE-induced increases in MUC5AC, TNF- α , IL-1 β , CCL-2 mRNA levels in the epithelial cells.

Mucus hypersecretion is a distinguishing feature of COPD, which is defined physiologically by limitations of airflow and pathologically by repetitive injury and inappropriate epithelial repair in airways [44]. MUC5AC appears most frequently in studies of human secretions and is regulated by many mediators that are likely to be present in the airways of COPD patients. The increase of MUC5AC expression will lead high secretion of sputum, small airway fibrosis and progressive airflow limitation [44]. The studies with acrolein and cigarette smoke have suggested that MUC5AC is inducible (accompanied by epidermal growth factor (EGF) ligand formation and the activation of EGF receptor-dependent pathways) [45]. The prevalence of patient presentation with cough and expectoration of sputum and the perceived importance of mucus in the pathophysiology of COPD, have led to development of drugs intended to treat airway mucus hypersecretion [46]. Essentially, the associated pharmacotherapy divides into two sections. The first is anti-inflammatory treatment of airway inflammation, probably the most beneficial therapy overall. The second is therapies directed specifically at different aspects of the pathophysiology of mucus hypersecretion, from inhibition of the secretagogue effects of specific endogenous inflammatory secretagogues to aiding expectoration of sputum by use of mucolytics [47, 48]. Amb is widely used in clinical practice, because it combines the effects of both anti-inflammatory and reducing mucus hypersecretion.

Substantial evidences have been presented that the activation of Erk is involved in various inflammatory conditions. For example, D'Armiento laboratory demonstrated a rapid and long-lasting cigarette smoke-induced activation of Erk-1/2 MAP kinase in cultured pulmonary epithelial cells [49]. In addition, they also found the elevated phosphorylation of Erk-1/2 (p44/42) in the lungs in mice exposed to cigarette smoke for 10 days [49]. These results are consistent with ours. Most importantly, the relevance between these events and COPD was established by the discovery of a significantly elevated Erk-1/2 activity in airway and alveolar epithelial cells of patients with emphysema, compared with non-emphysematous controls [49]. Other studies reported an increased mRNA and protein expression of Egr-1, an Erk-1/2 substrates, in lung tissues of patients with emphysema, suggesting that the activation of Erk-1/2 signaling is involved in the pathogenesis [50]. It is also likely that the activation of different MAP kinases can induce the same transcription factor. For example, Erk-1/2 [51] and JNK pathways each have been shown to activate NF- κ B signaling in human monocytes and airway epithelial cells [52], demonstrating the potentials for crosstalk between these pathways.

In summary, our results demonstrate that i.h. administration of Amb attenuates the CS-induced accumulation of inflammatory cells in the BALF, reduces the infiltration of neutrophils and macrophages into the alveolar spaces in lung tissues. Inhalation administration of Amb inhibits the mRNA expression or protein release of pro-inflammatory cytokines and decreases the glycosaminoglycans level in the BALF. It also down regulated the expression of MUC5AC mRNA in lung tissues in the CS-exposed mice. Moreover, Amb showed a concentration-dependent inhibition on the CSE-induced increases in MUC5AC, TNF- α , IL-1 β mRNA levels in epithelial cells by inhibiting Erk signaling pathway. Our results reviewed a beneficial effect of Amb with inhalation administration for treating COPD.

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Figure Legends

Fig.1. Ambroxol decreases inflammation in the CS-induced lung injuries. Mice were repeatedly exposed to CS for 4 d. Then, they were treated with Amb (1.875, 3.75, or 7.5 mg/ml) for 20 min with a jet nebulizer (BARI Co. Ltd., Germany) (i.h) 10 min before daily CS exposure. Amb hydrochloride at 20 mg/kg was intravenously injected 10 min before the mice were exposed to cigarettes. Dexamethasone sodium phosphate at 0.5 mg/kg was intraperitoneally injected into mice 1 h before their exposure to cigarettes. Control and model mice received solvent inhalation. (A) The total inflammatory cells in the BALFs were counted. At least 200 cells were used to classify inflammatory cells as neutrophils, macrophages, and lymphocytes. (B) Lung tissues were stained with H&E. Representative images from three independent experiments are shown. Inflammatory cells were observed under microscopes. Mice with air exposure had no infiltration of inflammatory cells (Control). CS exposure induced an influx of macrophages (green arrowheads), neutrophils (black arrowheads) and lymphocytes (red arrowheads) into the alveolar spaces. (C) To determine the severity of inflammatory cells infiltration in the alveolar spaces, cell count was performed based on a 5-point scoring system described previously [32, 33]. Data are presented as mean $\pm$ SEM, n =10. Scale bar, 20 μ m. #p < 0.05, ##p < 0.01, ###p < 0.001 compared with control. *p < 0.05. **p < 0.01, ***p < 0.001 compared with mice with CS exposure. †p < 0.05 compared with mice was intravenously injected with Amb at 20 mg/kg.

Fig. 2. Ambroxol inhibits IL-1 β , TNF- α , CCL-2 and KC mRNA expression in lung tissues and their protein levels in the BALF. Lung homogenates were prepared for analyzing mRNA expression 18 h after the final CS exposure. The BALF was also harvested and tested in the meantime. (A) IL-1 β , TNF- α , CCL-2 and KC mRNA levels in lung tissues and (B) protein levels in the BALF are shown. Data are presented as mean $\pm$ SEM, n =10. #p < 0.05, ##p < 0.01, ###p < 0.001 compared with control. *p < 0.05. **p < 0.01, ***p < 0.001, compared with mice with CS alone

exposure (model). † $p < 0.05$ compared with mice was intravenously injected with Amb at 20 mg/kg.

Fig. 3. Ambroxol reduces glycosaminoglycans levels and MUC5AC expression.

To measure glycosaminoglycans levels, and MUC5AC mRNA and protein expression in vivo, the BALF and the lung tissues were harvested and tested 18 h after the final CS exposure. (A) glycosaminoglycans levels in the BALF are shown; (B and C) MUC5AC mRNA expression in lung tissues and its protein levels in the BALF. Data are presented as mean $\pm$ SEM, $n = 10$. ### $p < 0.001$ compared with control. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$, compared with mice exposed to CS alone (model).

Fig. 4. Ambroxol reduces the CSE-induced IL-1 β , TNF- α and MUC5AC mRNA expression in pulmonary epithelial cells.

To examine the effects of CSE on IL-1 β , TNF- α and MUC5AC mRNA expression in lung epithelia, NCI-H292 cells were pretreated with Amb (0.2, 2 or 20 μ M) or U0126 (10 μ M) for 15 min; then the cells were treated with CSE (2.5%) for 24 h. The culture supernatants were measured for IL-1 β , TNF- α and MUC5AC mRNA levels using Q-PCR. The effects of CSE exposure at various concentrations or for various time duration on IL-1 β , TNF- α and MUC5AC mRNA expression are shown (A and B). Treatment with Amb at 20 μ M significantly decreases CSE-mediated IL-1 β , TNF- α and MUC5AC mRNA expression (C). Data are presented as mean $\pm$ SEM, $n = 5$. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with control. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$ compared with the cells exposed to CSE alone.

Fig.5. Ambroxol reduces the CSE-induced inflammatory factors and MUC5AC expression by inhibiting the activity of Erk1/2 in pulmonary epithelial cells.

CSE (2.5%) time-dependently activates Erk and P38 signaling in NCI-H292 cells. The activity of Erk1/2 and P38 was determined in the indicated cell line by immunoblotting. Amb 20 μ M significantly reduces the CSE-induced activation of

ERK (A), but not P38 (B) in pulmonary epithelial cells. The pharmacological inhibition with an ERK inhibitor U0126 at 10 μ M significantly decreases CSE-mediated IL-1 β , TNF- α and MUC5AC mRNA expression (C). Data are expressed as mean $\pm$ SEM, n = 5. #p < 0.05, ##p < 0.01 compared with control. *p < 0.05. **p < 0.01, ***p < 0.001 compared with cells exposed to CSE alone.

Table 1. Primer sequences for measuring mRNAs using real-time PCR (The sequences are listed from 5' to 3').

Genes	Primer sequences (5'-3')
Mouse TNF- α	F: CAAGGGACAAGGCTGCCCCG R: GCAGGGGCTCTTGACGGCAG
Mouse IL-1 β	F: GCTTCAGGCAGGCAGTAT R: ACAAACCGCTTTTCCATCT
Mouse CCL-2	F: AGGTCCCTGTCATGCTTCTGG R: TGCTGCTGGTGATCCTCTTGT
Mouse KC	F: GCTGGGATTACCTCAAGAA R: TGGGGACAAATTTTAGCATC
Mouse MUC5AC	F: ACATTTCCTCCATGCTCCACAGC R: GTGGTGGTATTAGACTCCTGG
Mouse β -actin	F: GTATGGTCG TGGCTCTAAGC R: ATGTCACGCACGATTTC
Human TNF- α	F: ATCTATCTGGGAGGGGTCTTCC R: AGAGGTTGAGGGTGTCTGAAGG
Human IL-1 β	F:ATCAGCCAGGACAGTCAG R:CAGTAAGCGAGGGTGTAAG
Huma MUC5AC	F: GGGACAAGGAAACCTACAACA R: GACAGGGAAGGATACAGAGCA
Human GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC

Figure 1A

A

B

Control

Vehicle

Ambroxol 75 mg/ml (i.h.)

Ambroxol 20 mg/kg (i.v.)

Dexamethasone 0.5 mg/kg (i.p.)

Figure 1C

C

Inflammatory score in lung tissues

Figure 2

Figure 3

Figure 4

Figure 5