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Osthole inhibits ovalbumin (OVA) induced asthma through regulating TLR9/JNK pathway

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Osthole is an active coumarin extracted from *Cnidium monnieri* (L.) Cusson, a traditional Chinese herb which possesses multiple pharmacological activities. Asthma is a long term disease characterized by chronic airway inflammation and hyper-responsiveness. Worldwide, around 339 million people are reported to be suffering from asthma. Coumarin osthole has been known to exhibitanti-inflammatory and antiallergic activities. Here, we investigated mechanisms underlying the protective effects of osthole in ovalbumin (OVA) induced asthmatic mice. A total 40 C57BL/6 male mice were used as experimental model animals. The results showed that osthole treatment significantly reduces OVA-induced elevated levels of serum IgE and inflammatory cytokines (IL-4, IL-5, IL-6, IL-13) except IL-10 in bronchoalveolar lavage fluid (BALF), and decreased the recruitment of inflammatory cells in BALF. The CCK-8 assay was performed to examine the viability of cells. The enzyme linked immunosorbent assay (ELISA) was used to determine the expression of inflammatory factors. Western blot analysis was conducted to examine TLR9 and JNK protein expression. Results showed that osthole significantly inhibits the recruitment of inflammatory cells and Th1/Th2 cytokines expression in BALF and airway hyper-responsiveness. Furthermore, osthole attenuated upregulation of systemic immunoglobulin production derived from OVA sensitization. Results also suggest that osthole can reverse the imbalance of Thlfrh2 in asthma, thus alleviating the symptoms of asthma to a certain extent. In conclusion, the study demonstrated that osthole, activecoumarin extract of *Cnidium monnieri*, attenuated the allergic airway inflammation *via* inhibition of TLR9/JNK pathway in mice model indicating that it could be a potential therapeutic target for asthma.

Keywords: Airway hyper-responsiveness, Antiasthmatic, BALF, *Cnidium monnieri*, Chinese herb, Monnier's snowparsley, She Chuang Zi, Th1/Th2 cytokines, Toll-like receptor 9

Asthma is a chronic heterogeneous inflammatory disease caused by genetic and environmental factors, characterized by varying degrees of airway spasm and hyper-responsiveness, mucus secretion and chronic inflammation[1]. Among them, chronic airway inflammation is an important direct cause of repeated exacerbation and airway remodeling[2]. According to the World Health Organization (WHO) report 2018, asthma is estimated to affect 339 million people of all ages worldwide. Globally, asthma is ranked 16th among the leading causes of years lived with disability and 28th among the leading causes of burden of disease, as measured by disability adjusted life years[3].

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At present, the treatment of asthma by traditional Chinese medicine is generally divided into acute attack stage and chronic remission stage[4]. In traditional Chinese medicine, decoction of *Cnidium Monnieri* (commonly called Monnier's snowparsley, and locally She Chuang Zi), thecoumarin osthole being its main component, can effectively treat and control the acute attack of asthma, improve the lung function of patients, reduce airway resistance, and regulate the immune dysfunction of the body[5]. The mechanism of action of traditional Chinese medicine (osthole) needs to be understood for developing effective drugs to control airway inflammation, to improve the quality of life and prognosis of patients with bronchial asthma, and to reduce the proportion of patients with severe asthma and refractory asthma[6].

Osthole exhibits anti-asthmatic activity and prevents airway inflammation by producing Th2 cytokines, IgE, recruitment of eosinophils and mucus overproduction[6]. Studies indicate that osthole has noticeable protective effect on histamine and acetylcholine-induced drug-induced asthma, and directly expands isolated bronchial smooth muscle[7]. Although the mortality rate of severe asthma has declined with regular use of corticosteroids (ICS) based inhalers. However, the impact of asthma is still enormous and its incidence has gradually increased in recent years[8].

Heterogeneous chronic inflammatory asthma has multiple phenotypes. Allergic asthma is a type of T helper 2 (Th2)-associated asthma and responses to it are characterized by the involvement of allergen-specific immunoglobulin E (IgE) and Th2 cells[9]. The balance of T helper cell Th1/Th2 is closely related to the prognosis of asthma[10]. Reports suggest that Th1/Th2 imbalance is the immunological mechanism of bronchial asthma[11]. During the course of asthma, the synthesis of cytokines, such as interleukin 4 (IL-4), IL-5, IL-6 and IL-13 in Th2 cells increase, the expression of cytokines (such as IL-12, IFN-gamma) in Th1 cells decreases, and specific IgE increases resulting in airway inflammation, eosinophil proliferation, and immune activation jointly leading to the development of chronic airway inflammation[12,13]. Moreover, chronic inflammation induces mast cells, eosinophils and neutrophils which in turn lead to airway epithelial injury, smooth muscle hypertrophy, goblet cell metaplasia and mucous gland hyperplasia, all of which are important characteristics of bronchial asthma[14].

Sanchez-Zauco *et al*.[15] have reported that significant decrease of Toll-Like Receptor 9 (TLR9) expression may imbalance Th1/Th2 ratio, thereby could be used effectively for treating asthma. TLR9 plays an important role in the inflammatory and immune response, cell signal transduction in asthma, and is closely related to airway inflammation and airway remodeling in asthma[16]. TLR-9 signaling has been implicated as a critical component of the inflammatory response following lung injury by Suresh *et al*.[17]. In addition, it has been reported that JNK signaling pathway not only increases the number of eosinophils and lymphocytes, but, also promotes airway remodeling by stimulating inflammatory mediators in airway smooth muscle cells and altering inflammation of airway sub-mucosal airway cells[18,19].

The active ingredients of *C. monnieri* mainly contain compounds which are called total coumarins[20]. There are six monomers isolated from *C. monnieri*[7]. Among them, osthole content is the highest accounting for about 60% of the total coumarin[21]. Previous studies have confirmed that osthole has significant effects on vasodilation, sedation, analgesia, antagonism, anticancer and allergic reaction[22]. Whether osthole has anti-asthmatic and anti-inflammatory effects and its underlying mechanisms are not yet fully understood.

In this study, we investigated the effects of osthole on expression of Th1/Th2-related inflammatory factors in OVA-induced bronchial asthma in mice. In addition, we also studied the effect of osthole on airway resistance of asthma, and further explored its possible mechanism to provide theoretical basis for the treatment of asthma by osthole.

Materials and Methods

Animals

Total 40 C57BL/6 male mice procured from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China) were used in the study. Mice were housed for 3 days to adapt themselves to the environment and then divided into four groups: Control group, OVA group (25 μg/mouse), OVA + Osthole (5 mg/kg) group and dexamethasone group (DEX, 1.5 mg/kg). The study was approved by the Animal Experiments Committee of Yancheng TCM Hospital affiliated to Nanjing University of Chinese Medicine, Nanjing, China. Osthole, dexamethasone and ovalbumin (grade V) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

Cell culture and treatment

The bronchial epithelial cell line (BEAS-2B cells) was incubated with serial concentrations of osthole (2-80 mg/kg) for 24 h. Then, the treated cells were tested for the cell viability.

Cell counting Kit-8 (CCK-8) assay

Volume of 100uL cell suspension was added to 96-well plate for 24 h with 5% CO_2 at 37°C. The osthole was then administered at different concentrations for 24 h. Then, 10uL of CCK-8 solution was added to each well and incubated in incubator for 2 h. Absorbance was taken at 450 nm by enzyme marker.

ELISA assay

Bronchoalveolar Lavage Fluid (BALF) was collected from different mice groups. After collection,

the supernatant in each group was harvested at 48 h and stored at −80°C until it was used for cytokine measurements. The total serum IgE, IgG1 and IgG2a levels were measured by ELISA kits (Bioscience, CA, USA) in line with the manufacturer's instructions[23].

Cell flow cytometry

The cells purified from lungs of four mice groups were resuspended in staining buffer and was analyzed by cell flow cytometry to determine the number and proportion of different inflammatory cells. Flow cytometry acquisition was performed using a FACS-Calibur (BD Bioscience, CA, USA), and the results were analyzed using CellQuest software (BD Bioscience, CA, USA).

Western blot analysis

The proteins of different mice groups were extracted and detected, then boiled for 10 minutes. The proteins were separated by electrophoresis and transmembraned, and then incubated overnight with primary antibody of TLR9 (Abcam, CA, USA) and P-JNK (Abcam, CA, USA) at 4°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as internal reference (Abcam, CA, USA). Then, nitrocellulose membranes were incubated with second antibody for 2h and washed with TBST thrice, 20 min each time. The bands were detected by chemi-luminescence method (Thermo Scientific, USA).

Statistical analysis

Statistical software platform SPSS 17.0 (SPSS, USA) was used for statistical analysis. The data were analyzed as mean ± SEM. Statistical differences were determined by Student's t-test and one-way ANOVA. The $P<0.05$ was considered statistically significant.

Results and Discussion

Selection of suitable concentration of osthole

Osthole (7-methoxy-8 [3-methylpent 2-enyl] coumarin) is an active coumarin extracted from the dried fruits of the king of traditional Chinese medicine, the *C. monnieri*. The chemical structure of osthole is shown in Fig. 1A. To investigate the optimum concentration of osthole for activity, BEAS-2B cell were incubated with serial concentrations of osthole (2-80 mg/kg), and the result demonstrated that there was no visible toxicity until the concentration of osthole exceeded 50 mg/kg (Fig. 1B). Hence, 50 mg/kg osthole was selected as final concentration for further research. Another study also used three different concentrations of osthole (25, 50 and 100 mg/kg) for evaluating its effect on influx of inflammatory cells in BALF of OVA-induced asthma murine models[24].

Osthole inhibited recruitment of inflammatory cells in BALF and airway hyper-responsiveness

To assess the inhibitory activity of osthole on inflammation and airway hyper-responsiveness induced by OVA, the numbers of total inflammatory cells, macrophage, eosinophil, neutrophil and lymphocyte collected from bronchoalveolar lavage fluid (BALF) in OVA-induced asthma murine models given different treatments were monitored. There was a significant difference between control and OVA-challenged group in total inflammatory cells, macrophage, eosinophil, neutrophil and lymphocyte (Fig. 2A). Moreover, OVA-induced increase of total inflammatory cells, macrophage, eosinophil, neutrophil and lymphocyte were all dramatically reduced by osthole at a concentration of 5 mg/kg body wt. or DEX (1.5 mg/kg) treatment. In this study, such OVA-triggered elevation was evidently attenuated by oral administration of osthole. In a similar study, it was found that treatment of osthole (25, 50 and 100 mg/kg) and DEX (2 mg/kg) markedly prevented the increase in inflammatory cells in BALF in a dose-dependent manner[24]. Another study also showed that oral treatment of mice with osthole at medium (25 mg/mL) and high doses (50 mg/mL) markedly reduced the infiltration of these inflammatory cells, particularly eosinophils[9].

The airway hyper-responsiveness represented by airway resistance (R_L) was analyzed in OVA-induced asthmatic mice under osthole or DEX treatment to evaluate its triggered anti-allergic effects on asthma. Results indicated that after one exposure to a series of

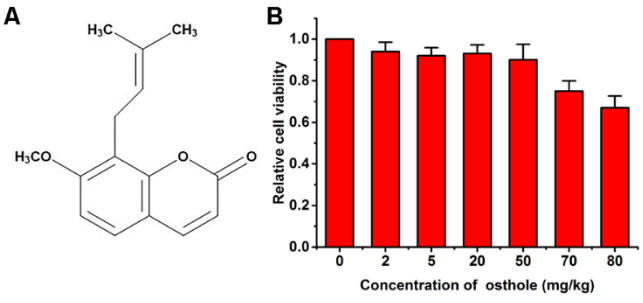

Fig. 1 — Selection of suitable concentration of osthole (A) The chemical structure of osthole; and (B) CCK-8 was used to detect the cell viability of BEAS-2B cell treated with various concentrations of osthole (2-80 mg/kg) for 24 h. [Each experiment were conducted in triplicates]

osthole concentration gradients (1-15 mg/mL), the relative R_L was evidently enhanced in OVA-induced group (Fig. 2B).

Asthma is an inflammatory disease regulated by a variety of cytokines, with eosinophils, lymphocytes and mast cells infiltrating mainly, and many kinds of cells participating in it[25]. Asthma is characterized by infiltration of inflammatory cells in the airway, exfoliation of airway epithelium and airway hyper-responsiveness[26]. The early pathological changes of asthma are mainly small airway inflammation. With the progression of asthma, long-term airway inflammation can lead to airway remodeling[27]. With the deepening understanding of asthma, the focus of treatment has shifted from simply relieving symptoms of airway smooth muscle spasm to comprehensive treatment based on asthma education and prevention and treatment of airway inflammation[28].

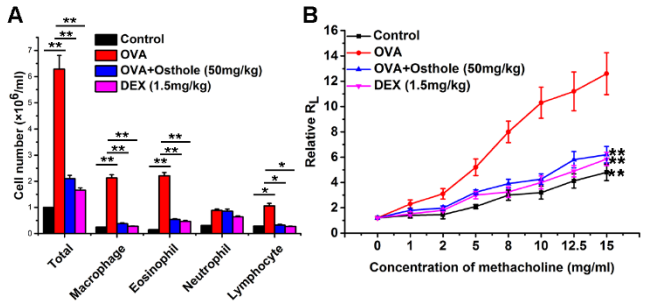

Fig. 2 — Effects of osthole on inflammation and airway hyper-responsiveness. (A) Number of total inflammatory cells, macrophage, eosinophil, neutrophil and lymphocyte collected from BALF in mice given different treatments; and (B) The airway resistance (R_L) of mice under different treatments upon exposure to stimulation of serial concentrations of osthole (1-15 mg/mL). [Each experiment was conducted in triplicates, **P*<0.05, ***P*<0.01]

Osthole suppress Th1/Th2 cytokines expression in BALF of OVA-induced asthma murine model

Imbalanced expression of Th1/Th2-related inflammatory factors, airway hyper responsiveness and changes in the number of airway inflammatory cells are consistent indicators of asthma attack and severity[10], and the imbalance in Th1/Th2 cells are involved in the pathogenesis of asthma[29]. Hence, to verify the therapeutic efficacy of osthole as a new strategy in the treatment of asthma, the relevant Th1/Th2 cytokines in the BALF of mice treated by varied strategies were analyzed by ELISA. Consequently, the results demonstrated that the levels of interleukin IL-4 (Fig. 3A), IL-5 (Fig. 3B), IL-6 (Fig. 3C), IL-10 (Fig. 3D), IL-13 (Fig. 3E) and eosinophil-specific chemokines eotaxin (Fig. 3F) were all distinctly increased via OVA-challenged mice as compared to the control group, while such upregulation was effectively attenuated by osthole or DEX treatment except for IL-10. As illustrated in Fig. 3D, the level of IL-10 in the BALF was

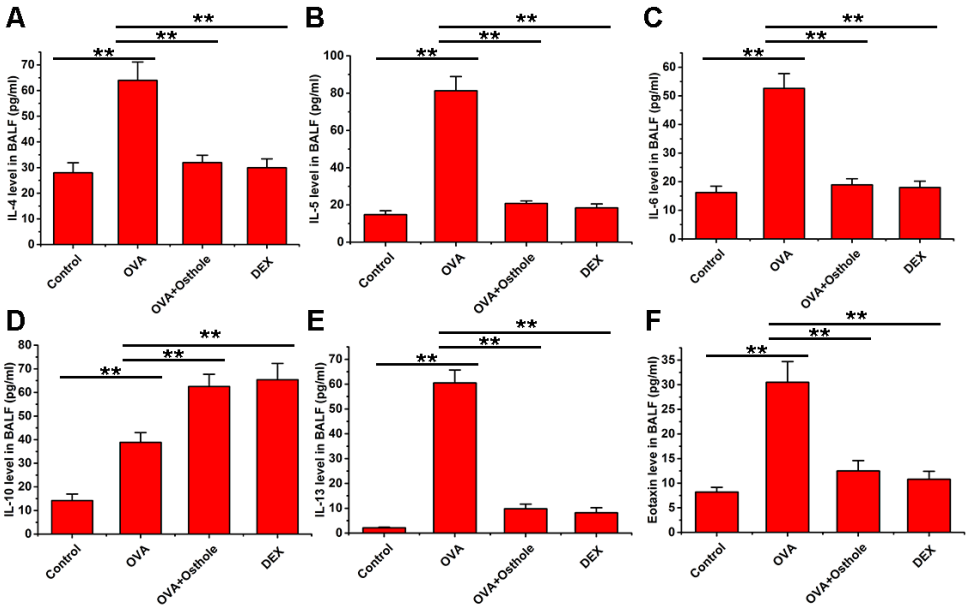

Fig. 3 — Osthole inhibits Th1/Th2 cytokines expression in BALF of OVA-induced asthma murine model: The levels of Th1/Th2 cytokines, including (A-E) IL-4, IL-5, IL-6, IL-10 and IL-13, respectively and eotaxin were all measured in the BALF of mice under described treatments by ELISA. [Each experiment was conducted three times independently, **P*<0.05, ***P*<0.01]

enhanced to a significant extent upon exposure to osthole or DEX treatment. Taken together, all these results indicated that osthole exert significant inhibition on Th1/Th2 cytokines expression in BALF of OVA-induced asthma murine model, thereby conferring its potential as new strategy to treat asthma. Similarly, administration of osthole and DEX dose-dependently suppressed the cytokine (IL-4, IL-5, and IL-13) elevation compared to the OVA-challenged group[24]. Chiang *et al*.[9] showed that oral administration of osthole to BALB/c mice sensitization with OVA suppressed the production of Th2-type cytokines including IL-4, IL-5 and IL-13. On contrary, IL-10 production was not inhibited and was even enhanced by osthole treatment.

In this study, it was found that osthole significantly inhibited recruitment of inflammatory cells and Th1/Th2 cytokines expression (IL-4, IL-5, IL-6, IL-13) in BALF and airway hyper-responsiveness. Data suggest that osthole can reverse the imbalance of Thl/Th2 in asthma, thus alleviating the symptoms of asthma to a certain extent.

Osthole attenuates upregulation of systemic immunoglobulin production derived from OVA sensitization

The high expression of systemic immunoglobulins, especially IgE and IgG1, is recognized as a primary feature of OVA-induced asthma. Therefore, to further validate the function of osthole in asthmatic inflammatory responses, the OVA-specific IgE and IgG1 levels in serum were analyzed. Another study also found that osthole and DEX treatment suppressed the levels of OVA-specific IgE in a dose dependent manner[24]. Results depicted that there was a measurable downward tendency of OVA-specific IgE and IgG1 levels upon exposure to osthole or DEX administration, which implied that osthole performance in improving OVA-induced asthmatic inflammation (Fig. 4). Furthermore, osthole attenuates up-regulation of systemic immunoglobulin production derived from OVA sensitization.

Osthole inhibits allergic airway injury through TLR9/JNK pathway

Although results of the present study demonstrated the therapeutic efficacy of osthole in asthma, however, the underlying mechanism is still not clear. It is also known that the Toll-like receptor 9 (TLR9) plays a pivotal role in the treatment of asthma due to its regulation of imbalance of Th1/Th2. Therefore, to validate the function of TLR9 pathway in the activity of osthole, the protein levels of TLR9 and p-Jun N-terminal kinase (JNK) were detected by western blot assays. Compared with control group, both TLR9 and p-JNK expression were upregulated via OVA sensitization (Fig. 5). Results indicated that the protein levels of TLR9 and p-JNK were significantly reduced by osthole or DEX administration. Thus, osthole can regulate TLR9/JNK pathway, preliminarily revealing

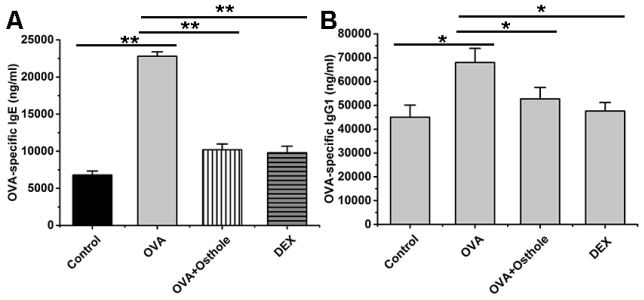

Fig. 4 — Osthole inhibits increased systemic immunoglobulin production caused by OVA sensitization. (A) The serum levels of OVA-specific IgE (A) and IgG1; and (B) in OVA-induced asthmatic murine model under described therapeutic treatments were detected by ELISA. [All the data are presented as mean value± SD. Each experiment was conducted three times independently, **P*<0.05, ***P*<0.01]

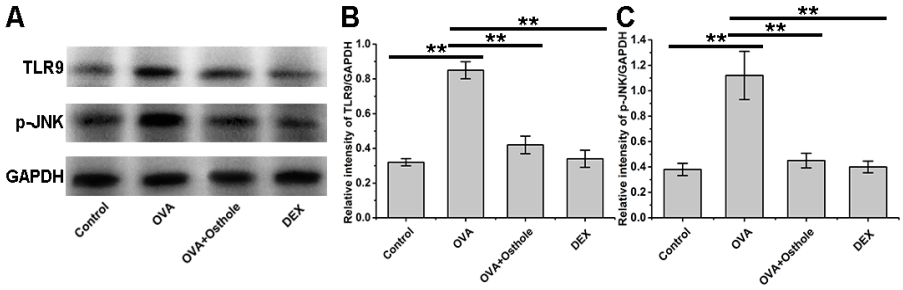

Fig. 5— Osthole in inhibits allergic airway inflammation via regulating TLR9/JNK pathway.(A) The protein levels of TLR9 and p-JNK in airway epithelial cells of mice under various approaches; and Quantification of grey levels of (B) TLR9; and (C) p-JNK in airway epithelial cells treated as depicted. [All the data are presented as mean value± SD. Each experiment was conducted three times independently, ***P*<0.01]

the therapeutic potential of *Cnidium monnieri* in treating the asthma.

Conclusion

Results of this study have confirm that osthole, the coumarin extract of the traditional Chinese herb *Cnidium monnieri*, locally called She chuang zi, alleviated airway resistance, regulate the expression of Th1/Th2-related inflammatory factors and reversed the imbalance of Th1/Th2 in asthmatic mice. Simultaneously, osthole reduced the content of eosinophils and macrophages, improved the inflammatory infiltration in lung tissue, thereby alleviating the asthmatic symptoms. Inhibition of TLR9/JNK pathway by osthole as observed here thus supports possible use of osthole as a therapeutic drug for patients with allergic asthma.

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Conflict of interest

The authors declare that they have no potential conflict of interests.

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