International Immunopharmacology 33 (2016) 90–98

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

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

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ARTICLE INFO

Article history:
Received 25 November 2015
Received in revised form 2 February 2016
Accepted 3 February 2016
Available online 13 February 2016

Keywords:
Ambroxol
Drug delivery
Mucokinetic activity
Airway inflammation

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 pro-inflammatory 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)-1β 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 μM inhibited the CSE-induced up-regulation of MUC5AC, TNF-α, IL-1β 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.

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-1β (IL-1β) 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-1β 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

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http://dx.doi.org/10.1016/j.intimp.2016.02.004
1567-5769/

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 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-1β, 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 7.5 mg/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 μl of SYBR GreenMasterMix, 0.4 μl of both the sense and antisense primers, 2.0 μl of the sample cDNA solution, and distilled water to obtain a final volume of 20 μl. 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.