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The anti-malaria drug artesunate inhibits cigarette smoke and ovalbumin concurrent exposure-induced airway inflammation and might reverse glucocorticoid insensitivity

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ABSTRACT

Background: The anti-malaria drug artesunate has been shown to attenuate experimental allergic asthma *via* inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. This study was to further determine the effects of artesunate on cigarette smoke and ovalbumin (OVA) concurrent exposure-induced airway inflammation, the related mechanism, and glucocorticoid insensitivity.

Methods and results: In vivo: Female BALB/c mice concurrently exposed to cigarette smoke and OVA developed mixed eosinophilic and neutrophilic airway inflammation. Airway hyper-responsiveness, total and differential cell counts, and pro-inflammatory cytokine levels (interleukin (IL)-4, IL-8, IL-13 and tumor necrosis factor (TNF)-α) in bronchoalveolar lavage fluid (BALF) were measured. Lung tissue sections were stained for histological analysis, and proteins were extracted for Western blotting. Artesunate reduced methacholine-induced airway hyper-responsiveness, suppressed pulmonary inflammation cell recruitment and IL-4, IL-8, IL-13 and TNF-α levels, selectively inhibited PI3Kδ/Akt pathway, and restored HDAC2 activity. *In vitro*: BEAS-2B cells were exposed to cigarette smoke extract (CSE) for 6 h and then stimulated with TNF-α overnight. Glucocorticoid sensitivity was evaluated by the inhibition of TNF-α-induced IL-8 production by dexamethasone. CSE reduced the effects of dexamethasone on TNF-α-induced IL-8 production in BEAS-2B cells, while artesunate reversed CSE-induced glucocorticoid insensitivity and restored HDAC2 deactivation induced by CSE.

Conclusion: Artesunate ameliorated cigarette smoke and OVA concurrent exposure-induced airway inflammation, inhibited the PI3Kδ/Akt pathway, restored HDAC2 activity, and reversed CSE-induced glucocorticoid insensitivity in BEAS-2B cells. These findings indicate that artesunate might play a protective role in asthma induced by cigarette smoke and glucocorticoid insensitivity.

1. Introduction

Asthma is a chronic airway inflammation characterized by inflammatory cell infiltrates, mucus hypersecretion, airway wall remodeling, and airway hyper-responsiveness (AHR) [1]. More than 300 million people have asthma worldwide [2]. The pathogenesis of asthma is not fully understood. Recently cumulative evidence suggests that asthma is a heterogeneous disorder with multiple phenotypes [3–4], T-helper type (Th) 1, Th2, Th17 and regulatory T cells are involved in the development of asthma [5]. Generally, the Th2 immune response with eosinophil influx is considered to play an essential role in the pathogenesis of allergic airway inflammation [6–8]. Most asthma patients with mild to moderate disease are of this type and can be managed by treatment with inhaled corticosteroids (ICS). Some patients have severe asthma, which cannot be managed even with state-of-the-art therapies. Studies have shown that these patients are of a mixed Th1/Th2 phenotype with a Th17 component, of which there is a high percentage of neutrophils, or neutrophils co-exist with eosinophils in their lung and sputum [3–4]. Although patients with severe asthma account for approximately 5% of the asthmatic population, they impose an enormous social and economic burden on health systems [9]. Therefore, there is an urgent need for the development of more effective treatments for the management of severe asthma.

A key to managing severe asthma is reversing corticosteroid insensitivity. Clinical evidence revealed that patients with severe asthma have poor responsiveness to glucocorticoid therapy [10–11]. Glucocorticoids exert their anti-inflammation action via the glucocorticoid receptor-α (GR-α). GR-α silences the expression of proinflammatory genes through the recruitment of histone deacetylase 2 (HDAC2) [12]. HDAC2, a member of the histone deacetylase (HDACs) family, regulates gene expression by deacetylating the hyperacetylated histones [13]. Reducing HDAC2 expression and/or loss of HDAC2 activity leads to the ineffectiveness of corticosteroids whereas improving HDAC2 levels restores glucocorticoid sensitivity [14–17].

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Besides HDAC2, the phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway is closely related with the anti-inflammation function of glucocorticoids [18]. PI3Ks generate secondary lipid messengers that regulate numerous cellular functions, including cell growth, differentiation, survival, and metabolism [19]. There are four classes (IA, IB, II, and III) of the PI3K family, of which class IA-PI3K p110δ (PI3Kδ) and class IB-PI3K p110γ (PI3Kγ) are mainly expressed in leukocytes and are thought to be involved in inflammation to a greater extent than the other isoforms [19]. Studies have shown that inhibition of PI3Kδ and PI3Kγ alleviates allergic airway inflammation, but their functions are different. Inhibition of PI3Kδ can completely prevent the development of steroid insensitivity but results in a limited anti-inflammation effect. In contrast, knockdown of PI3Kγ has markedly inhibited inflammation but has little effect on reversing corticosteroid sensitivity [18,20]. Differences between the roles of PI3Kδ and γ make them promising potential anti-inflammation targets.

Artesunate, a semi-synthetic derivative of artemisinin, has been mainly used to treat malaria, with high activation and rare toxicity. In recent years, scholars have found that it has extensive pharmacological action, such as anti-inflammation [21], cell proliferation inhibition [22], anti-angiogenesis [23], and anti-viral properties [24], in addition to its anti-malarial effect. Although recent studies have revealed that artesunate attenuates ovalbumin (OVA)-induced Th2-mediated allergic airway inflammation via negative regulation of the PI3K/Akt pathway [25], no study has focused on the effects of artesunate on the individual PI3K isoforms, HDAC2 activity and glucocorticoid insensitivity in asthma.

Cigarette smoke aggravates pulmonary dysfunction, induces mixed neutrophil and eosinophil inflammation response in patients with asthma, impairs HDAC2 activity, and attenuates the therapeutic effect of glucocorticoids [26]. In this study, we developed an animal model of OVA-challenged asthma in cigarette smoke exposure environment, not only to investigate the effect of artesunate on airway inflammation, but also to reveal for the first time the effect of artesunate on HDAC2 activity, and glucocorticoid insensitivity *in vitro*.

2. Materials and methods

2.1. Animals

Seventy-five female BALB/c mice (18–20 g) at 6 to 8 weeks of age from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences were divided equally into five groups: air-exposed and non-sensitized animals (Normal group); cigarette smoke (CS)-exposed and OVA-sensitized animals (CS + OVA group); CS-exposed and OVA-sensitized animals treated with vehicle for artesunate (Vehicle group); CS-exposed and OVA-sensitized animals treated with artesunate (Arts group); CS-exposed and OVA-sensitized animals treated with dexamethasone (Dex group). All mice were housed in a specific pathogen-free (SPF) residence, which satisfied the PRC National Standard (GB 14925-2010, China), and were provided with water and food. All experimental procedures were approved by the Animal Ethics Committee of China-Japan Friendship Hospital.

2.2. Cigarette smoke exposure

From day 1 to day 40, mice in the CS + OVA, Vehicle, Arts, and Dex groups were placed in a 70 × 45 × 30 cm organic glass box and exposed to the smoke of four commercial filtered cigarettes (11 mg of tar oil, 0.8 mg of nicotine, and 13 mg of carbon monoxide per cigarette, DaQianMen Factory, Shanghai, China) for 30 min, twice per day without interval, in the morning. There were two holes in the front and back walls of the smoke box to allow smoke in and out, ensuring circulation of the smoke and air in the box. Normal group mice were exposed to ambient air as control.

2.3. OVA-induced asthma model

Mice in the CS + OVA, Vehicle, Arts, and Dex groups were sensitized by intraperitoneal (i.p.) injections of aluminum hydroxide-absorbed OVA (Grade VI Sigma-Aldrich, St. Louis, MO, USA; 20 μg OVA + 4 mg $Al(OH)_3$ per mice) on days 10, 17, and 24. Seven days after the last sensitization, mice were challenged with 1% OVA aerosol for 30 min per day in the afternoon until day 40. Two hours before each aerosol challenge, vehicle (5% $NaHCO_3$ containing 5% DMSO), artesunate (Sigma-Aldrich, 30 mg/kg, dissolved in 5% DMSO and diluted with 5% $NaHCO_3$), and dexamethasone (Sigma-Aldrich, 1 mg/kg, dissolved in saline) was given by i.p. injection to mice in the Vehicle, Arts, and Dex groups, respectively. Normal group and CS + OVA group mice were saline sensitized and challenged as negative controls. A schematic drawing of the experimental protocol is depicted in Fig. 1.

2.4. Measurements of airway hyper-responsiveness

Six mice in each group were anesthetized with 1% pentobarbital sodium (0.1 ml/kg i.p.) 24 h after the last aerosol challenge, and tracheal intubation was performed. Airway hyper-responsiveness (AHR) to methacholine (Mch, Sigma-Aldrich) was measured. Each mouse was placed in a closed plethysmograph chamber and then connected to the AniRes 2005 animal lung function analysis system (Synol High-Tech, Beijing, China), with the tidal volume and frequency set at 120 μl and 90 breaths per min, respectively. Progressive Mch doses (0, 0.025, 0.05, 0.1, 0.2 mg/kg) were administered via the internal jugular vein. After each dose, the data were collected continuously for 150 s. Then, inspiratory and expiratory resistance and dynamic compliance were analyzed.

2.5. Total and differential cell counting in BALF

The remaining nine mice in each group were anesthetized, and tracheal intubation was performed. After removal of blood, the left lung was ligatured, and saline (0.8 ml × 8) was instilled into the right lung. Then, BALF (0.5–0.6 ml) was collected and centrifuged, and supernatants were collected and stored at −80 °C for subsequent analyses. Total and differential cell counts of the precipitates were determined for total and differential cell counts. Total cell counts were performed on cell counting chamber by trypan blue staining under light microscopy (Nikon DR-Ri1, Tokyo, Japan). Differential cell counts were performed on 500 cells to identify neutrophils (Neu), eosinophils (Eos), macrophages (Mac), and lymphocytes (Lym) by Giemsa staining. Differential counts were calculated based on their respective percentages.

2.6. Measurements of IL-4, IL-13, IL-8, TNF-α in BALF

Interleukin (IL)-4, IL-13, IL-8, and tumor necrosis factor (TNF)-α levels in BALF of each group were measured using quantitative enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, in

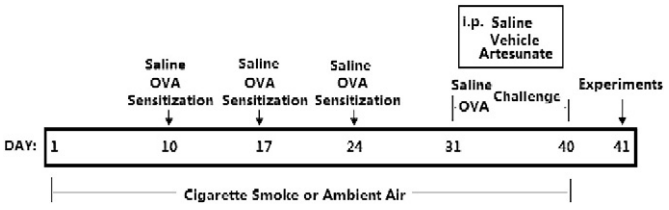

Fig. 1. Experimental protocol for the study: Mice were exposed to cigarette smoke or air in the afternoon from day 1 to day 40 in the morning. Three OVA or saline sensitization doses were injected on days 10, 17, and 24. From day 31, mice were exposed to aerosolized 1% OVA or saline (30 min per day) in the afternoon. Twenty-four hours later, mice were anesthetized and experiments were performed.