Anti-asthmatic activity of osthole in an ovalbumin-induced asthma murine model

Jingjing Wang^{a,1}, Yunhe Fu^{a,1}, Zhengkai Wei^a, Xuexiu He^a, Mingyu Shi^a, Jinhua Kou^a, Ershun Zhou^a, Weijian Liu^a, Zhengtao Yang^a, Changming Guo^{a,*}

Abstract

Osthole, an active coumarin extracted from the dried fruits of *Cnidium monnieri* (L.) Cusson, is known to possess a variety of pharmacological activities. In the present study, we investigated and illuminated the mechanisms underlying the protective effects of osthole in an experimental model of allergic asthma. Our results show that osthole treatment significantly reduced the OVA-induced increase in serum IgE and inflammatory cytokines (IL-4, IL-5, IL-13) in bronchoalveolar lavage fluid (BALF), and decreased the recruitment of inflammatory cells in BALF and the lung. It also effectively attenuated goblet cell hyperplasia and mucus overproduction in lung tissue. In addition, western blot analysis demonstrated that osthole blocked NF- κ B activation, which may be associated with a reduction in inflammatory cytokine production. These data suggest that osthole attenuated OVA-induced allergic asthma inflammation by inhibiting NF- κ B activation. The present study identified the molecular

mechanisms of action of osthole, which support the potential pharmaceutical application of osthole treatment for asthma and other airway inflammation disorders.

Keywords

Osthole; allergic asthma; inflammation; NF- κ B

1. Introduction

The prevalence of asthma has significantly increased over the past 3 decades (Wu et al., 2014). In China, more than 30 million people suffer from this disease (Chen, 2003). Asthma is a complex inflammatory disorder of the lung that is characterized by bronchoconstriction, lung tissues inflammation and airway hyperresponsiveness (AHR) (Mizgerd, 2006). Clinical and experimental investigations have demonstrated that CD4⁺ TH2 cells play a pivotal role in the pathophysiology of asthma (Zimmermann et al., 2003). Th2 cytokines, including IL-4, IL-5, and IL-13 are secreted by activated CD4⁺ TH2 cells, and are associated with asthma, IgE production, mucus secretion, mast cells, and activation of mast cells and eosinophils (Saenz et al., 2008). Both IL-4 and IL-13 regulate expression of IgE from B lymphocytes, whereas IL-5 plays a major role in eosinophilic inflammation. Despite effective therapies, exact pathophysiological mechanisms underlying asthma remain unclear and current treatments are not satisfactory.

Nuclear factor kappa B (NF- κ B), a well-known transcriptional factor that has a pivotal role in the recruitment of inflammatory cells, has numerous important functions in the production of Th2 cytokines in the airways of murine asthma models animals (Choi et al., 2004; Kang et al., 2009). Cytokines and other factors stimulate cell surface receptors to initiate a signaling cascade leading to the activation of NF- κ B, which mediates the release of cytokines to activate immune responses. Many studies have suggested that the NF- κ B pathway may be associated with asthma. According to Choi et al, pretreatment with NF- κ B

p65 antisense oligonucleotides significantly inhibited the asthmatic reaction in a murine model (Choi et al., 2004). Therefore, NF- κ B has emerged as a promising molecular target for the treatment of asthma.

Cnidii Monnieri Fructus, the dried fruits of *Cnidium monnieri* (L.) Cusson (Chinese name *Shechaungzi* and Japanese name *Jashoshi*) is an important crude drug and has been used as a traditional Chinese medicine to treat various diseases such as osteoporosis, sexual dysfunction, asthma and skin ailments (Baba et al., 1992). The extracts of *Cnidium monnieri* (L.) Cusson contain several ingredients, including isopimpinellin, bergapten, imperatorin, xanthotoxin, and osthole (Liu et al., 2004). Osthole (7-methoxy-8-isopentenoxycoumarin), a natural coumarin derivative, is one of the main constituents from *Cnidium monnieri* (L.) Cusson and has been used as a promising lead compound in drug discovery research due to its unique structural modification. Recently, osthole has received considerable interest because of its significant pharmacologic activities, including anti-oxidation (Zhang et al., 2011), anti-inflammatory (Liu et al., 2005), anti-allergic (Chiu et al., 2008; Matsuda et al., 2002), and anti-diabetic (Liang et al., 2009) effects. It has been reported that osthole inhibited immune inflammatory diseases including arthritis and hepatitis (Okamoto et al., 2003; Okamoto et al., 2001). Matsuda, H. et al found that osthole has an antipruritic effect in allergic model animals (Matsuda et al., 2002). Interestingly, recent studies have shown that osthole attenuated lipopolysaccharide induced acute lung injury in a murine model (Chen et al., 2013; Shi et al., 2013). In an alcoholic fatty liver model, osthole led to decreased oxidative stress and increased superoxide dismutase (SOD) activation (Zhang et al., 2011). Although osthole has been used in many clinical applications, limited data are available concerning its effect in

allergic asthma. In the present study, we sought to determine the effects of osthole on allergic asthma in an ovalbumin (OVA)-induced asthma murine model.

2. Materials and methods

2.1. Chemicals and reagents

Osthole (Fig. 1), dimethyl sulfoxide (DMSO) and Ovalbumin (OVA; grade V) were purchased from Sigma (St. Louis, MO, USA). Dexamethasone was obtained from Changle Pharmaceutical Co. (Xinxiang, Henan, China). Mouse IL-4 and IL-13 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Biolegend (USA), and a mouse IL-5 ELISA kit was obtained from eBioscience (USA). All western blot antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were provided by GE Healthcare (Buckinghamshire, UK). All other chemicals were of reagent grade.

2.2. Animals

Specific pathogen-free female BALB/c mice (6-8 weeks) were purchased from the Center of Experimental Animals of Baiqiu Medical College of Jilin University (Jilin, China). Before all experiments, all mice were maintained under standard conditions surviving on distilled water and standard chow ad libitum for 1 week. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Experimental protocols

Fig. 2 a schematically depicts the experimental protocol used in this study. Mice were divided into the following six groups (n = 8 for each): the control group, OVA group, OVA + osthole (25, 50, and 100 mg/kg) treatment groups, and OVA + dexamethasone (DEX) (2 mg/kg) group. On days 0 and 14, the mice were sensitized via an intraperitoneal injection of 20

μg of OVA emulsified with 2 mg aluminum hydroxide in a total volume of 200 μl PBS (pH 7.4) as an -adjuvant. On days 21, 22 and 23, the mice were challenged by intranasal inhalations with 100 μg OVA in a volume of 50 μl PBS once **per** day. Osthole (25, 50, and 100 mg/kg) and DEX were intraperitoneally given 1 h before the OVA challenge. For the control group, mice were given an equal volume of PBS. Mice were sacrificed to determine the pathophysiological features of asthma 24 h after the last challenge.

2.4. Collection of blood and bronchoalveolar lavage fluid (BALF)

Mice were anesthetized 24 h after the last challenge. Blood was collected by puncture of the vena cava. **Serum** was obtained by centrifugation of samples at 4°C (3000 rpm) for 10 min, and the serum was stored at -80°C for **the** IgE assay. The BALF was collected by cannulating the trachea and **lavaging** with two 0.8 ml aliquots of cold PBS. The samples were collected and immediately centrifuged at 1000 g at 4°C for 10 min. The supernatants were used for subsequent **cytokine measurements**, and the cell pellets were resuspended in PBS and used for total and differential cell counts.

2.5. Lung histopathology

Following BALF and serum collection, the lung tissues were fixed with 10% neutral formalin. The fixed sections were embedded in paraffin, sectioned at a thickness of 4 μm and stained with haematoxylin and eosin (H&E) to examine inflammatory cell infiltration. The inflammation cell infiltration was determined with a 5-point scoring system as previously described (**Duan et al., 2004; Myou et al., 2003**). Briefly, the scoring system was **the following**: grade 0 = no inflammatory cells; **grade 1** = a few inflammatory cells; **grade 2** = a ring of cells 1-2 cell layer deep; **grade 3** = a ring of cells 2- 4 cell layer deep and **grade 4** = a ring of cells > 4 cell layers deep. Periodic acid–Schiff (PAS) staining was used to observe airway goblet cells in the epithelium. The staining methods were scored as follows: 0 = no

goblet cells, 1 = < 25%, 2 = 25 – 50%, 3 = 50 – 75%, and 4 = > 75% goblet cells (Ma et al., 2016). Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Mean scores were assessed from three animals

2.6. Measurement of cytokine levels in BALF and OVA-specific IgE in serum

BALF levels of IL-4, IL-5, and IL-13 were quantified using enzyme-linked immunosorbent assays (ELISA). OVA-specific IgE levels in serum were measured using an enzyme-linked immunosorbent assay antibody. All ELISAs were performed according to the manufacturer's instructions.

2.7. Western blot

Lung tissue was homogenized with a lysis buffer including protease and phosphatase inhibitors and then centrifuged at 3000 g for 10 min at 4°C. The supernatant was collected and the total protein concentration was determined using the BCA protein assay kit. The samples were subjected to 10 % SDS-polyacrylamide gel electrophoresis and were electrotransferred to PVDF membranes.

The blots were incubated with the appropriate concentration of specific antibodies at 4°C overnight. Subsequently, the blots were washed with TBS with Tween (0.1% Tween 20, 100 mM Tris-HCl, and 150 mM NaCl, pH 7.5) followed by incubation with the appropriate secondary antibody conjugated with HRP at room temperature for 1 h. After washing, blots were analyzed by ECL plus Western Blotting Detection System (Amersham Life Science, UK).

2.8. Statistical analysis

The data were analyzed using GraphPad Prism 5 (GraphPad InStat Software, San Diego, CA, USA). Comparison between groups was made with ANOVA followed by Dunnett's test. Data were expressed as mean $\pm$ SEM. Statistical significance was set to $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Osthole inhibited the recruitment of inflammatory cells in BALF

To evaluate the inhibitory effect of osthole on the influx of inflammatory cells, the inflammatory cell counts were analyzed in the BALF of OVA-induced asthma murine models (Fig. 3). As expected, inflammatory cell levels in BALF were markedly increased in the OVA group compared with the control group. Treatment with osthole (25, 50, and 100 mg/kg) and DEX (2 mg/kg) prevented the increase in a dose-dependent manner.

3.2. Osthole reduced the levels of Th2 cytokines in BALF

To evaluate the effects of osthole on OVA-induced Th2 cytokine expression levels in BALF an enzyme-linked immunosorbent assay (ELISA) was performed. Compared with control, OVA challenge led to increases in the levels of IL-4, IL-5, and IL-13 in the BALF. Administration of osthole and DEX dose-dependently suppressed cytokine elevation in comparison to the OVA-challenged group (Fig. 4).

3.3. Osthole reduced the secretion of OVA-specific IgE in serum

An increase in blood IgE production is a primary feature of allergic asthma (Scirica et al., 2007). OVA-specific IgE levels in serum were elevated when measured via ELISA. These data showed that treatment with osthole and DEX dose-dependently suppressed the levels of OVA-specific IgE compared with the OVA group (Fig. 5).

3.4. Osthole ameliorated pathological changes of lung tissue

To directly observe the histological feature of lung tissue, H&E and PAS staining were performed. The lung tissue of OVA-challenged mice showed a massive inflammatory infiltration into the peribronchus, as well as mucus overproduction and goblet cell hyperplasia. However, pretreatment with osthole and DEX led to a reduction of inflammatory

infiltration, and inhibited goblet cell hyperplasia and mucus hypersecretion in a dose-dependent manner in lung tissue (Fig. 6).

5. Osthole suppressed NF- κ B activity in a mouse asthma model

We assessed the effects of osthole on NF- κ B activation by Western blot analysis, as shown in Fig. 7. In the OVA-challenged mice, the level of p-p65 was significantly increased compared with the control mice. In contrast, osthole dose-dependently reduced the level of phosphorylation of NF- κ B p65 in OVA-challenged mice.

2. Discussion

Over the last two decades, the morbidity and mortality of asthma has increased worldwide. New effective therapeutic agents are required to prevent or treat the disease. Here, our data suggest that osthole attenuated allergic inflammation by reducing NF- κ B activation, providing evidence for a potential therapeutic application of osthole in OVA-induced asthma in mice.

There is a long history of using traditional Chinese herbs to treat various diseases. *Cnidii monnieri* fructus, the dried fruit of *Cnidium monnieri* Cusson, has been used in traditional Chinese medicine as an external preparation for the treatment of cutaneous pruritus, eczema, and *Trichomonas vaginalis* infection (Ko et al., 1992). Osthole is the major component of *Cnidii monnieri* fructus extract. Pharmacological studies have revealed that osthole has anti-proliferative, anti-hepatitis, anti-inflammatory, and anti-allergic effects. In the present study, we investigated the protective effect of osthole on OVA-induced asthma in mice. Exposure to osthole reduced the infiltration of inflammatory cells into the lung, as well as the levels of OVA-specific IgE and Th2 cytokines in the BALF compared with the OVA-induced mice. Histopathologic analysis revealed that treatment with osthole substantially inhibited eosinophil infiltration into the airway and goblet cell hyperplasia. These findings clearly show

a significant role for treatment with osthole through a reduction in OVA-induced allergic airway inflammation.

Asthma is a complex inflammatory disease of the lung characterized by the recruitment of eosinophils, neutrophils, and lymphocytes to the lung, inflammatory mediator release, mucin hypersecretion, IgE production, and bronchoconstriction. Although asthma is multifactorial in origin, over the last decade it has become widely accepted that asthma is primarily an inflammatory disease. It has recently become appreciated that CD4⁺ TH2 cells play a pivotal role in the pathogenesis of asthma (Wills-Karp, 1999). IL-4, IL-5, and IL-13, which are cytokines produced by TH2 cells, are closely related to IgE production, airway infiltration, eosinophil activation and mucus secretion (Mosmann, 1996; Street and Mosmann, 1991). The blocking of IL-4 decreased serum IgE levels and airway eosinophilia in allergen-sensitized mice (Renz et al., 1995). IL-5 is a key cytokine for promoting proliferation and influx of eosinophils (Barnes, 2008; Kay, 2006). IL-13 potently induces mucus hypersecretion, airway inflammation, eotaxin expression, and AHR (Li et al., 1999). The present results clearly demonstrate that osthole strikingly reduced the levels of IL-4, IL-5, and IL-13 in a dose-dependent manner.

NF- κ B is an important participant in a broad spectrum of inflammatory signaling and plays a pivotal role in regulating the expression of genes for many inflammatory cytokines, including IL-4, IL-5 and IL-13 (Das et al., 2001). Several lines of evidence have indicated that allergic inflammation is modulated by NF- κ B activation (Alcorn et al., 2010). As in asthma, higher levels of activated NF- κ B are observed in the bronchial biopsies and inflammatory cells of COPD individuals (Caramori et al., 2003; Di Stefano et al., 2002). Additionally, neutrophils in the sputum of COPD donors show increased NF- κ B signaling following exposure to cigarette smoke (CS) extract. Activated NF- κ B induces transcription of

a variety of inflammatory genes that are abnormally expressed in asthma models (Barnes and Adcock, 1997). Our results **show** that osthole inhibited the activation of NF- κ B, thus preventing OVA-induced translocation of transcription factors into the nucleus. In addition, osthole suppressed the expression of inflammatory cytokines, in a process mediated by NF- κ B. This finding **suggests** that **osthole-mediated anti-inflammatory effects** against OVA-induced asthma may be associated with inhibition of the NF- κ B signaling pathway, which may explain the therapeutic **effects** of osthole.

3. Conclusion

In summary, our data **demonstrate** that osthole effectively suppressed airway inflammation, the release of Th2 **cytokines** and OVA-specific IgE, the recruitment of eosinophils and mucus overproduction in a mouse model of OVA-induced asthma. Moreover, osthole inhibited NF- κ B activity. Our findings thus support the possible use of osthole as a therapeutic drug for patients with allergic asthma.

Conflict of interest

All authors declare that they have no conflict of interest.

Acknowledgments

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Figure Legends

Fig. 1. Chemical structure of osthole.

Fig. 2. Experimental protocol of OVA challenge in mice as described in the materials and methods section.

Fig. 3. Effect of osthole on recruitment of inflammatory cells in BALF. 24 h after the last OVA-challenge. The total cell counts and differential cell counts were evaluated. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

Fig. 4. Effect of osthole on the levels of IL-4, IL-5 and IL-13 in BALF. 24 h after the last challenge BALF were collected and the levels of these cytokines were quantified by ELISA.

All values were given as mean $\pm$ SEM. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs.

OVA-challenged group.

Fig. 5. Effect of osthole on OVA-specific IgE levels in serum. 24 h after the last challenge serum was collected in mice. The levels of OVA-specific IgE were evaluated by ELISA. All values were given as mean $\pm$ SEM. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

Fig. 6. Effect of osthole on histopathologic changes in the lung tissues of OVA-challenged allergic mice. (A) Inflammation was evaluated in the lung tissues by analyzing inflammatory cell infiltration using H&E and goblet cell hyperplasia using PAS staining. A, PBS-challenged mice; B, OVA-challenged mice; C, OVA-challenged mice treated with osthole (25 mg/kg); D, OVA-challenged mice treated with osthole (50 mg/kg); E, OVA-challenged mice treated with osthole (100 mg/kg); F, OVA-challenged mice treated with DEX (2 mg/kg). H&E sections and PAS staining sections are magnified 400 $\times$.

(B) Areas of cellular infiltration in lung tissues were evaluated. (C) The percentage of goblet cells per bronchiole. The values were expressed as mean $\pm$ SEM. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.

Fig. 7. Effect of osthole on NF- κ B activation in OVA-induced mice asthma. Lung

homogenates were prepared 24 h after the last OVA challenge. Protein samples were assessed by western blot. β -actin was used as internal controls. Density ratio vs. β -actin was used as a control. The values were expressed as mean $\pm$ SEM. # $p < 0.05$ vs. control; * $p < 0.05$, ** $p < 0.01$ vs. OVA-challenged group.