



Anti-asthmatic effects of *Angelica dahurica* against ovalbumin-induced airway inflammation via upregulation of heme oxygenase-1

Mee-Young Lee^a, Chang-Seob Seo^a, Jin-Ah Lee^a, Nam-Hun Lee^a, Jung-Hoon Kim^a, Heykyung Ha^a, Ming-Shan Zheng^b, Jong-Keun Son^b, Hyeun-Kyoo Shin^{a,*}

^a Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine, Exporo 483, Yuseong-gu, Daejeon 305-811, Republic of Korea

^b College of Pharmacy, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Republic of Korea

ARTICLE INFO

Article history:

Received 19 July 2010

Accepted 3 December 2010

Available online 9 December 2010

Keywords:

Angelica dahurica Benth et Hooker

Asthma

Eosinophilia

Cytokine

Heme oxygenase-1

ABSTRACT

Asthma is a chronic immune inflammatory disease characterized by variable airflow obstruction. The present study was undertaken to assess the effects of an *Angelica dahurica* Benth et Hooker ethanolic extract (AD) on airway inflammation in an ovalbumin (OVA)-induced airway inflammation model. Mice that received AD displayed significantly lower airway eosinophilia, cytokine levels, including interleukin (IL)-4, IL-5, and tumor necrosis factor (TNF)-alpha levels, mucus production and immunoglobulin (Ig)E, compared with OVA-induced mice. In our experiments, AD treatment reduced airway inflammation and suppressed oxidative stress in the OVA-induced asthma model, partly via induction of heme oxygenase (HO)-1. The effects of AD on OVA-induced HO-1 induction were partially reversed by the HO-1 inhibitor, tin protoporphyrin (SnPP). Our results clearly indicate that AD is a suppressor of airway allergic inflammation, and may thus be effectively used as an anti-inflammatory drug in the treatment of asthma.

Crown Copyright © 2010 Published by Elsevier Ltd. All rights reserved.

1. Introduction

Asthma is a chronic inflammatory disease of the airways involving T-lymphocytes and eosinophil infiltration, mucus overproduction and airway hyper-responsiveness. Inflammatory mediators (including lipid mediators) play a critical role in the pathogenesis of chronic airway disease and facilitate the recruitment, activation, and trafficking of inflammatory cells in the airways. Spina and co-workers showed that the pathophysiology of asthma is related to T helper (Th)1/Th2 cell imbalance in the airways (Spina et al., 1998). Cytokines play an important role in airway inflammation in asthma by promoting the development, differentiation, recruitment, activation, and survival of inflammatory cells. Th2 cells participate in asthma pathogenesis by stimulating B cells that produce the allergen-specific immunoglobulin (Ig)E and inducing the infiltration of eosinophils and other inflammatory cells into the airways (Hamelmann et al., 1999; Zhang et al., 1999). While Th2 cytokines, including interleukins

(IL)-4, IL-5 and IL-13, are important in asthma (Foster et al., 1996), tumor necrosis factor (TNF)-α, a cytokine usually associated with Th1 responses, has also been implicated in the inflammatory response in asthma (Meiler et al., 2006). Inflammatory cells contribute to the generation of Th2 cytokines (IL-4, IL-5, and IL-13), chemokines (eotaxin and RANTES) and TNF-α, levels of which are increased in asthmatic lungs (Williams and Galli, 2000). TNF-α, a potent proinflammatory cytokine with immunoregulatory activities, is produced by several cell types, including monocytes, macrophages, lymphocytes, neutrophils, eosinophils and mast cells (Bazzoni and Beutler, 1996; Costa et al., 1993; Gordon and Galli, 1991).

Increased reactive oxygen species (ROS) generation, resulting in an imbalance between oxidative forces and the antioxidant defense systems, has been implicated in the pathogenesis of asthma (Dworski, 2000; Rahman and MacNee, 2000). Therefore, regulation of intracellular ROS during inflammation may have a potential advantage with regard to treatment of inflammatory diseases. Asthma is a chronic inflammatory disease of the airways associated with increased reactive oxygen species, nitric oxide (NO) production and increased levels of several inflammatory mediators in the airways, which may induce heme oxygenase (HO)-1 expression (Barnes, 1990; Kharitonov et al., 1996). HO-1 is emerging as an important phase II anti-inflammatory enzyme that is significantly upregulated under conditions of oxidative stress (Choi and Alam, 1996). HO is the rate-limiting enzyme in

Abbreviations: BALF, bronchoalveolar lavage fluid; AD, *Angelica dahurica* Benth et Hooker ethanolic extract; OVA, ovalbumin; ROS, reactive oxygen species; Ig, immunoglobulin; IL, interleukin; HO, heme oxygenase; NO, nitric oxide; SnPP, tin protoporphyrin; ELISA, Enzyme-linked immunosorbent assays; TNF, tumor necrosis factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

* Corresponding author. Tel.: +82 42 868 9464; fax: +82 42 864 2120.

E-mail addresses: cozy37@gmail.com, hkshin@kiom.re.kr (H.-K. Shin).

the heme-degradation reaction, converting heme to biliverdin IX, carbon monoxide, and iron. Three isoforms of HO have been fully characterized. Specifically, HO-2 and HO-3 are constitutive isozymes whereas HO-1 is induced by a variety of stimuli, including hemin and oxidative stress, in different cell types (Pae et al., 2003). Oxidative stress in ovalbumin-challenged guinea pigs is reduced when HO-1 expression and subsequent bilirubin production are induced by hemin (Almolki et al., 2004).

Several drugs for asthma therapy are currently available. However, most of these agents are relatively inefficient and induce adverse side effects. Therefore, novel drugs with fewer side effects are necessary for the effective treatment of asthma. We investigated the effects of the therapeutic agent *Angelica dahurica*, with a view to identifying safe lead compounds from natural sources with minimal side effects. The medicinal plant, *A. dahurica*, is a perennial herb abundant in Korea, China and Japan. The root of this species is used in Korea as a traditional folk medicine to treat headache, bleeding, menstrual disorders and neuralgia (Kimura et al., 1996). Previous studies focusing on the chemical constituents of *A. dahurica* led to the isolation of more than 20 coumarins (Baek et al., 2000; Kwon et al., 2002; Qiao et al., 1996; Kim et al., 1992). The major active constituents of this herb are simple coumarins and furocoumarins, including byakangelicoloxypeucedanin, imperatorin, phellopterin and isoimperatorin. These compounds have been reported to exhibit pharmacological effects such as inhibition of IL-1 β -induced cyclooxygenase-2 (COX-2) (Lin et al., 2002), inhibition of lipopolysaccharide-induced prostaglandin E2 (Ban et al., 2003), inhibition of acetylcholinesterase (Kim et al., 2002) and inhibitory effects on the GABA degradative enzyme (Choi et al., 2005). However, the effects of the *A. dahurica* ethanolic extract (AD) in patients with allergic diseases are yet to be established. Here, we investigated whether AD exerts anti-asthmatic effects in terms of inhibition of oxidative stress, inflammatory infiltration into the airways, mucus production, and expression of Th1 and Th2 cytokines and IgE, in a murine model of ovalbumin (OVA)-induced asthma, and whether HO-1 is involved in its mechanism of action.

2. Materials and methods

2.1. Preparation of the *A. dahurica* ethanolic extract (AD)

The *A. dahurica* root was purchased in October 2008 from HMAX (Jecheon, Korea). These materials were confirmed taxonomically by Professor Je-Hyun Lee of Dongguk University, Gyeongju, Republic of Korea. A voucher specimen (ST8) has been deposited at the Korea Institute of Oriental Medicine (KIOM), Daejeon, Republic of Korea. Fresh *A. dahurica* Benth et Hooker was washed three times with tap water to remove salts, epiphytes and sand, and stored at -20°C . Frozen

samples were lyophilized and homogenized in a grinder, prior to extraction. Extracts were prepared from dried roots of *A. dahurica* (200 g) with 70% EtOH (2 L $\times$ 3 times), followed by sonication for 1 h. The extract solution was filtered through filter paper and evaporated to dryness (62.19 g). The dried extract yield from starting crude material was 32.1%. Concentrated AD was freshly dissolved in phosphate-buffered saline (PBS), prior to use.

2.2. Animals

Specific 7 week-old pathogen-free inbred female BALB/c mice routinely screened serologically for relevant respiratory pathogens were purchased from Daehan Biolink Co. Ltd., (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to the conduction of experiments, and provided water and standard chow *ad libitum*. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

2.3. Sensitization and airway challenge

Mice were divided into groups receiving the following treatments: (1) sham sensitization plus challenge with phosphate-buffered saline (PBS; ipNeb) and PBS p.o., (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St. Louis, MO) (ipNeb), (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and AD (25, 50 or 100 mg/kg) or Montelukast (30 mg/kg) p.o. Briefly, mice were sensitized with an intraperitoneal injection of 20 μg OVA emulsified in 2 mg aluminum hydroxide in 200 μL PBS buffer (pH 7.4) on days 0 and 14. Mice were challenged through the airway with OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 21, 22, and 23 after initial sensitization. AD (25, 50 or 100 mg/kg) was administered orally once daily on days 19–23. Negative and positive control mice were orally treated with PBS and Montelukast (Mon; 30 mg/kg), respectively, once daily on days 19–23. Tin protoporphyrin (SnPP), an inhibitor of HO activity, was obtained from Porphyrin products (Logan, UT). SnPP treatment (10 mg/kg, i.p) was performed 12 h before AD administration. Montelukast, a positive control, was developed as a cysteinyl leukotriene-1 receptor antagonist, in which the clinical responses in patients with aspirin-sensitive asthma, nocturnal exacerbations of asthma and allergic asthma led to montelukast being successfully introduced into the market. Animals were sacrificed 48 h after the final challenge (day 25) to determine the suppressive effects of AD. A schematic diagram of the treatment schedule is shown in Fig. 1.

2.4. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were sacrificed with an overdose (50 mg/kg) of pentobarbital 48 h after the final challenge, and tracheostomy performed. Following instillation of ice-cold PBS (0.6 mL) into the lungs, BALF was obtained with three aspirations (total volume of 1.8 mL) via tracheal cannulation. BALF was centrifuged and the supernatant fractions collected and stored at -70°C . The total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells via trypan blue staining. BALF (100 μL) was pipetted onto a slide and centrifuged (200g, 4°C , 10 min) to fix cells using a cytospin machine (Hanil Science Industrial, Seoul, Korea). Cell pellets were suspended in 0.5 mL PBS, and 100 μL of each solution spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik[®] Staining reagent (B4132-1A; Dade Behring Inc., Deerfield, IL), according to the manufacturer's instructions.

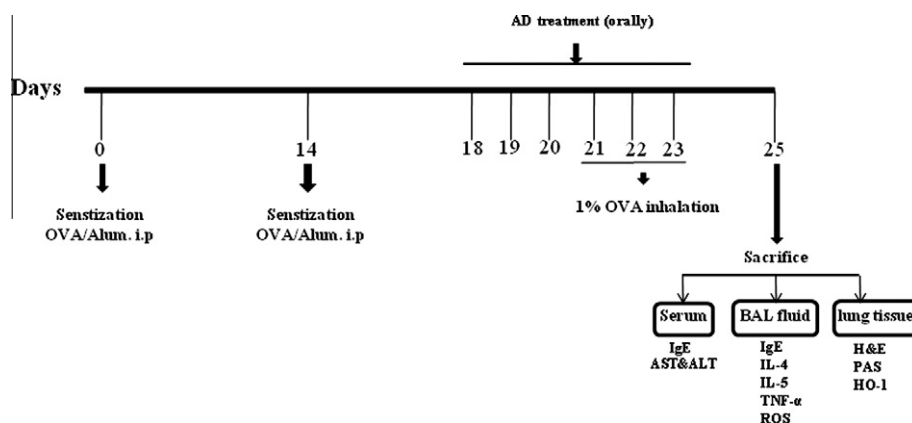


Fig. 1. Mouse model of airway inflammation and effects of treatment with the *A. dahurica* Benth et Hooker ethanolic extract (AD).

2.5. The 2',7'-dichlorofluorescein (DCF) fluorescence assay

Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. In brief, cells from BALF were washed with PBS and total cells (5×10^3) were counted. BALF cell was treated with 25 μ M DCF-DA for 10 min at 37 °C. Intracellular ROS activity was measured by measuring fluorescence at 488 nm excitation and 525 nm emissions on a fluorescence plate reader (Perkin–Elmer, Waltham, MA).

2.6. Measurement of total IgE levels in BALF and serum

BALF and serum were collected and stored at –70 °C after centrifugation (3000 rpm, 10 min). Total IgE levels were measured using Enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with 100 μ L/well IgE (10 μ g/mL; Serotec, Oxford, UK) in PBS-Tween 20. Antibodies in BALF and serum were detected using isotype-specific secondary antibodies (anti-mouse IgE; Serotec, Oxford, UK). After washing four times, 200 μ L *o*-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) was added to each well. The plate was incubated for 10 min in the dark and absorbance measured at 450 nm. Total IgE concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE (Serotec, Oxford, UK).

2.7. Measurement of cytokines in BALF

ELISA experiments were performed according to the manufacturer's instructions. TNF- α , IL-4 and IL-5 levels in BALF were measured using mouse-specific ELISA kits (BioSource International, Camarillo, CA).

2.8. Heme oxygenase (HO) enzyme activity

HO-1 enzyme activity in the mouse lung was quantified by assessing bilirubin generation with the aid of modified established techniques. Briefly, lungs were homogenized on ice in one volume of 100 mmol/L phosphate buffer with 2 mmol/L MgCl₂, and centrifuged for 15 min at 18,800g. The supernatant was used to measure HO-1 enzyme activity. ELISA assays were performed according to the manufacturer's instructions. The HO-1 level in lung tissue was estimated using a specific mouse HO-1 ELISA kit (DAKARA, Japan). Values are expressed per mg protein.

2.9. Western blotting

Lung tissue was homogenized in lysis buffer containing protease inhibitors [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS, 1 mM EGTA, 100 μ g/mL PMSF, 10 μ g/mL pepstatin A, and 100 μ M Na₃VO₃]. Homogenates were centrifuged at 12,000g for 25 min at 4 °C and the protein concentrations in the supernatant fractions determined using Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein (25 μ g) were subjected to 10% SDS/PAGE. After electrophoresis at 100 V for 90 min, separated proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% non-fat dry milk dissolved in TBST buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20] overnight at 4 °C and incubated with mouse anti-HO-1 (1:1000 dilution; Abcam Inc., MA) overnight at 4 °C. After removal of the primary antibody, membranes were washed three times with TBST buffer at room temperature, and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution; Santa Cruz Biotechnology, CA) for 1 h at room temperature. Membranes were subsequently re-washed with TBST buffer, and immunoreactive bands visualized using ECL reagent (Amersham Biosciences).

2.10. Lung tissue histopathology

After BALF was obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h at 4 °C. Tissues were embedded in paraffin, sectioned at 4 μ m thickness, and stained with H&E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110–1-32) and periodic acid-Schiff (PAS) (IMEB Inc., San Marcos, CA) to assess mucus production. Tissues were subsequently mounted and coverslipped using Dako mounting medium (Dakocytomation, Denmark, CA). For immunohistochemistry, paraffin sections were deparaffinized, dehydrated, washed in PBS with 0.3% Triton X-100, and preincubated for 10 min at room temperature with 10% goat serum to block non-specific staining. Subsequently, slides were incubated with primary mouse anti-rabbit HO-1 antibody (1/200 dilution; Abcam) overnight at 4 °C. After removal of primary antibodies, sections were washed and incubated with biotinylated secondary antibody at 37 °C for 1 h, followed by the avidin–biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Excess secondary antibody was removed, and sections washed with PBS and incubated with 0.05% diaminobenzidine (1:200; Millipore, Billerica, MA) for a further 10 min. Sections were counterstained, rinsed in PBS to terminate the reaction, and coverslipped for microscopic examination.

2.11. Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum

ALT and AST levels were determined to assess liver and kidney function using commercial kits (BECKMAN coulter, Inc., Fullerton, CA, USA) and an auto-analyzer (BECKMAN CX4, CA, USA).

2.12. Image capture and Photomicrography

Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and montages assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but not otherwise manipulated.

2.13. Chromatographic system

Analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a PDA detector. The data processor employed LCSolution software (Version 1.24). The analytical column used was a Gemini C18 (250 $\times$ 4.6 mm; particle size 5 μ m, Phenomenex, Torrance, CA, USA). The mobile phases were solvent A (H₂O) and solvent B (Acetonitrile). The gradient flow was as follows: (A)/(B) = 80/20 (0 min) $\rightarrow$ (A)/(B) = 60/40 (10 min) $\rightarrow$ (A)/(B) = 30/70 (30 min; hold for 5 min) $\rightarrow$ (A)/(B) = 80/20 (40 min; hold for 10 min). The column temperature was maintained at 40 °C. The analysis was carried out at a flow-rate of 1.0 mL/min with PDA detection from 190–400 nm. The injection volume was 10 μ L.

2.14. Chemicals and reagents

Oxypeucedanin, oxypeucedanin hydrate and byakangelicin were isolated from *A. dahurica* by a series of chromatography procedures. The purity of each compound was determined to be above 95% by HPLC analysis. Nodakenin was obtained from Wako (Osaka, Japan). Imperatorin and isoperimorin were purchased from Chroma-Dex (Santa Ana, CA, USA). HPLC-grade reagents, methanol, acetonitrile, and water were obtained from J.T.Baker (Phillipsburg, NJ, USA). Other chemicals were of analytical grade.

2.15. Preparation of standard and sample solution

Standard stock solutions of oxypeucedanin (500 μ g/mL), oxypeucedanin hydrate (500 μ g/mL), byakangelicin (500 μ g/mL), nodakenin (500 μ g/mL), imperatorin (1000 μ g/mL) and isoperimorin (1000 μ g/mL) were prepared in methanol and held below 4 °C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. The sample powder was weighed (1.0 g) into a 50 mL flask and 70% methanol was added to the volumetric mark. The mixture was sonicated for 60 min at room temperature. After extraction, the mixture was filtered through a 0.2 μ m membrane filter. The resulting solution was used HPLC analysis.

2.16. Statistical analysis

Data are expressed as means $\pm$ standard deviations (SD). Statistical comparisons were made with one-way analysis of variance (ANOVA) using SPSS 16.0, followed by Duncan's new multiple range test, using $P < 0.05$ as the level of significance.

3. Results

3.1. Effects of AD on OVA-induced eosinophilia and ROS generation in BALF

To determine whether AD has protective effects on airway inflammation, the total cell and eosinophil numbers in BALF were measured in OVA-challenged mice either left untreated or treated with AD. The numbers of total cells, eosinophils and other inflammatory cells in BALF were significantly increased in OVA-challenged mice compared with the negative control group. Oral administration of AD significantly attenuated the increase in total cells, eosinophils and other inflammatory cells in BALF compared with those of OVA-challenged mice (Fig. 2A). Moreover, ROS levels in BALF were markedly increased in OVA-challenged mice, as shown in Fig. 2B. Pre-administration of AD led to a significant decrease in the ROS level. The effects of AD on the ROS generation in BALF cell were similar to those of montelukast (positive control).

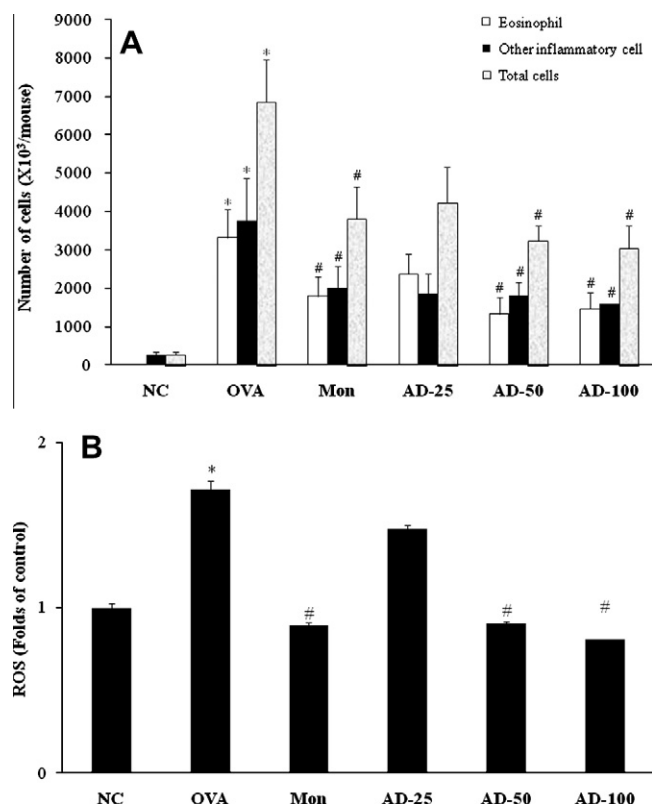


Fig. 2. Effects of AD on recruitment of inflammatory cells and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. (A) Cells were isolated by centrifugation, and stained with Diff-Quik® Stain reagent. Cell numbers were determined by counting within at least five squares of a hemocytometer under a light microscope. Dead cells, stained with trypan blue, were excluded from the total cell number. (B) Oxidative stress in BALF was quantified based on DCF fluorescence. The values represent relative fluorescence units per milligram protein. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AD-25, AD (25 mg/kg) + OVA-sensitized/challenged mice; AD-50, AD (50 mg/kg) + OVA-sensitized/challenged mice; AD-100, AD (100 mg/kg) + OVA-sensitized/challenged mice. AD or montelukast treatment was performed 1 h before challenge. Values are expressed as means $\pm$ SD. *Significant difference from NC, $P < 0.05$, and # significant difference from OVA, $P < 0.05$.

3.2. Effects of AD on cytokine levels in BALF

The levels of Th2 cytokines, including IL-4 and IL-5, in BALF were significantly decreased in AD-treated mice compared with their OVA-induced counterparts (Figs. 3A and B). Moreover, the TNF- α level in BALF was markedly decreased in the AD-treated group compared with OVA-challenged mice (Fig. 3C).

3.3. Effects of AD on airway goblet cell hyperplasia and mucus production in lung tissue

Light microscopic analysis revealed that inflammatory cells infiltrated peribronchiole and perivascular connective tissue of OVA-challenged mice. Eosinophils were among the most highly infiltrating inflammatory cells. Administration of AD (50 or 100 mg/kg) markedly attenuated this eosinophil-rich leukocyte infiltration compared with that observed in OVA-challenged mice (Fig. 4A).

To examine possible AD-induced suppression of mucus overproduction caused by goblet cell hyperplasia, we stained lung sections with PAS. Mucus overproduction was clearly observed as a violet color in the bronchial airways of OVA-challenged mice, compared with those of negative control mice. Notably, the mucus

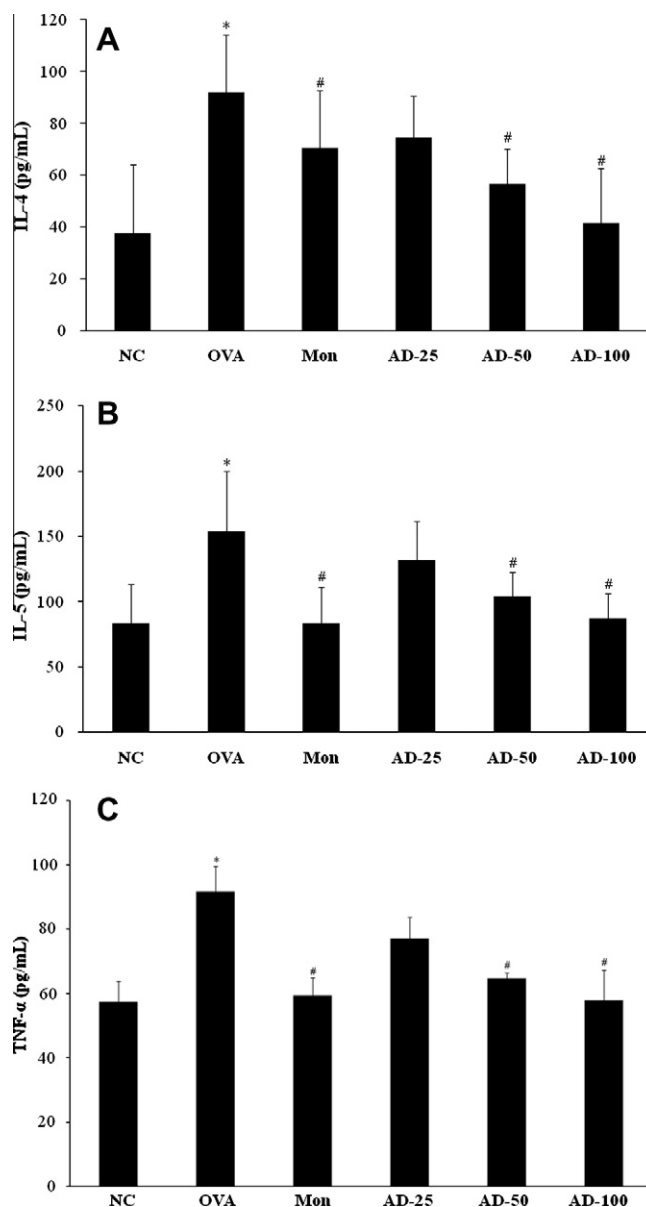


Fig. 3. Effects of AD on cytokine levels in BALF. BALF was collected 48 h after the last OVA challenge of mice. Individual samples were analyzed using ELISA. (A) IL-4; (B) IL-5; (C) TNF- α . NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AD-25, AD (25 mg/kg) + OVA-sensitized/challenged mice; AD-50, AD (50 mg/kg) + OVA-sensitized/challenged mice; AD-100, AD (100 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. AD or montelukast treatment was performed 1 h before challenge. Values are expressed as means $\pm$ SD. *Significant difference from NC, $P < 0.05$, and # significant difference from OVA, $P < 0.05$.

stain was markedly diminished in a dose-dependent manner upon administration of AD (Fig. 4B).

3.4. Effects of AD on total IgE

Total IgE levels in BALF and serum were markedly increased in OVA-challenged mice, compared with those in negative control mice. AD pretreatment led to significant reductions in the total IgE levels to 2754 ± 368.6 ng/mL (AD-100, plasma), in a dose dependent manner (Fig. 5A) and 10.6 ± 2.2 ng/mL (AD-100, BALF). In the serum (systemic), AD at 50 and 100 mg/kg similar reduced the total IgE level (Fig. 5B).

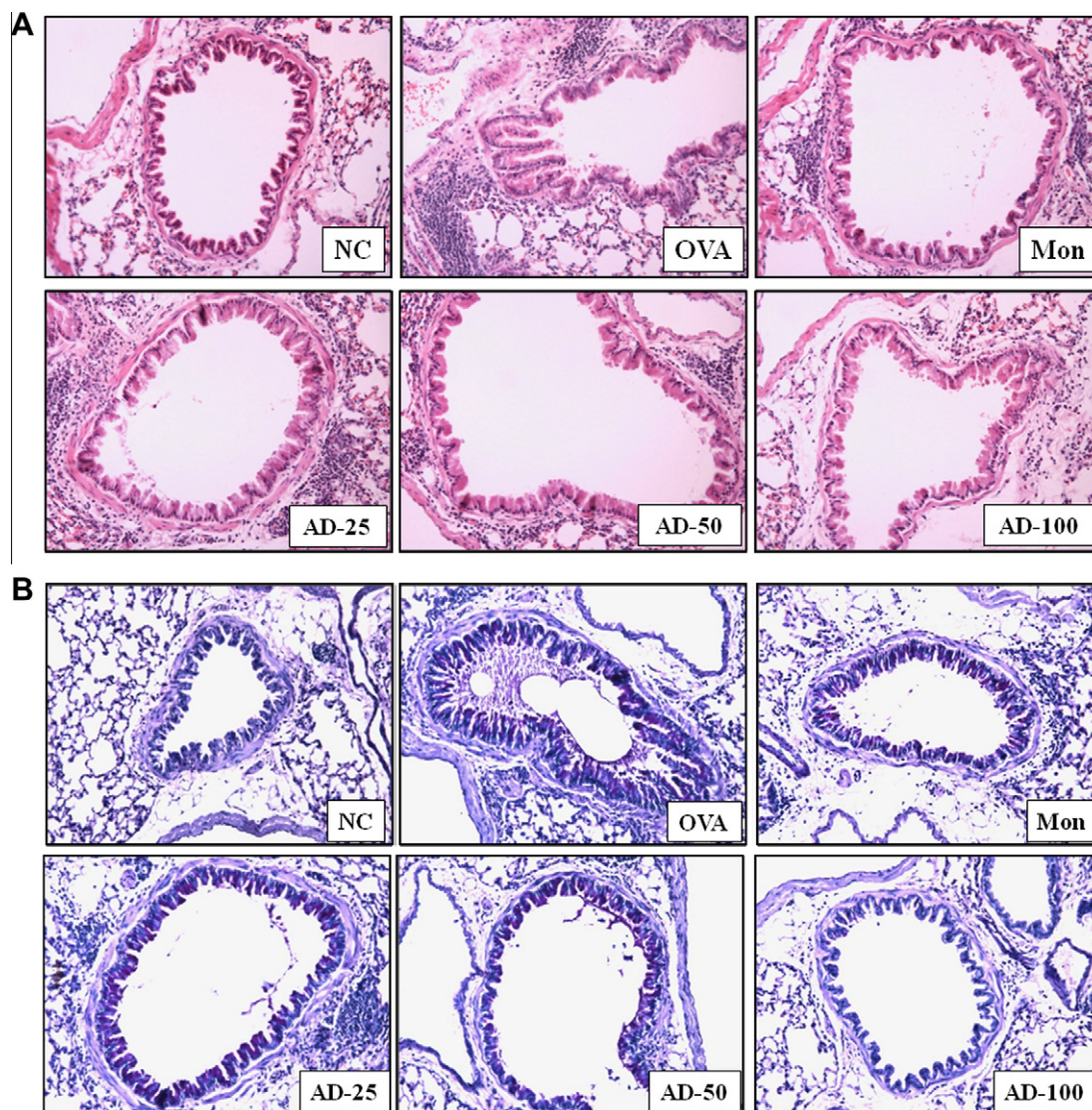


Fig. 4. Effects of AD on recruitment of leukocytes and mucus production in lung tissue. Histological examination of lung tissue was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (A) and Periodic Acid Schiff (PAS) (B). (magnification 200 $\times$). NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AD-25, AD (25 mg/kg) + OVA-sensitized/challenged mice; AD-50, AD (50 mg/kg) + OVA-sensitized/challenged mice; AD-100, AD (100 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. AD or montelukast treatment was performed 1 h before challenge.

3.5. Effects of AD on HO-1 expression

Next, we investigated whether AD upregulates HO-1 expression as part of the mechanism underlying protection against OVA-challenged lung inflammation. To determine whether the inhibitory effects of AD on airway inflammation are related to HO-1 induction, expression was assessed using ELISA, Western-blot, and immunohistochemical analyses. As shown in Fig. 6A, AD treatment led to increased HO-1 activity in lung tissue. After treatment of AD at 50 and 100 mg/kg, the HO-1 activity induction is similar.

Compared with OVA-challenged mice, AD induced the greatest increases in activity at doses of 25, 50, and 100 mg/kg. Consistent with this increase in activity, HO-1 protein levels were markedly increased in AD-treated mice (Fig. 6B). Moreover, immunohistochemistry analyses revealed higher HO-1 expression in both OVA-challenged and negative control mice. When treated with AD (25, 50, 100 mg/kg), however, the high level expression of HO-1 was observed in a number of cells. They appeared to be cells

that infiltrated to the lung (Fig. 6C). As shown in Fig. 6D, SnPP treatment partly attenuated the activity HO-1.

3.6. Cytotoxicity of AD in a murine model of allergic asthma

AST and ALT kits were used for the measurement of AST and ALT, respectively, in serum, according to the manufacturer's protocol. As shown Fig. 7, AD did not affect the activities of serum toxicity marker enzymes (ALT, AST), indicating normal liver function.

3.7. HPLC analysis

In our method, satisfactory separation was obtained using mobile phases consisting of solvent A (H_2O) and solvent B (Acetonitrile). The HPLC chromatogram of reference compounds and AD was shown in Fig. 8. Using optimized chromatography conditions,

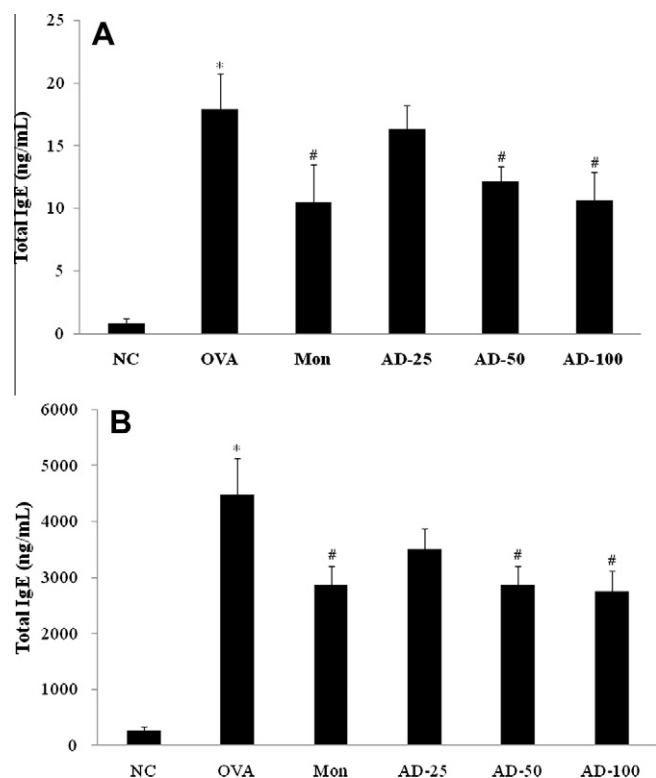


Fig. 5. Effects of AD on total IgE levels in BALF and serum. BALF and serum were collected 48 h after the final OVA challenge in mice. Each sample was analyzed using ELISA. (A) Total IgE level in BALF; (B) Total IgE level in serum; NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AD-25, AD (25 mg/kg) + OVA-sensitized/challenged mice; AD-50, AD (50 mg/kg) + OVA-sensitized/challenged mice; AD-100, AD (100 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. AD or montelukast treatment was performed 1 h before challenge. Values are expressed as means $\pm$ SD. *Significant difference from NC, $P < 0.05$, and # significant difference from OVA, $P < 0.05$.

six compounds were eluted before 35 min, and showed good resolution in sample analysis.

4. Discussion

Asthma is a chronic inflammatory disease of the airways characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyper responsiveness to inhaled allergens and non specific stimuli (Kay, 1991). The inflammatory response involves recruitment and activation of inflammatory cells and changes in the structural cells of the airway (D'Ambrosio et al., 2001; Holgate et al., 2000). Recently, Wen and co-workers highlighted the usefulness of anti-asthma herbal medicine for the treatment of adult patients with moderate–severe allergic asthma (Wen et al., 2005). Therefore, a new approach for asthma therapy has been developed within the framework of herbal remedies as a complementary or alternative medicine. This study demonstrates that AD suppresses OVA-induced airway inflammation, accompanied by reduction of oxidative stress, cytokine and IgE levels, and these effects are mediated, at least in part, via induction of HO-1.

An important cell type in the inflammation of allergic asthma is eosinophil leukocytes, present not only in the airway wall but also uncontrolled asthma and contained in large numbers in the sputum and BALF (Lemiere et al., 2006). Allergic reactions in the airways are well known to be mediated via two pathways. The first pathway involves induction of IgE by B cells via IL-4 released from Th2 cells and mast cells. The other pathway involves recruitment

of eosinophils via release of IL-5 and eotaxin from Th2 cells or mast cells. Th2-type cytokines, such as IL-4, IL-5, and IL-13, produced by activated CD4⁺ T cells, play a central role in the pathogenesis of asthma (Renz et al., 1993) by controlling the key processes of IgE production, growth of mast cells, and differentiation and activation of mast cells and eosinophils (Punnonen et al., 1994; Saito et al., 1998). Blockage of the IL-4 receptor in mice sensitized and challenged with antigen markedly decreased airway goblet cell metaplasia. In the absence of IL-4R alpha in recipient mice, no goblet cell metaplasia or mucus hypersecretion was observed in response to OVA, even in the presence of Th2 cells and substantial eosinophilic infiltration (Kelly-Welch et al., 2001). In our experiments, AD significantly prevented mucus overproduction in allergic mice, together with reductions in allergen-induced IL-4 levels. Infiltration of eosinophils into the airways is linked to the production of IL-5, which is important for eosinophil proliferation, activation, and migration (Foster et al., 1996). Our data show that AD inhibits the pulmonary accumulation of eosinophils upon injection in mice, in parallel with a decrease in the IL-5 level in BAL fluid. The results suggest that AD plays a key role in blocking mucus secretion and recruitment of eosinophils in the lung via partially inhibition of IL-4- and IL-5-dependent pathways.

IgE, a key target for developing anti-asthma strategies, is one of the most important factors responsible for the progression of allergic reactions (Hamelmann et al., 1999). AD downregulates BALF and serum levels of total IgE, further supporting its potential as an effective anti-asthma agent.

Inflammatory cells recruited to the asthmatic airways display exceptional ROS production ability. Specifically, stimulated cells, such as neutrophils, eosinophils, lymphocytes and macrophages, produce substantial levels of ROS (Babior, 1999; Conner and Grisham, 1996; Leusen et al., 1996). Consistent with these findings, our present results show that ROS generation is substantially reduced upon administration of AD.

HO-1, a protective gene, has a remarkable anti-inflammatory effect in various diseases. Its precise mechanism of action is still under investigation (Chapman et al., 2001; Otterbein et al., 2000; van Scott et al., 2000). HO-1 catalyzes the initial and rate-limiting steps in the oxidative degradation of heme to bilirubin. Bilirubin is an antioxidant and CO has several biological activities, including stimulation of guanylate cyclase (Stocker et al., 1987). HO-1 induction is therefore suggested as a protective mechanism against oxidant-mediated cellular injury (Vogt et al., 1995). According to recent *in vivo* studies, induction of HO-1 expression leads to improved airway inflammation, mucus hypersecretion, oxidative stress and hyper responsiveness in an asthma guinea pig model, and exogenous bilirubin or low-dose CO ameliorates airway inflammation in a murine asthma model via inhibition of leukocyte migration as well as production of cellular ROS and IL-5 (Ameredes et al., 2003; Keshavan et al., 2005). Our findings demonstrate that AD attenuated OVA-induced production of ROS, suggesting that the protective effect of AD is related to its reduction of oxidative stress. In this study, we tested whether AD-mediated HO-1 expression correlated with inhibition of OVA-induced IL-4 and IL-5. Our results indicate that the inhibition of HO activity by the HO inhibitor SnPP, partially reversed. Therefore, HO-1 plays an important role in preventing airway inflammation. Thus, these data indicate that AD-induced HO-1 expression was at least partly responsible for the resulting anti-asthmatic effects of these materials.

We analyzed the six coumarins such as nodakenin, oxypeucedanin hydrate, byakangelicin, oxypeucedanin, imperatorin and isomeratorin using HPLC in AD. Our result showed good separation in AD. Therefore, we must be preformed to activity test for major compounds. To determine the activity of each batch, it need to the quality control of AD and its pharmaceutical preparation.

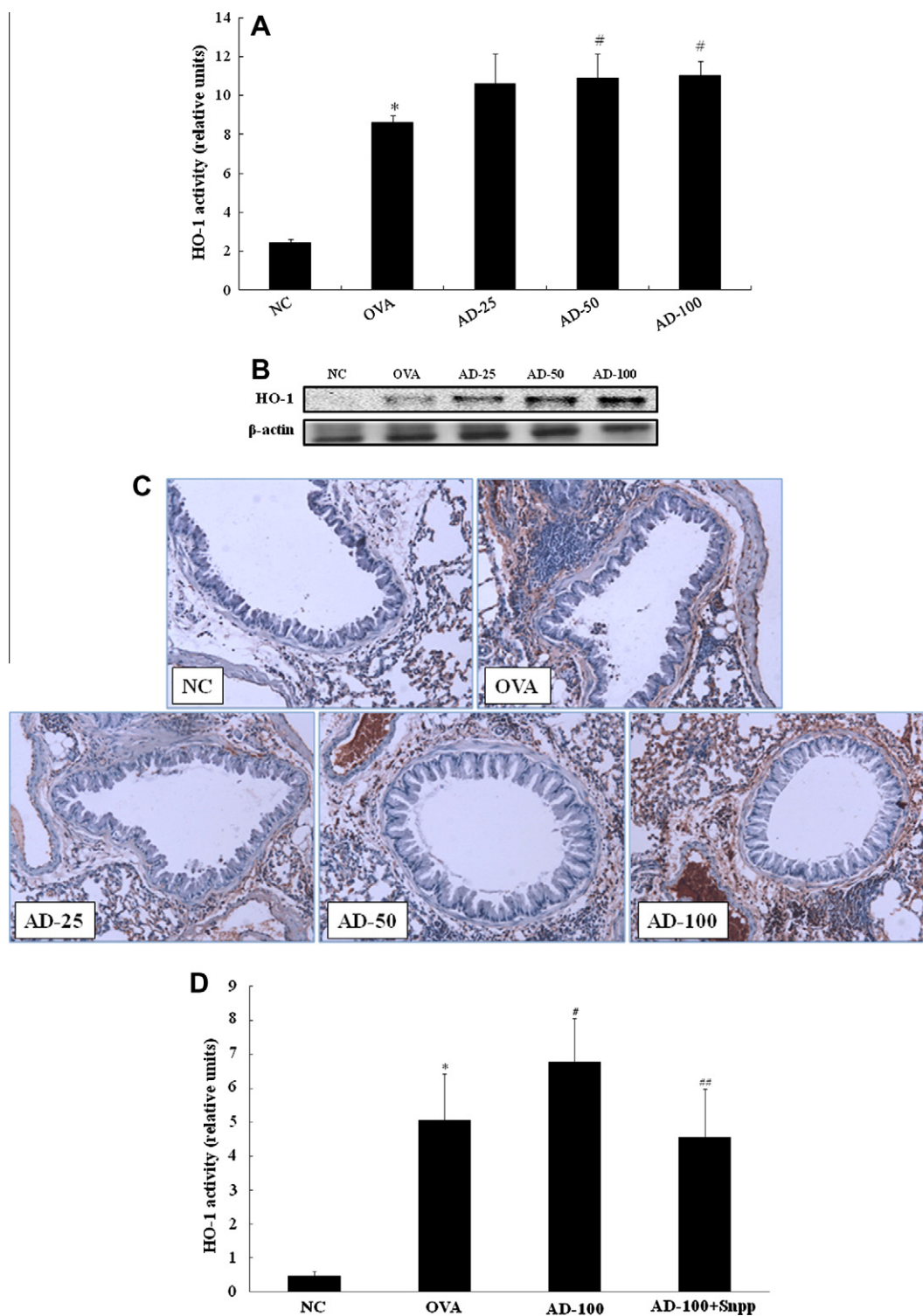


Fig. 6. Effects of AD on HO-1 activity and protein levels. Lung tissue was collected 48 h after the final OVA challenge in mice. Each sample was analyzed using ELISA, Western blotting and immunohistochemistry. (A) HO-1 enzyme activity in lung tissue (B) HO-1 protein expression in the lung was determined using Western blot analysis after OVA challenge with or without AD. The HO-1 protein level was normalized to that of β -actin. (C) HO-1 immunoreactivity in lung tissue. (D) Effect of SnPP on inhibition of HO-1 activity; NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AD-25, AD (25 mg/kg) + OVA-sensitized/challenged mice; AD-50, AD (50 mg/kg) + OVA-sensitized/challenged mice; AD-100, AD (100 mg/kg) + OVA-sensitized/challenged mice; AD-100 + SnPP, SnPP (10 mg/kg, i.p.) + AD (100 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as means $\pm$ SD. *Significant difference from NC, $P < 0.05$, and # significant difference from OVA, and ## significant difference from AD, $P < 0.05$.

In summary, our data strongly indicate that AD could play a pivotal role in controlling allergic airway inflammation in a murine asthma model by reducing OVA-induced oxidative stress, eosino-

phil infiltration, cytokine production, attenuating mucus secreting goblet cell hyperplasia and, at least in part, by upregulation of HO-1.

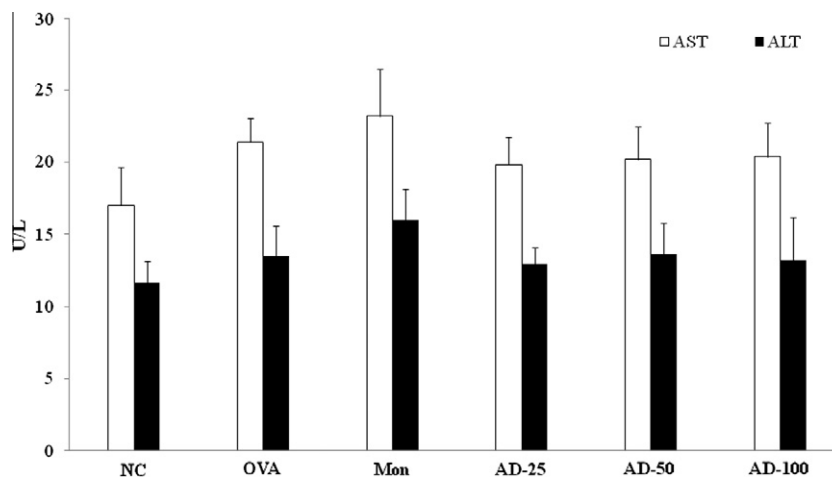


Fig. 7. Levels of ALT and AST in sera. Serum was collected 48 h after the final OVA challenge of mice. Each sample was analyzed using commercial kits (Beckman Coulter, Inc., Fullerton, CA). AD or montelukast treatment was performed 1 h before challenge.

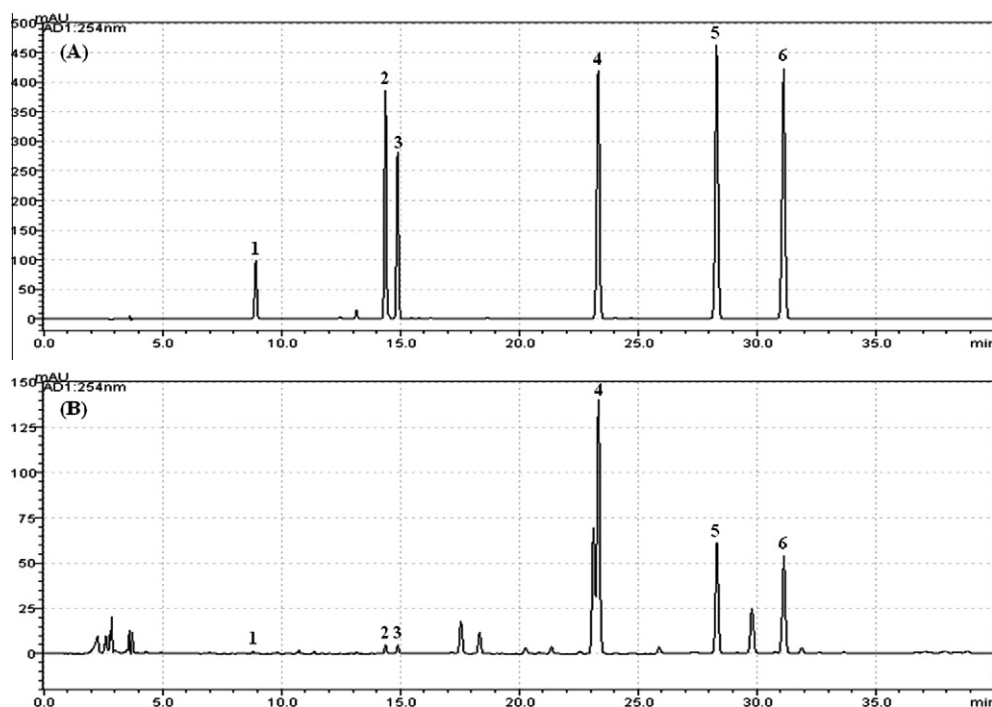


Fig. 8. HPLC chromatogram of (A) standard mixture and (B) AD at 254 nm (1, nodakenin; 2, oxypeucedanin hydrate; 3, byakangelicin; 4, oxypeucedanin; 5, imperatorin; 6, isoimperatorin).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

This research was supported by a grant 'The Evidence-based Medicine for herbal formulae' from Korea Institute of Oriental Medicine (KIOM).

References

Almolki, A., Taillé, C., Martin, G.F., Jose, P.J., Zedda, C., Conti, M., Megret, J., Henin, D., Aubier, M., Boczkowski, J., 2004. Heme oxygenase attenuates allergen-induced airway inflammation and hyperreactivity in guinea pigs. *Am. J. Physiol. Lung Cell Mol. Physiol.* 287, L26–L34.

Ameredes, B.T., Otterbein, L.E., Kohut, L.K., Gligonic, A.L., Calhoun, W.J., Choi, A.M., 2003. Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 285, 1270–1286.

Babior, B.M., 1999. NADPH oxidase: an update. *Blood* 93, 1464–1476.

Baek, N.I., Ahn, E.M., Kim, H.Y., Park, Y.D., 2000. Furanocoumarins from the root of *Angelica dahurica*. *Arch. Pharm. Res.* 23, 467–470.

Ban, H.S., Lim, S.S., Suzuki, K., Jung, S.H., Lee, S., Lee, Y.S., Shin, K.H., Ohuchi, K., 2003. Inhibitory effects of furocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E2 production. *Planta Med.* 69, 408–412.

Barnes, P.J., 1990. Reactive oxygen species and airway inflammation. *Free Radic. Biol. Med.* 9, 235–243.

Bazzoni, F., Beutler, B., 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* 334, 1717–1725.

Chapman, J.T., Otterbein, L.E., Elias, J.A., Choi, A.M., 2001. Carbon monoxide attenuates aeroallergen-induced inflammation in mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 281, L209–L216.

Choi, A.M., Alam, J., 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell Mol. Biol.* 15, 9.

- Choi, S.Y., Ahn, E.M., Song, M.C., Kim, D.W., Kang, J.H., Kwon, O.S., Kang, T.C., Baek, N.I., 2005. *In vitro* GABA-transaminase inhibitory compounds from the root of *Angelica dahuricae*. *Phytother. Res.* 19, 839–845.
- Conner, E.M., Grisham, M.B., 1996. Inflammation, free radicals, and antioxidants. *Nutrition* 12, 272–277.
- Costa, J.J., Matossian, K., Resnick, M.B., Beil, W.J., Wong, D.T., Gordon, J.R., Dvorak, A.M., Weller, P.F., Galli, S.J., 1993. Human eosinophils can express the cytokines tumor necrosis factor- α and macrophage inflammatory protein-1 α . *J. Clin. Invest.* 91, 2673–2684.
- D'Ambrosio, D., Mariani, M., Panina-Bordignon, P., Sinigaglia, F., 2001. Chemokines and their receptors guiding T lymphocyte recruitment in lung inflammation. *Am. J. Respir. Crit. Care Med.* 64, 1266–1275.
- Dworski, R., 2000. Oxidant stress in asthma. *Thorax* 55, S51–S53.
- Foster, P.S., Hogan, S.T., Ramsay, A.J., Matthaei, K.I., Young, I.G., 1996. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* 183, 195–201.
- Gordon, J.R., Galli, S.J., 1991. Release of both preformed and newly synthesized tumor necrosis factor α (TNF- α)/cachectin by mouse mast cells stimulated via the Fc ϵ 1 receptor. A mechanism for the sustained action of mast cell-derived TNF- α during IgE-dependent biological responses. *J. Exp. Med.* 174, 103–107.
- Hamelmann, E., Tieda, K., Oshiba, A., Gelfand, E.W., 1999. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness—a murine model. *Allergy* 54, 297–305.
- Holgate, S.T., Lackie, P., Wilson, S., Roche, S., Davies, D., 2000. Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma. *Am. J. Respir. Crit. Care Med.* 162, S113–S117.
- Kay, A.B., 1991. Asthma and inflammation. *J. Allergy. Clin. Immunol.* 87, 893–910.
- Kelly-Welch, A.E., Melo, M.E., Smith, E., Ford, A.Q., Haudenschild, C., Noben-Trauth, N., Keegan, A.D., 2001. Complex role of the IL-4 receptor α in a murine model of airway inflammation: expression of the IL-4 receptor α on nonlymphoid cells of bone marrow origin contributes to severity of inflammation. *J. Immunol.* 172, 4545–4555.
- Keshavan, P., Deem, T.L., Schwemberger, S.J., Babcock, G.F., Cook-Mills, J.M., Zucker, S.D., 2005. Unconjugated bilirubin inhibits VCAM-1-mediated transendothelial leukocyte migration. *J. Immunol.* 174, 3709–3718.
- Kharitonov, S.A., Chung, K.F., Evans, D., O'Connor, B.J., Barnes, P.J., 1996. Increased exhaled nitric oxide in asthma is mainly derived from the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 153, 1773–1780.
- Kim, S.H., Kang, S.S., Kim, C.M., 1992. Coumarin glycosides from the root of *Angelica dahurica*. *Arch. Pharm. Res.* 15, 73–77.
- Kim, D.K., Lim, J.P., Yang, J.H., Eom, D.O., Eun, J.S., Leem, K.H., 2002. Acetylcholinesterase inhibitors from the roots of *Angelica dahuricae*. *Arch. Pharm. Res.* 25, 856–859.
- Kimura, T., But, P.P.H., Guo, J.X., Sung, C.K., 1996. International Collection of Traditional and Folk Medicine: part 1. World Scientific, Singapore (pp. 117–118).
- Kwon, Y.S., Shin, S.J., Kim, M.J., Kim, C.M., 2002. A new coumarin from the stem of *Angelica dahurica*. *Arch. Pharm. Res.* 15, 73–77.
- Lemiere, C., Ernst, P., Olivenstein, R., Yamauchi, Y., Govindaraju, K., Ludwig, M.S., Martin, J.G., Hamid, Q., 2006. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. *J. Allergy Clin. Immunol.* 118, 1033–1039.
- Leusen, J.H., Verhoeven, A.J., Roos, D., 1996. Interactions between the components of the human NADPH oxidase: intrigues in the phox family. *J. Lab. Clin. Med.* 128, 461–476.
- Lin, C.H., Chang, C.W., Wang, C.C., Chang, M.S., Yang, L.L., 2002. Byakangelicol, isolated from *Angelica dahuricae*, inhibits both the activity and induction of cyclooxygenase-2 in human pulmonary epithelial cells. *J. Pharm. Pharmacol.* 54, 1271–1278.
- Meiler, F., Zimmermann, M., Blaser, K., Akdis, C.A., Akdis, M., 2006. T-cell subsets in the pathogenesis of human asthma. *Curr. Allergy Asthma Rep.* 6, 91–96.
- Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R.J., Flavell, R.A., Choi, A.M., 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* 6, 422–428.
- Pae, H.O., Oh, G.S., Choi, B.M., Chae, S.C., Chung, H.T., 2003. Differential expressions of *hemin oxygenase-1* gene in CD25⁺ and CD25⁺ subsets of human CD4⁺ T cells. *Biochem. Biophys. Res. Commun.* 306, 701–705.
- Punnonen, J., Aversa, G., Cocks, B.G., de Vries, J.E., 1994. Role of interleukin-4 and interleukin-13 in synthesis of IgE and expression of CD23⁺ by human B cells. *Allergy* 49, 576–586.
- Qiao, S.Y., Qao, X.S., Wang, Z.Y., 1996. Coumarins of the roots of *Angelica dahurica*. *Planta Med.* 62, 584.
- Rahman, I., MacNee, W., 2000. Oxidative stress and regulation of glutathione in lung inflammation. *Eur. Respir. J.* 16, 534–554.
- Renz, H., Bradley, K., Saloga, J., Loader, J., Larsen, G.L., Gelfand, E.W., 1993. T cells expressing specific V β elements regulate immunoglobulin E production and airways responsiveness *in vivo*. *J. Exp. Med.* 177, 1175–1180.
- Saito, H., Hatake, K., Dvorak, A.M., Leiferman, K.M., Donnenberg, A.D., Arai, N., Ishizaka, K., Ishizaka, T., 1998. Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc. Natl. Acad. Sci.* 85, 2288–2292.
- Spina, D., Shah, S., Harrison, S., 1998. Modulation of sensory nerve function in the airways. *Trends Pharmacol. Sci.* 19, 460–465.
- Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N., 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235, 1043–1046.
- van Scott, M.R., Justice, J.P., Bradfield, J.F., Enright, E., Sigounas, A., Sur, S., 2000. IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. *Am. J. Physiol.* 278, L667–L674.
- Vogt, B.A., Alam, J., Croatt, A.J., Vercellotti, G.B., Nath, K.A., 1995. Acquired resistance to acute oxidative stress. Possible role of heme oxygenase and ferritin. *Lab. Invest.* 72, 474–483.
- Wen, M.C., Wei, C.H., Hu, G.Q., Srivastava, K., Ko, J., Xi, S.T., Mu, D.Z., Du, J.B., Li, G.H., Wallenstein, S., Sampson, H., Kattan, M., Li, X.M., 2005. Efficacy and tolerability of anti-asthma herbal medicine intervention in adult patients with moderate-severe allergic asthma. *J. Allergy Clin. Immunol.* 116, 517–524.
- Williams, C.M., Galli, S.J., 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192, 455–462.
- Zhang, D.H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P., Ray, A., 1999. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 11, 473–482.