



Pulmonary, gastrointestinal and urogenital pharmacology

Protective effect of forsythiaside A on OVA-induced asthma in mice

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ABSTRACT

Forsythiaside A (FSA), an active constituent isolated from the Chinese medicinal herb *Forsythia suspensa*, has been known to have anti-inflammatory effect. However, the effect of FSA on allergic airway inflammation remains unclear. The aim of this study was to investigate the effect of FSA on OVA-induced asthma in mice. Mice model of asthma was induced by OVA. OVA-induced airway hyperresponsiveness (AHR) and inflammatory cells in BALF were detected. The production of IgE, IL-4, IL-5, IFN-γ, and IL-13 were detected by ELISA. The effects of FSA on Nrf2 and NF-κB signaling pathways were detected by western blot analysis. The results showed that treatment of FSA significantly attenuated OVA-induced lung histopathological changes. FSA inhibited OVA-induced AHR and inflammatory cells in BALF. OVA-induced IgE, IL-4, IL-5, and IL-13 production were also inhibited by FSA. Western blot analysis showed that treatment of FSA inhibited OVA-induced NF-κB activation. Treatment of FSA dose-dependently up-regulated the expression of Nrf2 and HO-1. In addition, we found that FSA up-regulated the expression of Nrf2 and HO-1 both in A549 cells and MS-H cells. Taken together, FSA suppressed inflammatory responses in OVA-induced asthma through activating Nrf2/HO-1 signaling pathway. FSA may be a promising potential preventive agent for asthma treatment.

1. Introduction

Allergic asthma, often caused by allergen inhalation, is a heterogeneous inflammatory disorder characterized by airway hyper-responsiveness (AHR), remodeling, and inflammation (Agrawal and Shao, 2010; Anderson, 2008). Although it is a well-known inflammatory lung disease, the specific underlying mechanism remains unclear. Chronic inflammation has attracted much attention for its contribution to asthma (Barnes, 2008). Now, asthma treatment is usually dependent on the use of corticosteroids (Barnes and Pedersen, 1993). However, it often suffers from a series of adverse effects, such as suppression of the hypothalamic-pituitary axis, reduced bone growth, and increased risk of opportunistic infections (Agertoft and Pedersen, 1994; Buchman, 2001). Thus, there is an urgent need to seek safe and effective therapeutic drugs for the treatment of asthma. Based on previous studies, remedies with anti-inflammatory properties based on modification of NF-κB signaling pathway may represent a potentially useful alternative (Bureau et al., 2000; Edwards et al., 2009).

Forsythiaside A (FSA), an active constituent isolated from the Chinese medicinal herb *Forsythia suspensa*, has been reported to have anti-inflammatory effect (Cheng et al., 2014). FSA has been reported to inhibit LPS-induced inflammatory cytokine production in BV2 microglia cells (Wang et al., 2015). FSA also inhibited hydrogen peroxide-induced oxidative stress in PC12 cells (Huang et al., 2015).

Furthermore, FSA has been reported to protect against LPS/D-galactosamine-induced liver injury in mice (Pan et al., 2015). However, it remains unclear whether FSA has a protective effect on lung disease, such as asthma. Here, we established an OVA-induced asthma model to investigate the protective effects of FSA on OVA-induced asthma.

2. Materials and methods

2.1. Animals

BALB/c mice were purchased from the Center of Experimental Animals of Beijing University (Beijing, China). Animals were housed in a temperature-controlled room under a 12-hr light/dark cycle. Food and water were provided *ad libitum*. All the animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health.

2.2. Reagents

FSA (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). OVA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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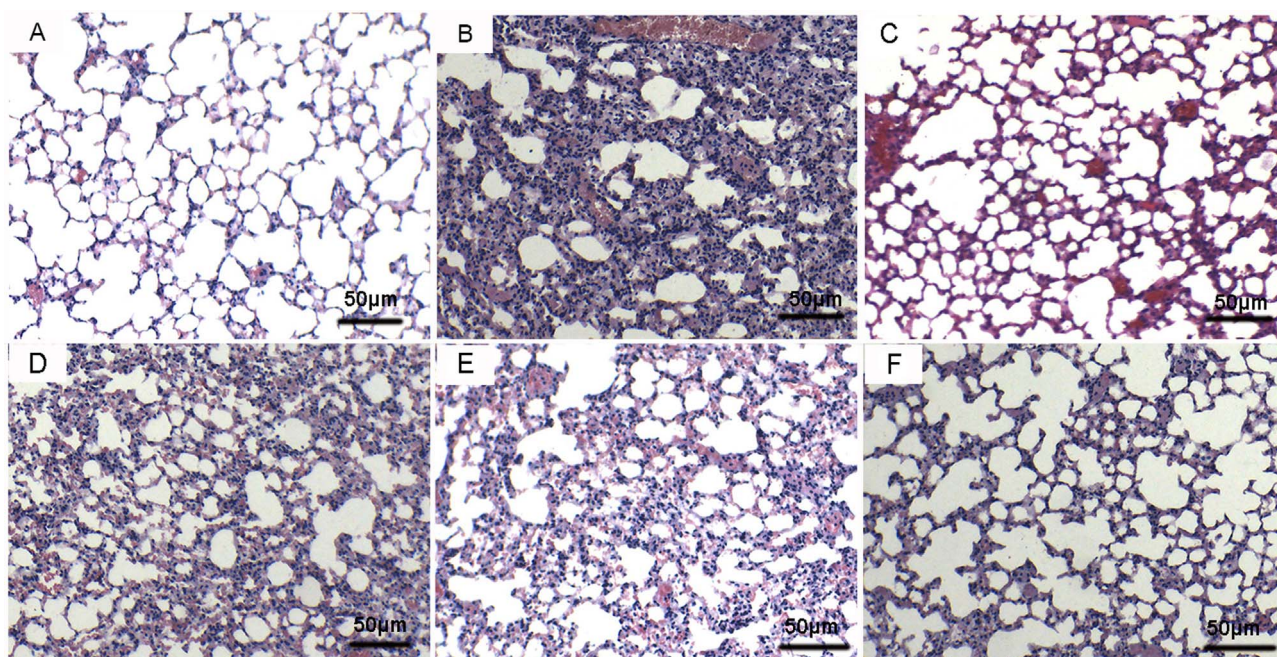


Fig. 1. Effects of FSA on histopathological changes in lung tissues in OVA-induced asthma. Lungs tissues from each experimental group were processed for histological evaluation. Representative histological changes of lung obtained from mice of different groups. A: Control group, B: OVA group, C: OVA + DEX group, D: OVA+ FSA (15 mg/kg) group, E: OVA + FSA (30 mg/kg) group, F: OVA+ FSA (60 mg/kg) group (Hematoxylin and eosin staining, magnification 200 $\times$).

Mouse IgE, IL-4, IL-5, IFN- γ , and IL-13 ELISA kits were purchased from R & D Systems (Minneapolis, MN). Antibodies against Nrf2, HO-1, NF- κ B, I κ B α , and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.3. OVA-induced mice asthma model

The mice were randomly divided into six groups: normal control group, OVA group, OVA + FSA (15, 30, 60 mg/kg) group, and OVA+ Dexamethasone (DEX) group. Mice were sensitized on day 1 and 14 by intraperitoneal injection with 20 μ g OVA with 1 mg aluminum hydroxide adsorbed in 200 μ l PBS. On days 21–23, mice were challenged with 100 μ g OVA adsorbed in 20 μ l PBS by intranasal inhalations. Forsythiaside was dissolved in 50 μ l PBS to the doses of 15, 30 and 60 mg/kg. The control mice were injected the same volume of PBS (50 μ l). FSA was administered by oral gavage 1 h before the OVA treatment on day 21–23. The mice were sacrificed 24 h after the last challenge.

2.4. H & E staining

The lungs were collected and fixed in 4% paraformaldehyde. Then, the tissues were dehydrated with graded alcohol, embedded in paraffin, and sectioned. The sections were stained with H & E staining. Finally, the histopathologic changes of the lung tissues were examined under a light microscopy.

2.5. Cytokine and OVA-specific IgE production

BALF and serum were harvested as described previously. The levels of IL-13, IL-4, IL-5, IFN- γ in BALF, and IgE in serum were detected by ELISA kits (R & D Systems, Minneapolis, MN) following the manufacturer's instructions.

2.6. Collection of BALF and cell counting

24 h after the last OVA challenge, the mice were killed and the lungs were washed twice by intratracheal instillation with cold PBS (0.5 mL)

to collect BALF. The BALF was centrifuged 1000 rpm for 10 min and the supernatants were collected for cytokines detection. The cell pellets were resuspended with 30 μ l cold PBS and the numbers of inflammatory cells were measured with a hemocytometer.

2.7. Evaluation of AHR

Twenty-four hours after the last challenge, mice were anesthetized using ketamine/xylazine. AHR was measured with a whole-body and invasive plethysmography (Buxco Electronics Inc., NY, USA) according to previous studies (HuangFu et al., 2006).

2.8. Cells and treatment

A549 lung epithelial cell line was purchased from the China Cell Line Bank (Beijing, China) and cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 $^{\circ}$ C with 5% CO $_2$. Media was changed once every 48 h. MH-S alveolar macrophage was purchased from the ATCC and cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 $^{\circ}$ C with 5% CO $_2$. Media was changed once every 48 h. A549 cells and MH-S cells were treated with FSA (2.5, 5, 10 μ g/mL) for 12 h. Then, the expression of Nrf2 and HO-1 were detected by western blot analysis.

2.9. Western blot analysis

Total proteins from lung tissues were extracted by using T-PER Tissue Protein Extraction Reagent Kit (Thermo) according to the manufacturer's instructions. The concentration of the protein was determined by BCA method. The protein samples were separated through 12% SDS polyacrylamide gels, then transferred onto PVDF membranes. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies: Nrf2, HO-1, NF- κ B, I κ B α , and β -actin. Then, the membranes were washed three times with TBST and incubated with peroxidase-conjugated secondary antibody. Antibody binding was visualized with the ECL Plus Western Blotting Detection

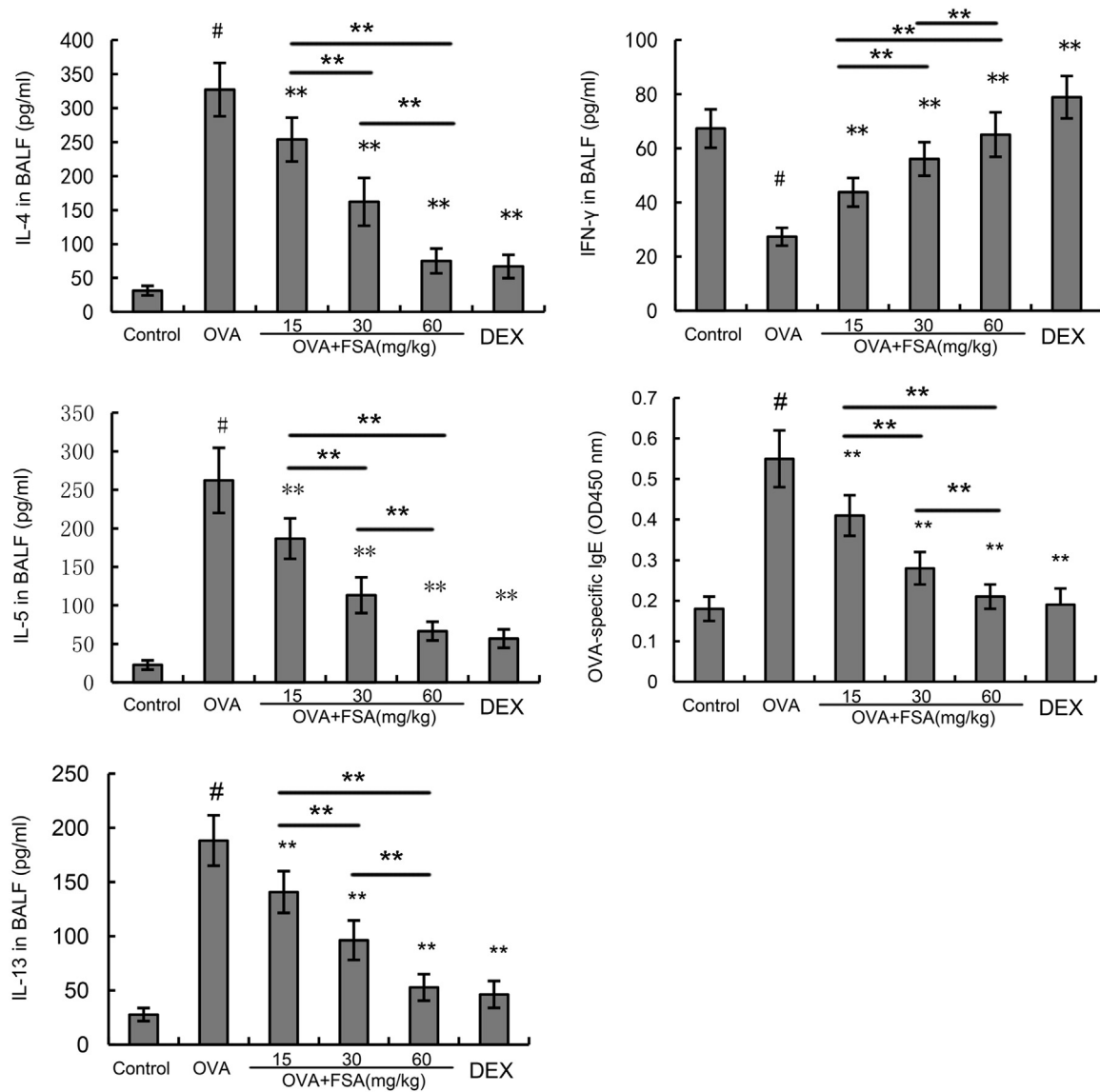


Fig. 2. Effects of FSA on OVA-induced IgE in serum, IL-4, IL-5, IL-13, and IFN-γ productions in BALF were detected by ELISA. The values presented are the means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.05$ vs. control group; ^{**} $p < 0.01$ vs. OVA group.

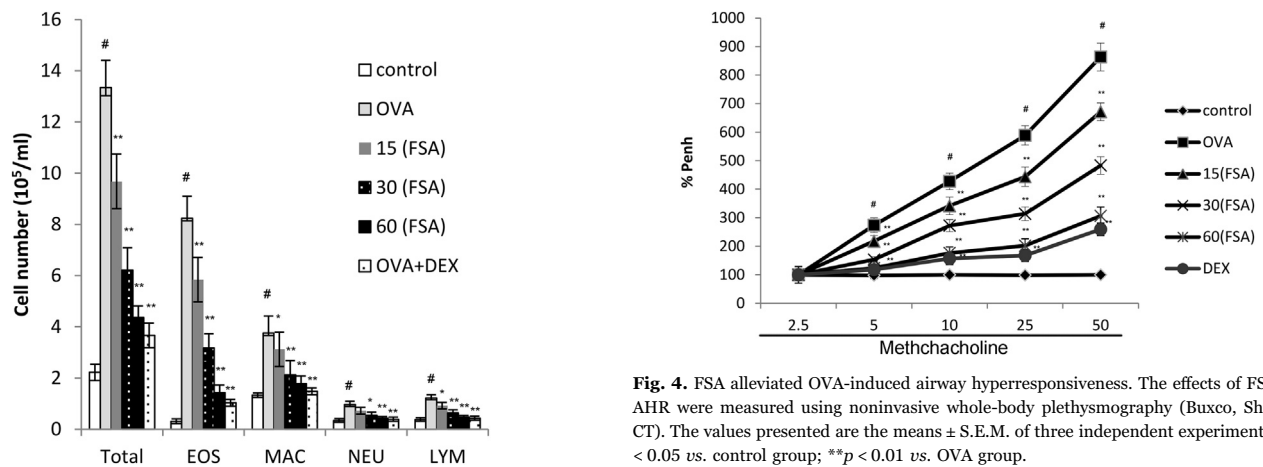


Fig. 3. Effects of FSA on OVA-induced inflammatory cells count in the BALF. FSA inhibited OVA-induced macrophages (MAC), neutrophils (NEU), eosinophils (EOS), and lymphocytes (LYM) infiltration. The values presented are the means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. OVA group.

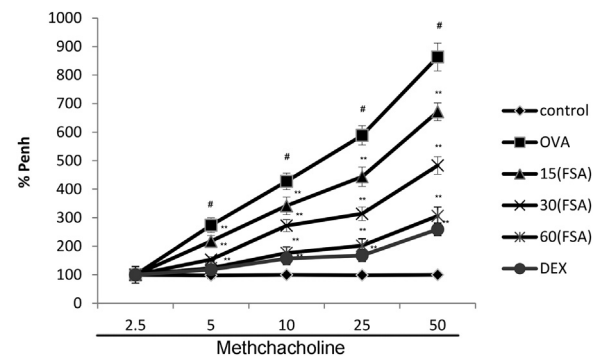


Fig. 4. FSA alleviated OVA-induced airway hyperresponsiveness. The effects of FSA on AHR were measured using noninvasive whole-body plethysmography (Buxco, Sharon, CT). The values presented are the means $\pm$ S.E.M. of three independent experiments. [#] $p < 0.05$ vs. control group; ^{**} $p < 0.01$ vs. OVA group.

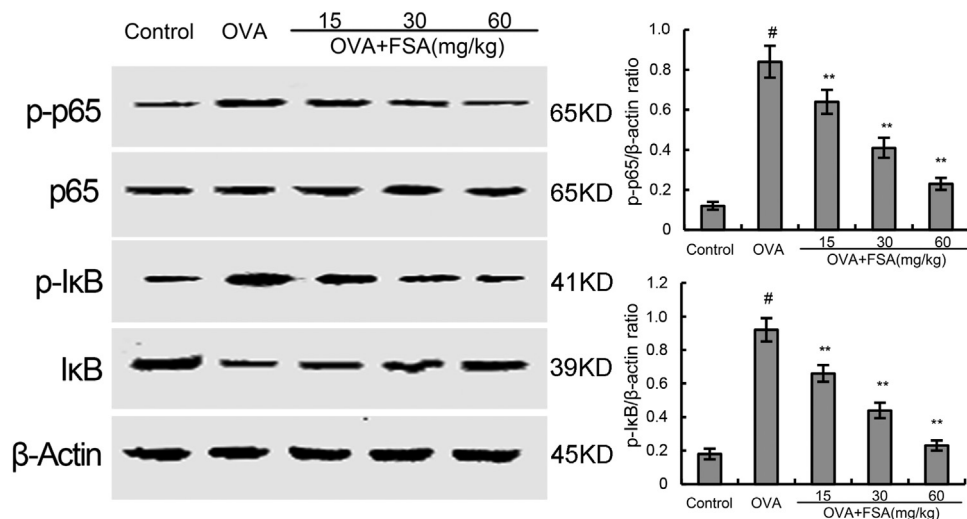


Fig. 5. Effects of FSA on OVA-induced NF-κB activation. The expression of NF-κB and IκBα were detected by western blotting. The values presented are the means ± S.E.M. of three independent experiments. [#]*p* < 0.05 vs. control group; ^{**}*p* < 0.01 vs. OVA group.

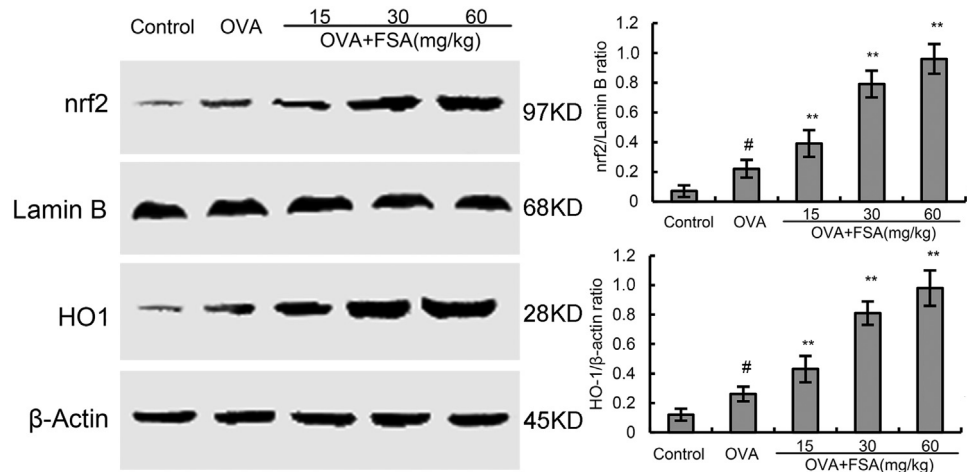


Fig. 6. Effects of FSA on Nrf2 signaling pathway. The expression of Nrf2 and HO-1 were detected by western blotting. The values presented are the means ± S.E.M. of three independent experiments. [#]*p* < 0.05 vs. control group; ^{**}*p* < 0.01 vs. OVA group.

System (GE Healthcare, Chalfont St Giles, UK). Band intensity was quantitated using Image J software.

2.10. Statistical analysis

The experimental data were expressed as the mean ± S.E.M. and analyzed using Student's *t*-test and Mann-Whitney-*U* test. Statistical analyses were performed using the SPSS 19.0 software, and *P* < 0.05 was considered statistically significant.

3. Results

3.1. Effect of FSA on lung histopathological changes

Lung sections were stained with H & E, and the effects of FSA on lung histopathologic changes were detected in this study. As shown in Fig. 1, OVA-challenged mice showed marked inflammatory cell infiltration in the perivascular areas and airway epithelial thickening. However, treatment of FSA or DEX attenuated OVA-induced pathological changes in a dose-dependent manner (Fig. 1).

3.2. Effect of FSA on cytokine production and IgE level in serum

The abnormal Th2 response is the key feature of asthma. In this

study, the results showed that OVA challenge significantly increased the production of Th2 cytokines, including IL-4, IL-5, and IL-13. FSA or DEX treatment significantly inhibited OVA-induced IL-4, IL-5, and IL-13 production (Fig. 2). Compared with the control group, the level of IgE in serum increased markedly in OVA group. However, FSA treatment dose-dependently inhibited OVA-induced IgE production. In addition, OVA challenge significantly decreased the level of IFN-γ. And FSA treatment dose-dependently increased the level of IFN-γ (Fig. 2).

3.3. Effect of FSA on inflammatory cells in BALF

The effects of FSA on inflammatory cells infiltration in BALF were detected in this study. The results showed that the numbers of total cells, macrophages, neutrophils, eosinophils, and lymphocytes increased significantly in OVA group. However, FSA or DEX significantly attenuated OVA-induced inflammatory cells in BALF (Fig. 3).

3.4. Effect of FSA on OVA-induced AHR

AHR was measured by Penh in response to increased doses of methacholine. The results showed that Penh value of OVA-challenged mice was higher than that of the control group. However, a significant reduction of Penh value was observed in mice treated with FSA or DEX (Fig. 4).

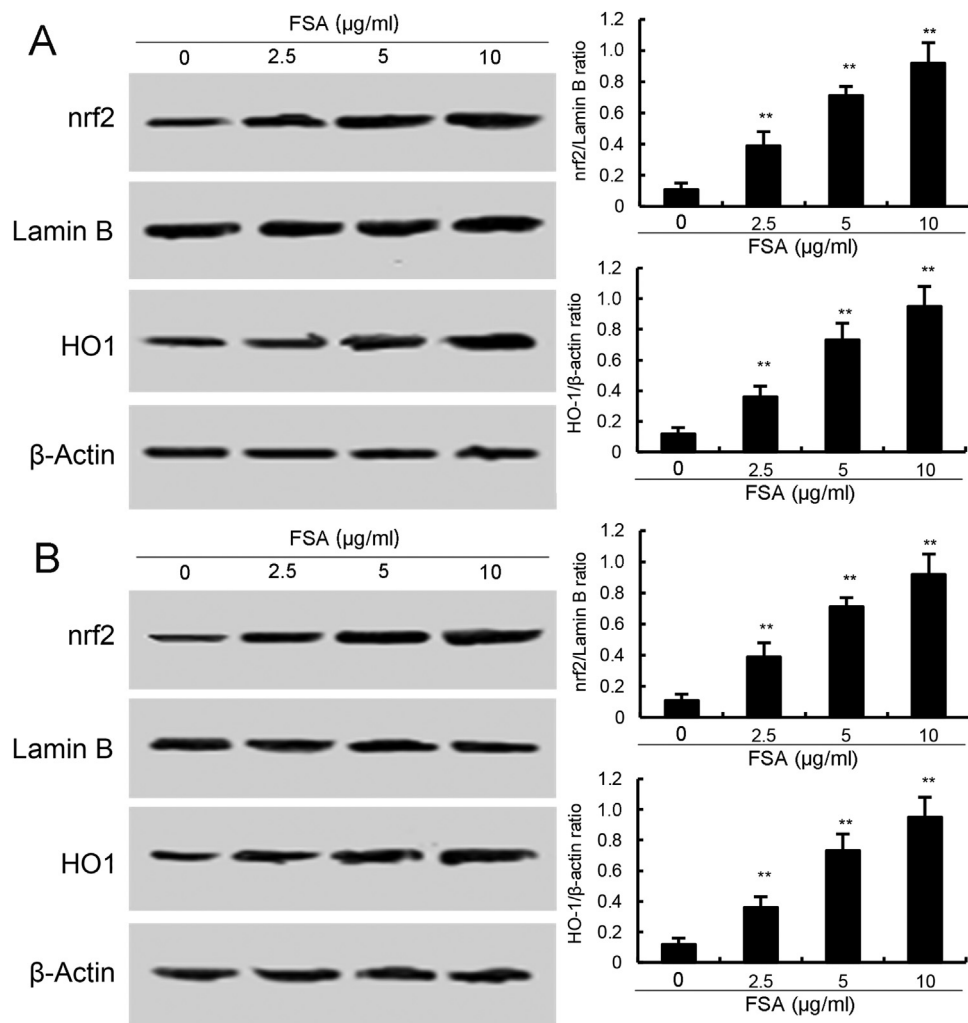


Fig. 7. Effects of FSA on Nrf2 signaling pathway in lung epithelial cells (A) and macrophages (B). The expression of Nrf2 and HO-1 were detected by western blotting. The values presented are the means $\pm$ S.E.M. of three independent experiments. ** $p < 0.01$ vs. control group.

3.5. Effect of FSA on OVA-induced NF- κ B activation

The effects of FSA on NF- κ B activation were detected by western blotting. As shown in Fig. 5, compared with the control group, the levels of phosphorylation of NF- κ B and I κ B α increased markedly in OVA group. However, FSA treatment dose-dependently inhibited OVA-induced NF- κ B activation.

3.6. Effect of FSA on Nrf2 signaling pathway

The effects of FSA on Nrf2 signaling pathway were detected by western blotting. As shown in Fig. 6, the expression of Nrf2 and HO-1 were decreased by OVA challenge. However, FSA significantly increased the expression of Nrf2 and HO-1 in OVA-challenged mice.

3.7. Effects of FSA on Nrf2 signaling pathway in lung epithelial cells and macrophages

To investigate the cellular targets of FSA, the effects of FSA on Nrf2 signaling pathway in A549 cells and MS-H cells were detected by western blotting. As shown in Fig. 7, the expression of Nrf2 and HO-1 were up-regulated by FSA both in A549 cells and MS-H cells.

4. Discussion

Over the last decades, the prevalence of asthma has markedly

increased worldwide (Beasley et al., 2008). Nowadays, corticosteroids are still regarded as the best way to treat asthma. However, corticosteroids have been known to have several adverse effects (Boulet, 1998). Therefore, it is urgent to seek novel therapeutic options that both safe and effective in asthma management. Previous study showed that the LD50 of FSA is 1976.5 mg/kg. Thus, the doses used in this study did not have adverse effects. In this study, our results showed that FSA attenuated OVA-induced allergic airway inflammation by activating Nrf2 signaling pathway.

A large number of studies showed that inflammation played an important role in the development of asthma (Al-Shami et al., 2005; Jember et al., 2001). During the development of asthma, inflammatory cells infiltration were observed in patients of asthma (Fahya et al., 1995). Eosinophils are the major inflammatory cells that contribute to asthma (Minshall et al., 1997). The infiltration of eosinophils caused AHR and airway remodeling (Gleich, 2000). In this study, our results showed that FSA significantly attenuated the infiltration of inflammatory cells and OVA-induced AHR. Th2 cytokine, such as IL-4, IL-5, and IL-13, plays an important role in the development and progression of asthma (Komai et al., 2003). Furthermore, studies showed that the pathogenesis of allergic asthma was mediated by the Th2 immune response (Truyen et al., 2006). In this study, our results showed that the production of IL-4, IL-5, and IL-13 were significantly inhibited by FSA. Taken together, these results indicated that FSA exerted its anti-asthmatic effects *via* down-regulation of Th2 cytokines and eosinophil infiltration.

NF- κ B, a critical transcriptional factor, is considered to play a vital role in inflammatory phenotypic changes in various pathophysiological conditions (Baker et al., 2011). NF- κ B activation has been demonstrated in lung tissues of OVA-challenged mice (Li et al., 2009). Studies showed that inhibition of NF- κ B activation could protect against OVA-induced asthma (Desmet et al., 2004). Therefore, there is growing likelihood that NF- κ B has emerged as a promising molecular target for the treatment of asthma. In this study, we found that treatment of FSA significantly inhibited OVA-induced NF- κ B activation. Nrf2, a critical transcription factor, has been known to regulate detoxifying enzymes (Itoh et al., 2004). Recent studies showed that Nrf2 was involved in the regulation of immune and inflammatory response (Thimmulappa et al., 2006). Previous study showed that there was a link between Nrf2 and NF- κ B signaling pathways and activation of Nrf2 could inhibit NF- κ B activation (Buelna-Chontal and Zazueta, 2013; Sun et al., 2015). Furthermore, activation of Nrf2 could regulate the Th1/Th2 balance in the lung and protect against OVA-induced asthma (Hsu et al., 2012; Kikuchi et al., 2010). To investigate the protective mechanism of FSA, the effects of FSA on Nrf2 signaling pathway were detected in this study. The results showed that FSA dose-dependently up-regulated the expression of Nrf2 and HO-1. The results indicated that FSA protected against OVA-induced asthma by activating Nrf2 signaling pathway. To investigate the cellular targets of FSA, the effects of FSA on Nrf2 signaling pathway in lung epithelial and macrophage cells were detected. Our results showed that FSA exhibited its anti-inflammatory mechanism both in lung epithelial and macrophage cells.

In conclusion, the present findings showed that FSA has a protective effect against OVA-induced asthma. The protective mechanism of FSA is related to the activation of Nrf2 signaling pathway. FSA may be a promising potential preventive agent for asthma treatment.

Conflict of interest

The authors have no conflict of interest to declare.

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References

- Agertoft, L., Pedersen, S., 1994. Effects of long-term treatment with an inhaled corticosteroid on growth and pulmonary function in asthmatic children. *Respir. Med.* 88, 373–381.
- Agrawal, D.K., Shao, Z., 2010. Pathogenesis of allergic airway inflammation. *Curr. Allergy Asthma Rep.* 10, 39–48.
- Al-Shami, A., Spolski, R., Kelly, J., Keane-Myers, A., Leonard, W.J., 2005. A role for TSLP in the development of inflammation in an asthma model. *J. Exp. Med.* 202, 829–839.
- Anderson, G.P., 2008. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 372, 1107–1119.
- Baker, R.G., Hayden, M.S., Ghosh, S., 2011. NF- κ B, inflammation, and metabolic disease. *Cell Metab.* 13, 11–22.
- Barnes, P.J., 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *J. Clin. Invest.* 118, 3546–3556.
- Barnes, P.J., Pedersen, S., 1993. Efficacy and safety of inhaled corticosteroids in asthma. *Am. Rev. Respir. Dis.* 148, S1–S26.
- Beasley, R., Pearce, N., Crane, J., 2008. International trends in asthma. *Rising Trends Asthma* 786, 140.
- Boulet, L.-P., 1998. Perception of the role and potential side effects of inhaled corticosteroids among asthmatic patients. *CHEST J.* 113, 587–592.
- Buchman, A.L., 2001. Side effects of corticosteroid therapy. *J. Clin. Gastroenterol.* 33, 289–294.
- Buelna-Chontal, M., Zazueta, C., 2013. Redox activation of Nrf2 & NF- κ B: a double end sword? *Cell. Signal.* 25, 2548–2557.
- Bureau, F., Delhalle, S., Bonizzi, G., Fiévez, L., Dogné, S., Kirschvink, N., Vanderplassen, A., Merville, M.-P., Bours, V., Lekeux, P., 2000. Mechanisms of persistent NF- κ B activity in the bronchi of an animal model of asthma. *J. Immunol.* 165, 5822–5830.
- Cheng, G., Zhao, Y., Li, H., Wu, Y., Li, X., Han, Q., Dai, C., Li, Y., 2014. Forsythiaside attenuates lipopolysaccharide-induced inflammatory responses in the bursa of Fabricius of chickens by downregulating the NF- κ B signaling pathway. *Exp. Ther. Med.* 7, 179–184.
- Desmet, C., Gosset, P., Pajak, B., Cataldo, D., Bentires-Alj, M., Lekeux, P., Bureau, F., 2004. Selective blockade of NF- κ B activity in airway immune cells inhibits the effector phase of experimental asthma. *J. Immunol.* 173, 5766–5775.
- Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M., Johnston, S.L., 2009. Targeting the NF- κ B pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol. Ther.* 121, 1–13.
- Fahya, J.V., Kimb, K.W., Liub, J., Bousheya, H.A., 1995. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J. Allergy Clin. Immunol.* 95, 843–852.
- Gleich, G.J., 2000. Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* 105, 651–663.
- Hsu, W.-H., Lee, B.-H., Huang, Y.-C., Hsu, Y.-W., Pan, T.-M., 2012. Ankaflavin, a novel Nrf-2 activator for attenuating allergic airway inflammation. *Free Radic. Biol. Med.* 53, 1643–1651.
- Huang, C., Lin, Y., Su, H., Ye, D., 2015. Forsythiaside protects against hydrogen peroxide-induced oxidative stress and apoptosis in PC12 cell. *Neurochem. Res.* 40, 27–35.
- HuangFu, T., Lim, L.H., Chua, K.Y., 2006. Efficacy evaluation of Der p 1 DNA vaccine for allergic asthma in an experimental mouse model. *Vaccine* 24, 4576–4581.
- Itoh, K., Tong, K.I., Yamamoto, M., 2004. Molecular mechanism activating Nrf2–Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic. Biol. Med.* 36, 1208–1213.
- Jember, A.G.-H., Zuberi, R., Liu, F.-T., Croft, M., 2001. Development of allergic inflammation in a murine model of asthma is dependent on the costimulatory receptor OX40. *J. Exp. Med.* 193, 387–392.
- Kikuchi, N., Ishii, Y., Morishima, Y., Yageta, Y., Haraguchi, N., Itoh, K., Yamamoto, M., Hizawa, N., 2010. Nrf2 protects against pulmonary fibrosis by regulating the lung oxidant level and Th1/Th2 balance. *Respir. Res.* 11, 31.
- Komai, M., Tanaka, H., Masuda, T., Nagao, K., Ishizaki, M., Sawada, M., Nagai, H., 2003. Role of Th2 responses in the development of allergen-induced airway remodelling in a murine model of allergic asthma. *Br. J. Pharmacol.* 138, 912–920.
- Li, J., Luo, L., Wang, X., Liao, B., Li, G., 2009. Inhibition of NF- κ B expression and allergen-induced airway inflammation in a mouse allergic asthma model by andrographolide. *Cell. Mol. Immunol.* 6, 381.
- Minshall, E.M., Leung, D.Y., Martin, R.J., Song, Y.L., Cameron, L., Ernst, P., Hamid, Q., 1997. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 17, 326–333.
- Pan, C.-w., Zhou, G.-y., Chen, W.-l., Zhuge, L., Jin, L.-x., Zheng, Y., Lin, W., Pan, Z.-z., 2015. Protective effect of forsythiaside A on lipopolysaccharide/d-galactosamine-induced liver injury. *Int. Immunopharmacol.* 26, 80–85.
- Sun, C.C., Li, S.J., Yang, C.L., Xue, R.L., Xi, Y.Y., Wang, L., Zhao, Q.L., Li, D.J., 2015. Sulforaphane attenuates muscle inflammation in Dystrophin-deficient mdx Mice via NF-E2-related factor 2 (Nrf2)-mediated inhibition of NF- κ B signaling pathway. *J. Biol. Chem.* 290, 17784–17795.
- Thimmulappa, R.K., Lee, H., Rangasamy, T., Reddy, S.P., Yamamoto, M., Kensler, T.W., Biswal, S., 2006. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J. Clin. Invest.* 116, 984–995.
- Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L.J., Ceuppens, J.L., Bullens, D.M., 2006. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 61, 202–208.
- Wang, Y., Zhao, H., Lin, C., Ren, J., Zhang, S., 2015. Forsythiaside A exhibits anti-inflammatory effects in LPS-stimulated BV2 microglia cells through activation of Nrf2/HO-1 signaling pathway. *Neurochem. Res.* 1–7.