



Contents lists available at ScienceDirect

International Immunopharmacology

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

Inhibition of airway inflammation, hyperresponsiveness and remodeling by soy isoflavone in a murine model of allergic asthma

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ARTICLE INFO

Article history:

Received 2 October 2010

Received in revised form 9 November 2010

Accepted 1 February 2011

Available online xxxx

Keywords:

Soy isoflavone

Asthma

Airway hyperresponsiveness

Inflammation

Remodeling

ABSTRACT

Epidemiologic studies have associated higher dietary consumption of soy isoflavones with decreased self-report of cough and allergic respiratory symptoms, but the pharmacodynamic effects of soy isoflavone on asthmatic model have not been well-described. Here, we hypothesized that soy isoflavone may have potential effects on airway hyperresponsiveness, inflammation and airway remodeling in a murine of asthma. Mice sensitized and challenged with ovalbumin developed airway inflammation. Bronchoalveolar lavage fluid was assessed for inflammatory cell counts, and for cytokine levels. Lung tissues were examined for cell infiltration, mucus hypersecretion and airway remodeling, and for the expression of inflammatory biomarkers. Airway hyperresponsiveness was monitored by direct airway resistance analysis. Oral administration of soy isoflavone significantly reduced ovalbumin-induced airway hyperresponsiveness to intravenous methacholine, and inhibited ovalbumin-induced increases in eosinophil counts. RT-PCR analysis of whole lung lysates revealed that soy isoflavone markedly suppressed ovalbumin-induced mRNA expression of eotaxin, interleukin(IL)-5, IL-4 and matrix metalloproteinase-9, and increased mRNA expression of interferon (IFN)- γ and tissue inhibitor of metalloproteinase-1 in a dose-dependent manner. Soy isoflavone also substantially recovered IFN- γ /IL-4 (Th1/Th2) levels in bronchoalveolar lavage fluid. In addition, histologic studies showed that soy isoflavone dramatically inhibited ovalbumin-induced lung tissue eosinophil infiltration, airway mucus production and collagen deposition in lung tissues. Our findings suggest that soy isoflavone as nutritional supplement may provide a novel means for the treatment of airway inflammatory disease.

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1. Introduction

Isoflavonoids from soybeans include the isoflavones genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone), which occur mainly as the glycosides genistin and daidzin. The soy isoflavone, genistein, is a small molecule inhibitor of tyrosine kinases. Recent study showed that increasing dietary intake of soy isoflavone is associated with better lung function in patients with asthma [1]. The chronic obstructive pulmonary disease (COPD) patients had significantly lower habitual intakes of isoflavones (genistein and daidzein) than control subjects. Lung function measures were found to be positively associated with isoflavones [2]. In patients with asthma, following 4 weeks of dietary soy isoflavone supplementation, inhibits 5-lipoxygenase and decreases leukotriene C₄ synthesis by eosinophils at concentrations similar to

those attained in clinical studies of soy isoflavone supplementation [3,4].

Early studies demonstrated that genistein, a main flavonoid component, concentration-dependently inhibited ovalbumin-induced anaphylactic contraction of the bronchi, as well as release of histamine and leukotrienes from chopped lung preparations *in vitro* model of guinea pig allergic asthma. Genistein significantly suppressed bronchial contraction to leukotriene D₄ and to histamine. Daidzein, another flavonoid component, did not alter OA-induced anaphylactic contraction, however, it slightly reduced bronchial contraction to leukotriene D₄ and ovalbumin-stimulated release of leukotrienes [5]. Genistein 15 mg/kg via intraperitoneal injection markedly inhibited ovalbumin-induced, but not histamine- and methacholine-induced acute bronchoconstriction. In addition, genistein significantly reduced ovalbumin-induced increases in total cell counts and eosinophils recovered in bronchoalveolar lavage fluid, airway eosinophilia, and eosinophil peroxidase activity in cell-free bronchoalveolar lavage fluid and markedly attenuated ovalbumin-induced airway hyperresponsiveness to inhaled methacholine. Immunoblot analysis of lung lysates

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isolated from genistein-pretreated animals showed that epidermal growth factor-induced tyrosine phosphorylation in lung tissues was inhibited by genistein [6].

Dietary intake of the soy isoflavone may be associated with reduced severity of asthma, but as a potential effect for inflammation, airway hyperresponsiveness and remodeling effect are not completely known. To extend our understanding of soy isoflavone in asthma, in the present study, we evaluate the roles of soy isoflavone on secretion of airway mucus, production of proinflammatory mediators, airway responsiveness and remodeling in a murine asthma model.

2. Materials and methods

2.1. Reagents

Soy isoflavone as a test substance and genistein as a control were kindly provided by JF-Natural of Tian Jin Co., China (Tianjin, China). The main components of soy isoflavone are over 90% isoflavone that consist of genistein (more than 40%), daidzein (more than 20%), genistin (10%), daidzin (10%) and other isoflavone (10–20%). The purity of genistein is more than 98%. Ovalbumin (OVA) and methacholine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRIzol and Taq DNA polymerase were from GIBCO (Carlsbad, CA, USA). Reverse Transcription System kit was provided by MBI Fermentas (Vilnius, Lithuania). Oligonucleotide primers for eotaxin, interleukin 4 (IL-4), IL-5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). IFN- γ and IL-4 ELISA kits were from eBioscience Co. (San Diego, CA, USA). Superoxide dismutase (SOD) and myeloperoxidase (MPO) determination kits were provided by Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). All other chemicals were of reagent grade.

2.2. Animals

Ten-week-old female specific-pathogen-free ICR mice (Certificate No. SCXK 2007-0029), purchased from Laboratory Animal Center of Zhejiang University (Hangzhou, China), were maintained in the animal facility under standard indoor conditions with a 12-h light–dark cycle (lights on from 8:00 am to 8:00 pm) and were given free access to water and rodent chow. In all experiments, all animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

2.3. Sensitization and treatment

On days 0 and 10, mice were systemically sensitized with a subcutaneous injection of 10 μ g/mouse OVA emulsified in 2 mg aluminum hydroxide adjuvant at footpad, neck, back, and groin. From days 0 to 28, OVA-sensitized mice were orally administered with soy isoflavone or genistein once a day, at the doses of 10, 30 and 100 mg/kg/day for soy isoflavone and 30 mg/kg/day for genistein. Control mice were orally administered with the same volume of 1% hydroxymethylcellulose vehicle. One hour after administration of soy isoflavone, genistein or vehicle, mice were challenged with antigen for 7 consecutive days. After the treatment, all animals were placed in a plastic box and challenged with an antigen via the airways with aerosolized ovalbumin (10 mg/ml in saline) or control (equal volume saline) in a jet nebulizer (BARI Co. Ltd, Germany) for 30 min once a day, on days 22–28, as previously described [7].

2.4. Measurement of respiratory function

Airway resistance (R_L) and dynamic compliance (C_{dyn}) were determined as changes of airway function after intravenous methacholine

challenge. The lung function of anesthetized and tracheostomized mice was assessed as described previously [8]. Briefly, each anesthetized mouse was placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fisher tube connected to the airways in a pressure transducer, and the changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure pleural pressure, a needle (No. 12) with a multiholed tip was directly inserted into pleural cavity through a port in the connecting tube with a differential pressure transducer. Transpulmonary pressure was calculated as the difference between mouth and pleural pressure. The signals from all pressure transducers were continuously processed (MedLab, Nanjing Biotech Instruments, China) by fitting flow, volume, and pressure to an equation of motion. Changes in R_L and C_{dyn} from methacholine provocation were analyzed by using a dose–response curve to calculate the PD₁₀₀ and PD₅₀ values of methacholine (the provocative doses of methacholine required to increase R_L by 100% and decrease C_{dyn} by 50%, as compared with the baseline). Methacholine dosages (0, 0.0625, 0.125, 0.25 and 0.5 mg/kg, i.v.) were described previously [9]. The effects of soy isoflavone and genistein were determined by comparing the changes in R_L and C_{dyn} after drug treatment with the mean of methacholine response alone in the same mouse on previous and successive control periods.

2.5. Preparation of bronchoalveolar lavage fluids

After the last OVA challenge, mice were anesthetized with urethane (2 g/kg, i.p.), and bronchoalveolar lavage fluids (BALF) were obtained via tracheal tube and washing the lung with 0.5 ml of sterilized normal saline containing 1% bovine serum albumin (BSA) and 5000 IU/l heparin three times. BALF was diluted with 1.5 ml of Hank's balanced salt solution (HBSS) containing 2% fetal calf serum, and was centrifuged at 500 $\times$ g at 4 °C for 10 min. The supernatants were harvested and stored at –80 °C until measurement of cytokines, MPO and SOD, the pellets were resuspended with HBSS for cell count and classification. Two hundred cells from the cell suspension were stained by Wright–Giemsa and classified under light microscope. The results are expressed as the numbers of each type of cell population in one liter of BALF.

2.6. Measurement of MPO and SOD activity

The levels of MPO and SOD activity in BALF were measured by the kits according to the manufacturer's instructions (Jiancheng Bioengineering Institute of Nanjing, China).

2.7. Assay for cytokine in BALF

The concentrations of IFN- γ and IL-4 in BALF were measured by sandwich ELISA using paired matched antibodies according to the manufacturer's instructions. The limits of detection were 4 pg/ml for IL-4 and 15.6 pg/ml for IFN- γ .

2.8. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung homogenates in 1 ml TRIzol (Takara Bio, Dalian, China) per the manufacturer's instructions. 1 μ g aliquots of the total RNA were reverse transcribed to cDNA using reverse transcriptase moloney murine leukemia virus (RTase M-MLV, Takara Bio, Dalian, China) according to the manufacturer's instructions. The PCR amplification was performed in a total volume of 25 μ l (1.25 U of LA Taq DNA polymerase; Takara, JP), 0.2 μ M sense and antisense primers, and 400 μ M dATP, dTTP, dGTP and dCTP. All PCR reactions were performed in a gradient thermal cycler PCR machine (Eppendorf, Germany). For PCR amplification, the following mouse-specific sense and antisense primers were used in Table 1. PCR samples and markers were separated in 1.5%

Table 1
The gene primers (5′–3′), annealing temperature and cycle.

Subjects	Gene primers	Annealing temperature (°C)	Cycle
Eotaxin	Forward-AGAGGCTGAGATCCAAGCAG Reverse-ACAGATCTCTTTGCCAACCT	61	32
IL-4	Forward-ACGGCAGACAGCTATTGATG Reverse-ATGGTGGCTCAGTACTACGA	55	35
IL-5	Forward-ATGACTGTGCTCTGTGCTGGAGC Reverse-CTGTTTTCTGGAGTAACTGGGG	61	35
IFN- γ	Forward-CCTCAGACTCTTTGAAGTCT Reverse-CAGCGACTCTTTCTCTCTT	58	35
MMP-9	Forward-GTATGGTCGTGGCTCTAAGC Reverse-AAAACCTCTTGGTCTGCGG	60	35
TIMP-1	Forward-GACCACCTATACCAGCGTT Reverse-GTCACTCTCCAGTTTGCAAG	55.5	32
β -actin	Forward-CTGTCCCTGTATGCCTCTG Reverse-ATGTCACGCACGA TTTC	55	32

agarose gels containing 0.5 μ g/ml ethidium bromide. Photographs of UV-transilluminated ethidium bromide-stained agarose gels were visualized with an imaging system (UVP Company Limited, England).

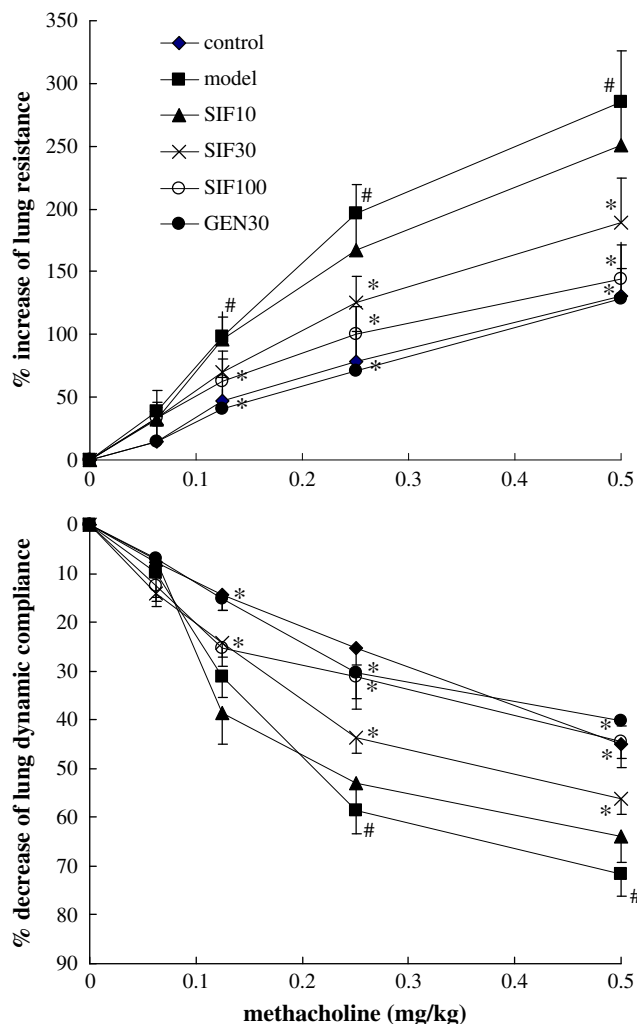


Fig. 1. Dose-dependent response of R_L and C_{dyn} to methacholine. OVA-sensitized mice were orally administered with soy isoflavone at the doses of 10 (SIF10), 30 (SIF30) and 100 mg/kg (SIF100), genistein at the dose of 30 mg/kg (GEN30) or vehicle once a day for 28 days. One hour after administration of soy isoflavone, genistein or vehicle, mice were challenged with aerosolized antigen or normal saline (NS) for 30 min daily. Twenty-four hours after the last OVA challenge, R_L and C_{dyn} against intravenous methacholine was determined as described in Materials and Methods. Data are expressed as mean $\pm$ SEM. #: $P < 0.05$, versus model group ($n = 10$) and control group ($n = 10$); *: $P < 0.05$, versus model and SIF10 group ($n = 9$), SIF30 ($n = 10$), SIF100 ($n = 9$) and GEN30 ($n = 9$).

Table 2
Effects of oral administration of soy isoflavone and genistein on methacholine-induced increase of airway resistance (PD_{100}) and reduction of lung dynamic compliance (PD_{25}) in actively sensitized mice. Sensitized mice react at smaller doses of intravenous methacholine, indicating airway hyperresponsiveness. Data are given as mean (95% confidence limits).

Groups	n	R_L PD_{100} (95% CI) mg/kg, i.v.	C_{dyn} PD_{25} (95% CI) mg/kg, i.v.
Control	10	0.321 (0.266–0.387)	0.648 (0.434–0.967)
model	10	0.109 (0.099–0.120)*	0.229 (0.196–0.268)*
SIF10	9	0.127 (0.114–0.143)	0.257 (0.213–0.309)
SIF30	10	0.163 (0.144–0.184)*	0.365 (0.276–0.483)*
SIF100	9	0.234 (0.197–0.286)*	0.676 (0.386–1.187)*
GEN30	9	0.345 (0.284–0.420)*	0.693 (0.442–1.030)*

$P < 0.05$, versus control group.

* $P < 0.05$, versus model group.

Results were expressed as a ratio of OD signal for eotaxin and cytokines to that for β -actin. All primers were checked against GenBank for selectivity.

2.9. Tissue processing and histological analysis

Right lungs were taken from mice under terminal anesthesia, inflated with 10% formalin, and immersed in the same solution before tissue processing in paraffin-embedded blocks. Five-micrometer sections were stained with H&E to evaluate general morphology. To determine the severity of inflammatory cell infiltration, peribronchial cell counts were performed blind based on a 5 point scoring system described by [10]. Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells 4–6 cells deep; 5, a ring of cells >6 cells deep. Mucus-secreting goblet cells were visualized on periodic acid-Schiff (PAS). Goblet cell and airway epithelium were counted by class optical microscope. To determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium was semi-quantified blind using a 5 point grading system described by [11]. Briefly, the adopted grading system was: 0, no goblet cells; 1, <15%; 2, 15–30%; 3, 30–45%; 4, >45–60%; and 5, >60%. Masson trichrome stain was used for assessment of subepithelial fibrosis. Masson-trichrome stain was used for assessment of subepithelial fibrosis using an Image-Pro Plus image analysis system (Media Cybernetics, Silver Spring, MD, USA). Four to eight specimens of Masson trichrome stain histologic preparations of the right lobe were selected. Digital photographs were taken at 440 magnification. The area of collagen deposition (AC) and the perimeter of basement

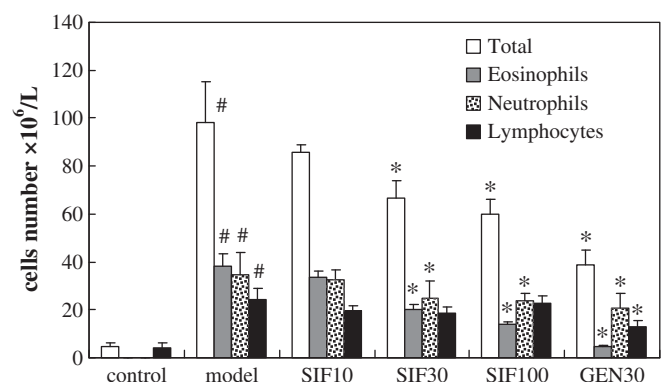


Fig. 2. Effects of soy isoflavone and genistein on inflammatory cell levels in BALF. Mice were processed as described in Fig. 1, and BALF was harvested 24 h after the last OVA challenge. Total inflammatory cell numbers in BALF were counted, and cell classification was performed on a minimum of 200 cells to classify eosinophils, neutrophils, and lymphocytes. Data are expressed as mean $\pm$ SEM. #: $P < 0.05$, versus model group ($n = 12$) and control group ($n = 12$); *: $P < 0.05$, versus model and SIF10 group ($n = 11$), SIF30 ($n = 9$), SIF100 ($n = 12$) and GEN30 ($n = 9$).

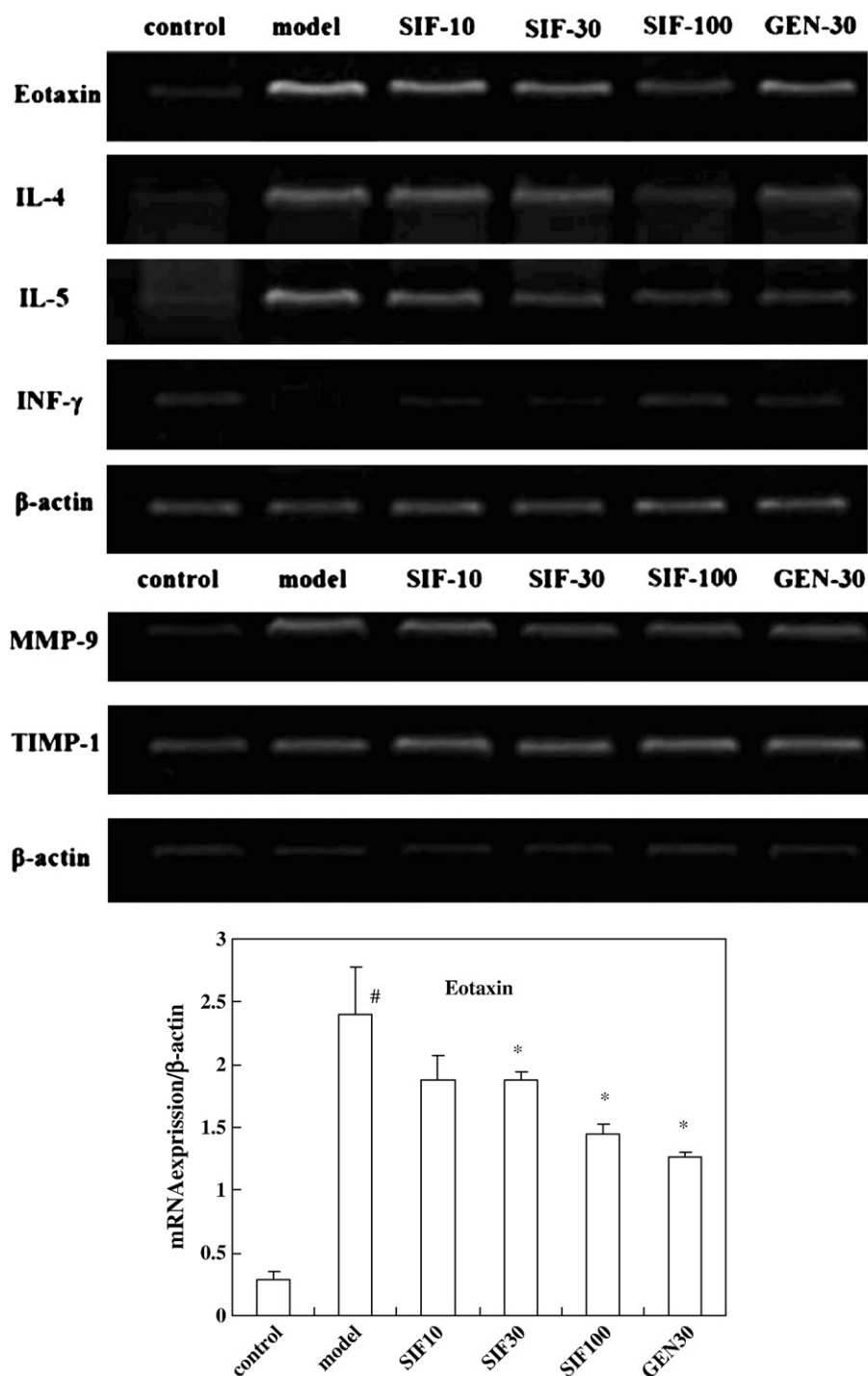


Fig. 3. Effects of soy isoflavone and genistein on mRNA expression of eotaxin, IL-4, IL-5, INF- γ , MMP-9 and TIMP-1 in the lungs of asthmatic mice. Mice were processed and treated as described in Fig. 1. Twenty-four hours after the last OVA challenge, 100 mg of lung tissues from each mouse was harvested for isolation of total RNA, and 1 μ g aliquots of the total RNA sample were used for reverse transcriptional synthesis of first-strand that was used as template of PCR amplification. The PCR product was analyzed by electrophoresis on 1.5% agarose gels and specific bands were digitized using imaging system. PCR reactions were done in triplicates, and the relative expression of mRNAs from independent triplicate experiments was normalized by dividing the eotaxin and cytokine values by the respective β -actin values, and the mean levels of mRNA expression from NS-vehicle mice were defined as 1. Results are shown as mean $\pm$ SEM. #: $P < 0.05$, versus control group; *: $P < 0.05$, versus model group. $n = 6$ (each group).

membrane of bronchioles (Pbm) beneath the basement membrane at 20 μ m depth were measured and described by McMillan and Lloyd [12]. Results are expressed as the area of collagen deposition (AC) per the perimeter of basement membrane of bronchioles (WAc/Pbm μ m²/ μ m). At least 10 bronchioles were counted in each slide. The adopted grading system was: 0, <5 WAc/Pbm (μ m²/ μ m); 1, 5–10 WAc/Pbm (μ m²/ μ m); 2, 10–15 WAc/Pbm (μ m²/ μ m); 3, 15–20 WAc/Pbm (μ m²/ μ m); 4, >20–25 WAc/Pbm (μ m²/ μ m); and 5, >25 WAc/Pbm (μ m²/ μ m). Mean scores of

inflammatory cells, goblet cells and collagen deposition were obtained from 6 animals. Analyses were performed in a blind fashion, and slides were presented in random order for each examination.

2.10. Data analysis

Numerical data are presented as means $\pm$ S.E.M. Statistical calculations were performed using SigmaStat software (SigmaStat 2.0, SPSS

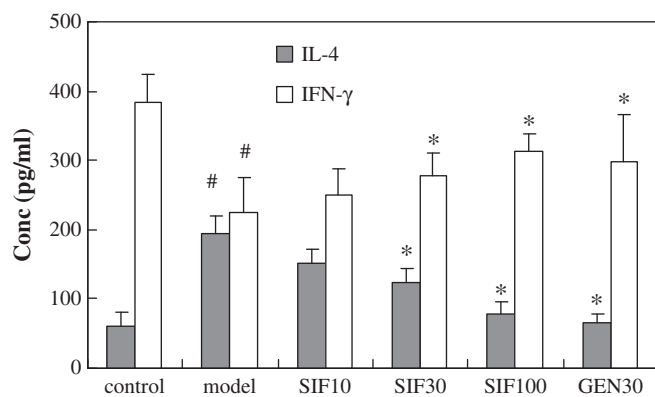


Fig. 4. Effects of soy isoflavone and genistein on cytokine levels in BALF. Mice were processed and treated as described in Fig. 1, and BALF was harvested 24 h after the last OVA challenge. Levels of IL-4 and IFN- γ in BALF were analyzed by ELISA. $n = 10$ (each group). Results are shown as mean $\pm$ SEM. #: $P < 0.05$, versus control group; *: $P < 0.05$, versus model group.

Inc., Chicago, IL, USA), ANOVA and Student–Newman–Keuls multiple comparisons test were used for calculating the difference of respiratory function (Fig. 1 and Table 2), inflammatory cells (Fig. 2), cytokines mRNA expression (Fig. 3) in lungs and cytokine levels (Fig. 4), MPO and SOD levels (Fig. 5) in BALF between each group. A non-parametric test, Mann–Whitney U -test was used to compare the difference of infiltration of inflammatory cells, goblet cell hyperplasia and collagen deposition in airway (Table 3). Significance was assessed at the $p < 0.05$ level. Experiments were performed independently at least twice; results were qualitatively identical, and representative results are shown.

3. Results

3.1. Effects of soy isoflavone on allergen-induced airway hyperresponsiveness to methacholine

The airway responsiveness to intravenous methacholine at the doses of 0, 0.0625, 0.125, 0.25 and 0.5 mg/kg was assessed 24 h after the last challenge. The responsiveness to methacholine in control mice (NS-challenged) showed weak change in airway resistance (R_L) and lung dynamic compliance (C_{dyn}); however, model (OVA-challenged) mice exhibited an obvious AHR with a significant and dose-dependent increase in R_L and decrease in C_{dyn} as compared with

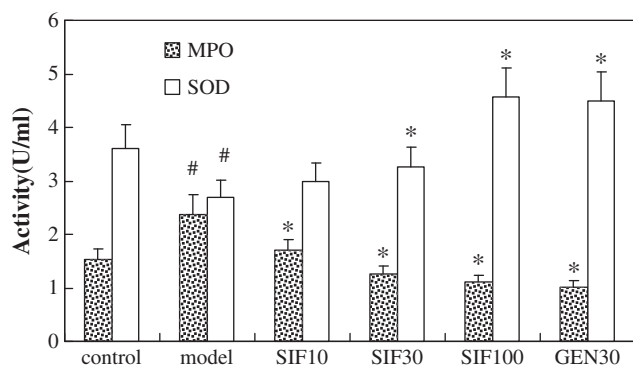


Fig. 5. Effects of soy isoflavone and genistein on MPO and SOD activity in BALF. Mice were processed and treated as described in Fig. 1, and BALF was harvested 24 h after the last OVA challenge. SOD activity in BALF was quantified by oxidase-hydroxylamine method using SOD determination kit, and MPO activity in BALF was determined with a kit by measurement of the H_2O_2 -dependent oxidation of o -dianisidine solution. $n = 10$ (each group). Results are shown as mean $\pm$ SEM. #: $P < 0.05$, versus control group; *: $P < 0.05$, versus model group.

Table 3

Effects of soy isoflavone and genistein on eosinophil infiltration, mucus production and collagen deposition in lungs. Mean scores of inflammatory cells, goblet cells and collagen deposition were obtained from 6 animals. Analyses were performed in a blind fashion, and slides were presented in random order for each examination. Results are shown as mean $\pm$ SEM.

Groups	n	Inflammation	Goblet cell hyperplasia	Collagen deposition
Control	6	0.52 $\pm$ 0.08	0.80 $\pm$ 0.06	0.36 $\pm$ 0.10
model	6	3.66 $\pm$ 0.42 [#]	3.84 $\pm$ 0.48 [#]	3.44 $\pm$ 0.41 [#]
SIF10	6	3.18 $\pm$ 0.48	3.35 $\pm$ 0.44	2.74 $\pm$ 0.37
SIF30	6	2.74 $\pm$ 0.33 [*]	2.80 $\pm$ 0.41 [*]	2.25 $\pm$ 0.32 [*]
SIF100	6	2.44 $\pm$ 0.29 [*]	1.75 $\pm$ 0.34 [*]	1.65 $\pm$ 0.26 [*]
GEN30	6	2.41 $\pm$ 0.30 [*]	2.21 $\pm$ 0.29 [*]	1.71 $\pm$ 0.28 [*]

[#] $P < 0.05$, versus control group.

^{*} $P < 0.05$, versus model group.

control mice (Fig. 1 and Table 2). Oral pretreatment with soy isoflavone at the doses of 10, 30 and 100 mg/kg significantly reduced AHR provoked by methacholine in a dose-dependent manner. Additionally, genistein at the dose of 30 mg/kg also had a better effect on reduction of AHR (Fig. 1 and Table 2). The potency of SIF at the dose of 100 mg/kg on inhibition of AHR was similar to that of GEN at the dose of 30 mg/kg.

3.2. Effects of soy isoflavone on airway eosinophilia and lung inflammation

The total number of inflammatory cells in BALF of model mice was 15-fold greater than that in control mice. Oral pretreatment with soy isoflavone at the doses of 10, 30 and 100 mg/kg significantly inhibits the infiltration of inflammatory cells in airways in a dose-dependent manner. Genistein at the dose of 30 mg/kg also had a remarked effect on inhibition of the infiltration of inflammatory cells in airways. The potency of soy isoflavone at the dose of 30 and 100 mg/kg on inhibition of the infiltration of inflammatory cells in BALF was slightly smaller to that of genistein at the dose of 30 mg/kg. Classification of these inflammatory cells indicated that in model mice, numbers of eosinophils, neutrophils and lymphocytes in the BALF increased 150-, 2- and 18-fold, respectively, as compared with those in control mice (Fig. 2). Pretreatment of OVA-sensitized and -challenged mice with varying doses of soy isoflavone for 28 days significantly decreased eosinophil and neutrophil numbers in dose-dependent manners, but not lymphocytes. The inhibitory effect of soy isoflavone at 100 mg/kg on eosinophils and neutrophil was smaller than that of genistein at 30 mg/kg (Fig. 2).

3.3. Effects of soy isoflavone on chemokine, cytokine and matrix metalloproteinase mRNA expression in the lung tissues

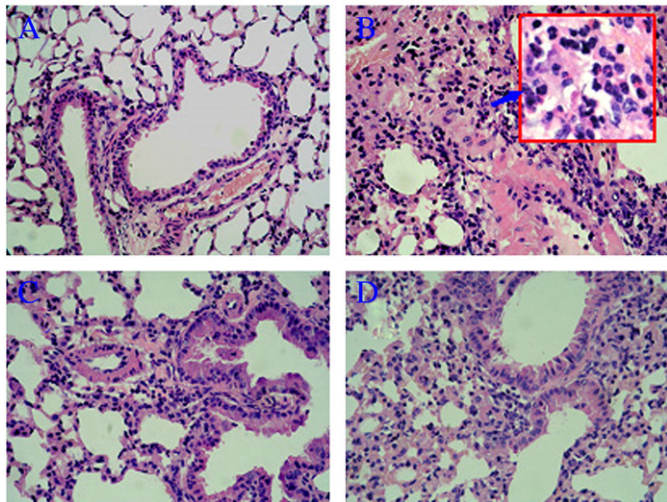
To measure cytokine and chemokine mRNA expression in vivo, lung tissues were harvested 24 h after the last OVA challenge. Eotaxin, IL-5, IL-4 and IFN- γ , metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 mRNA expression were measured by RT-PCR. As shown in Fig. 3, both soy isoflavone and genistein significantly inhibited the up-regulation of eotaxin, IL-4, IL-5 and MMP-9 mRNA expression, and the down-regulation of IFN- γ and TIMP-1 mRNA expression as compared with model mice. The inhibitory effect of SIF and at 30 and 100 mg/kg on eotaxin, IL-4, IL-5 and MMP-9 mRNA expression was similar to that of genistein at 30 mg/kg, and up-regulation of IFN- γ and TIMP-1 mRNA expression also was similar to that of genistein at 30 mg/kg.

3.4. Effects of soy isoflavone on cytokines levels in BALF

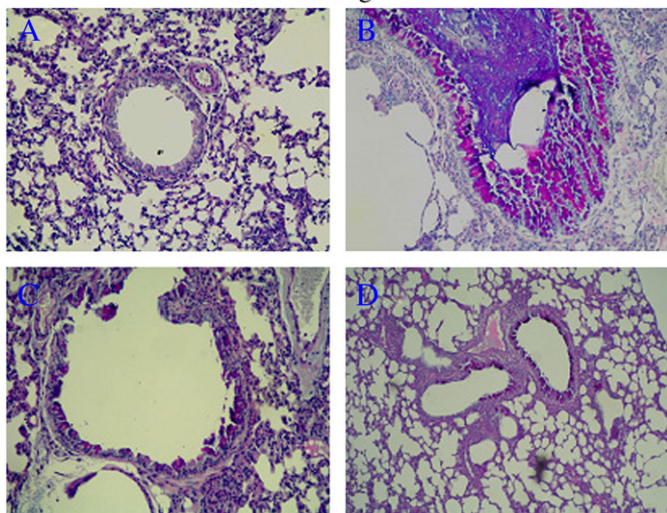
IL-4 and IFN- γ levels were measured by ELISA. As shown in Fig. 4, in model mice, IL-4 levels in BALF increased to 10-fold compared to those

in control mice. In contrast, IFN- γ level in BALF had a significant downregulation compared to those in control mice. Treatment of OVA-sensitized and -challenged mice with either soy isoflavone or genistein significantly increased IFN- γ level; however, had a significant inhibitory effect to IL-4 level.

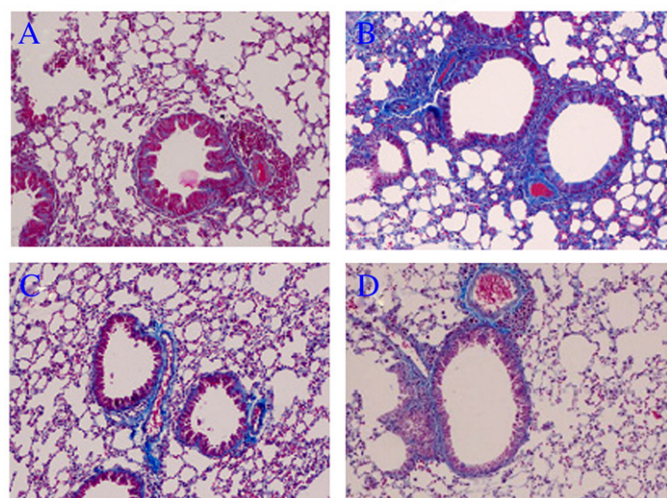
H & E staining $\times 400$



PAS staining $\times 200$



Masson's trichrome staining $\times 200$



3.5. Effects of soy isoflavone on MPO and SOD levels in BALF

Oxidative stress plays an important role in the development of asthma, and soy isoflavone may have potential antioxidative effects. To evaluate the effects of soy isoflavone on oxidative stress, SOD and MPO activity in BALF were determined. As shown in Fig. 5, 24 h after last antigen challenge, antigen challenge resulted in significant decreases of SOD activity and significant increases of MPO activity in BALF ($P < 0.05$) in model group as compared with control group, however, treatment with soy isoflavone at the dose of 10, 30 and 100 mg/kg significantly increased MPO activity in BALF in a dose-dependent manner, and also significantly increased the SOD activity in BALF. Induction of SOD activity and inhibition of MPO activity in BALF were maximally achieved at dose of 100 mg/kg, which are as comparable as genistein treatment at dose of 30 mg/kg.

3.6. Eosinophil infiltration, mucus production and collagen deposition in lung tissues

Lung tissue was harvested 24 h after the last OVA challenge. Model mice (Fig. 6B) exhibited an obvious infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues, goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lungs, and a dramatic increase in both the extent of collagen deposition and intensity of staining as compared with control mice (Fig. 6A). Soy isoflavone at the dose of 30 and 100 mg/kg (Fig. 6C) and genistein at the dose of 30 mg/kg (Fig. 6D) markedly attenuated OVA-induced eosinophil infiltration, mucus production and collagen deposition in lung tissues as compared with that in model mice (Table 3). Soy isoflavone at the dose of 10 mg/kg did also show any obvious effect on mucus production and collagen deposition, but not eosinophil infiltration in lung tissues.

4. Discussion

In the present study, we investigated the effects of soy isoflavone on changes of airway hyperresponsiveness, inflammation, mucus secretion and remodeling in a model of mouse asthma. In this mouse model of allergic asthma, antigen sensitization and repeated challenge induced airway inflammation, pathologic oxidizing process, airway goblet cell hyperplasia, collagen deposition, airway hyperresponsiveness and an increase in mRNA expression of eotaxin, IL-4, IL-5 and MMP-9, and a decrease in mRNA expression for IFN- γ and TIMP-1. A parallel change in the levels of IL-4, and IFN- γ in the bronchoalveolar lavage fluids was also shown. These changes were reversed by soy isoflavone and genistein by oral administration.

Th2 cells play an important role in the modulation of the allergic airway inflammation [13,14]. There is much evidence that Th2 cytokines can be produced by alveolar macrophages, tissue mast cells, and airway epithelial cells as well as infiltrated inflammatory cells such as lymphocytes, eosinophils and even neutrophils [14]. Our present data show that soy isoflavone and genistein significantly inhibited mRNA expression of eotaxin, IL-4, and IL-5 in the lung tissues in a dose-dependent manner. In contrast, expression of IFN- γ mRNA, a Th1 cytokine was increased by soy isoflavone and genistein. In addition, soy

Fig. 6. Effects of soy isoflavone and genistein on eosinophil infiltration, mucus production and collagen deposition in lungs. Histological examination of lung tissue eosinophil infiltration, mucus secretion and collagen deposition (A–D) and were performed 24 h after the last allergen challenge. The pictures representative were showed in A: control; B: model; C: SIF100 mg/kg group; D: GEN30 mg/kg group. Semi-quantitative analysis of inflammatory cell infiltration, mucus production and collagen deposition in lung sections were performed as described in Materials and methods. Lung tissues were fixed, sectioned at 5 μ m thickness, and stained with H&E for tissue eosinophils infiltration, periodic acid-Schiff for mucus production (red are positive for mucus) and Masson's trichrome for collagen deposition (blue are positive for collagen deposition), respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isoflavone and genistein also significantly increased the levels of IFN- γ and reduced protein level of IL-4 in BALF.

Eosinophil is a key effector cell for the pathogenesis of allergic inflammation. However, evidence supports an important role for eosinophils in asthma. Eosinophils are the first cells recruited to the site of the allergic reaction. Their presence may influence clinical presentation and has been linked to the development of severe chronic asthma and sudden severe attacks [15]. Our present findings showed that soy isoflavone and genistein inhibited eosinophil infiltration into the airways, as shown by a significant decrease in total cell counts and eosinophil counts in BAL fluid. Similarly, airway tissue infiltration of eosinophil was also attenuated, as revealed by a significant reduction of inflammatory cell infiltration in histological examination. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines such as IL-5 and coordinated by specific chemokine such as eotaxin in combination with adhesion molecules such as VCAM-1 and VLA-4 [16,17]. Our result showed that mRNA expression of eotaxin and IL-5 in the lung tissues from soy isoflavone and genistein-treated mice was substantially reduced. Taken together, the observed reduction in airway eosinophilia by soy isoflavone and genistein may be a result of composite effects of reduction in Th2 cytokine production and eotaxin formation.

An imbalance in reducing and oxidizing (redox) systems favoring a more oxidative environment is present in asthma and linked to the pathophysiology of the defining symptoms and signs including airflow limitation, hyper-reactivity, and airway remodeling [18,19]. Neutrophil mediators, such as myeloperoxidase MPO, have been studied less actively. However, neutrophils have an increased propensity to release MPO in patients with asthma [20], and MPO has been suggested to have epithelial cell damaging capacity and even pro-inflammatory properties [21]. The essential first line antioxidant enzymes superoxide dismutases (SOD) and catalase are reduced in asthma as compared to healthy individuals, with lowest levels in those patients with the most severe asthma [22]. In the present study, we demonstrated a dramatic parallel increase of MPO activity level and neutrophil numbers in BALF of OVA-vehicle mice. In contrast, SOD activity level was reduced. Soy isoflavone and genistein significantly reversed increase of MPO activity level and neutrophil numbers, and reduction of SOD activity level. The results indicated present effective antioxidant effects. Genistein and other isoflavonoids can interact with cellular and vascular space oxidants, thereby protecting critical biochemical targets [23,24].

Our results also demonstrated a dramatic reduction in mucus production with less goblet cell hyperplasia and collagen deposition in soy isoflavone and genistein -treated mice as compared with untreated control. In addition, mucus plug was not detectable within the bronchial tissue in lungs of soy isoflavone and genistein-treated mice, whereas it is common in lungs from OVA-challenged group. In addition, the mRNA expression of MMP-9 in the lung tissues from soy isoflavone and genistein-treated mice was significantly reduced, and the mRNA expression of TIMP-1 was substantially increased. Airway remodeling is a major change responsible for irreversible asthmatic airflow restriction. In asthmatic condition, MMP-9 is the most relevant among the 23 kinds of human MMPs at present detected [25,26]. Although the mechanism is still under investigation and not accurately known, the imbalance between MMP-9 and TIMP-1 is considered a major theory to explain the progression of airway remodeling. TIMPs are specific inhibitors of MMPs that bind non-covalently in a 1:1 molar ratio with activated MMPs [27].

It is believed that inflammatory mediators released and airway remodeling during the allergic inflammation play a critical role in AHR development [28,29]. Our data showed that soy isoflavone significantly inhibited OVA induced AHR to inhaled methacholine in a dose-dependent manner. It has been established that IL-5 and eotaxin plays a critical role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products such as major basic protein and

cysteinyl-leukotrienes, which are closely associated with AHR [30]. In addition, IL-5, eotaxin and IL-13 have been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated to play a major role in AHR [31]. Moreover, airway remodeling refers to airway structural changes also plays a critical role in persistent AHR; these changes include epithelial alteration, subepithelial fibrosis, increased smooth muscle mass, goblet and mucous gland hyperplasia [32]. As such, the observed reduction of AHR by soy isoflavone may be associated with reduction in expression of IL-5, eotaxin and MMP-9, tissue eosinophilia, goblet gland hyperplasia and subepithelial fibrosis by soy isoflavone.

Genistein inhibits human blood eosinophil LTC₄ synthesis results from blockade of 5-LO activation, in association with inhibition of p38 and MAPKAP-2 [3]. Our data do not clarify whether soy isoflavone also blocks activation of p38 directly or acts upstream in the p38 activation cascade. We remain to further study on soy isoflavones of the signal pathway to inhibit airway inflammation and AHR.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report in this work for the first time that soy isoflavone effectively reduced ovalbumin-induced Th2 cytokine production, pathologic oxidizing process, pulmonary eosinophilia, MMP-9 expression in lung tissues, mucus hypersecretion, airway remodeling and AHR in a mouse asthma model. These findings support an effective role for soy isoflavone as dietary intake treatment for asthma and airway inflammatory disease.

Acknowledgements

This project was supported by the grants from Taizhou Science and Technology Bureau of Zhejiang Province (No. 081KY48) and the Medicine and Health Science Research Foundation of Zhejiang Province (No. 2009A114). We thank Katherine Theken, Pharm. D. from the University of North Carolina at Chapel Hill in USA for improving the manuscript.

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