



Alpinia katsumadai seed extract attenuate oxidative stress and asthmatic activity in a mouse model of allergic asthma

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ABSTRACT

Allergic asthma is a chronic inflammatory disorder of the airways characterized by biphasic airway obstruction. Oxidative stress plays an important role in the development of asthmatic conditions. Thus, identification of oxidative stress markers in bronchoalveolar lavage fluid (BALF) and lung tissue from ovalbumin (OVA)-sensitized mice could provide new insights into both the pathogenesis of the disease and the possible use of anti-oxidants to alleviate disease severity.

In this study, we investigated the effect of an ethanolic extract of *Alpinia katsumadai* seeds (AK) on a murine model of OVA-induced asthma model. The anti-oxidant and anti-asthmatic effects of AK on OVA-induced murine airway reaction were determined through observation of Th2-type cytokine levels, eosinophil recruitment, and lung histopathology. AK was found to significantly inhibit increases in Th2-type cytokines and mRNA expression such as IL-4 and IL-5 in BALF and lung tissue, and effectively suppressed IgE, IgG2a, eosinophilia, and mucus hypersecretion in the asthmatic mouse model. Also, the generation of reactive oxygen species (ROS) in BALF was diminished by AK treatment. These findings indicate that oxidative stress may play a crucial role in the pathogenesis of OVA-induced asthma model and that AK may have applications in the treatment of asthma.

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1. Introduction

The prevalence of allergic lung inflammation has significantly increased over recent decades. This increase is related to changes in the environment acting on susceptible individuals, influencing both the induction and progression of the disease (Holgate, 1999). Airway inflammation is associated with the infiltration of eosinophils, neutrophils, and T- and B-lymphocytes into airway and lung tissues. Asthma is a chronic inflammatory disease of the airway, characterized by the presence of increased numbers of T-helper 2 (Th2) lymphocytes, eosinophils, (Pascual and Peters, 2005) and airway inflammation (Robinson et al., 1996). Asthma patients also exhibit high serum levels of immunoglobulin (Ig)E, as well as intrapulmonary production of interleukin-4 (IL-4), IL-5, and IL-13 by allergen-specific Th2 cells (Neurath et al., 2002). Infiltrating leukocytes potentiate the inflammatory process by the release of reactive oxygen species (ROS) into the surrounding

Abbreviations: BALF, bronchoalveolar lavage fluid; AK, an ethanolic extract of *Alpinia katsumadai* seeds; OVA, ovalbumin; ROS, reactive oxygen species; COPD, chronic obstructive pulmonary disease; Ig, immunoglobulin.

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tissue, resulting in increased oxidative stress (Chanez et al., 1990; Cluzel et al., 1987; Kelly et al., 1999; Nadeem et al., 2003). Oxidative stress occurs not only as a result of environmental exposure, but also because of inflammation, as both airway and intravascular inflammatory cells generate ROS (Bowler and Crapo, 2002; Dworski, 2000). ROS, such as the superoxide radical, hydrogen peroxide, hypochlorous acid, and the hydroxyl radical, are reportedly associated with many pathogenic features of asthma. Oxidative injury plays an important role in the inflammation observed both in individuals with asthma or COPD and in animal models of these conditions (Morcillo et al., 1999; MacNee, 2001; MacNee and Rahman, 2001). An increasing amount of clinical and experimental evidence suggests that ROS play essential roles in the pathogenesis of airway inflammation (Kwak et al., 2003; Hamelmann et al., 1997; Lee et al., 2002). Although the precise etiology of asthma remains unknown, the pathology is clearly multifactorial, including a possible genetic predisposition coupled with exposure to certain environmental triggers (Bukstein et al., 2006). Over thousands of years, an ethanolic extract of *Alpinia katsumadai* seeds (AK) has been widely used in traditional Chinese medicine to treat a variety of gastric disorders and emesis. Previous investigations of *A. katsumadai* have reported a variety of diarylheptanoids (Kuroyanagi et al., 1983), chalcones and flavonoids

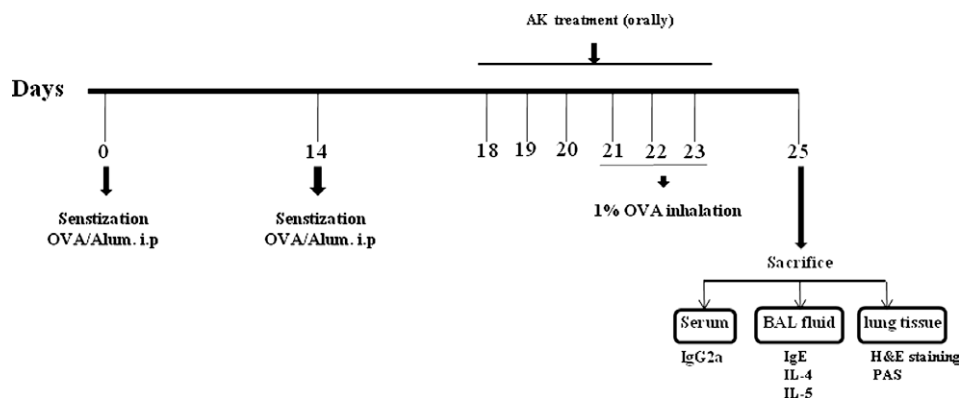


Fig. 1. A mouse model of airway inflammation and treatment with an ethanolic extract of *Alpinia katsumadai* seeds (AK).

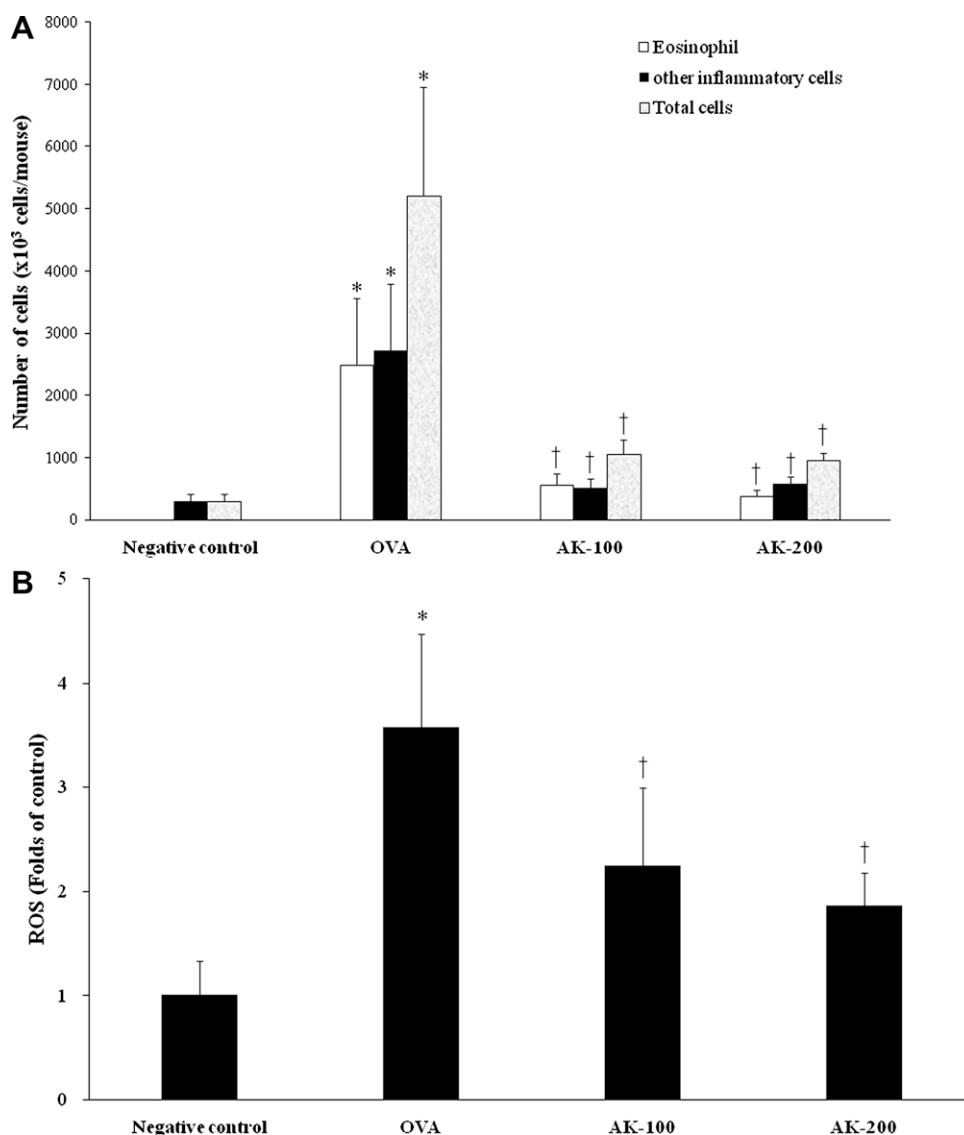


Fig. 2. Effect of AK on the recruitment of inflammatory cells and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. (A) Cells were isolated by centrifugation and stained with Diff-Quik® Stain reagents. Cell numbers were determined using a light microscope to count cells in at least five squares of a hemocytometer. Dead cells, stained with trypan blue, were excluded from the total. (B) Oxidative stress was quantified by DCF fluorescence in BALF. Values represent relative fluorescence units per milligram protein. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AK-1, AK (100 mg/kg) + OVA-sensitized/challenged mice; AK-2, AK (200 mg/kg) + OVA-sensitized/challenged mice. * Significant difference from NC, $P < 0.05$, and † significant difference from OVA, $P < 0.05$.

(Yushiro et al., 1968), monoterpenes, sesquiterpenoids (Saiki et al., 1978; Lawrence et al., 1972), stilbenes and labdanes (Brown and

Rice-Evans, 1998). Yang et al. (1999), also, isolated two novel diarylheptanoids from the seeds of *A. katsumadai*. A pair of unique

sesquiterpene–chalcone conjugates and two novel monoterpene–chalcone conjugates were isolated from the seeds of *A. katsumadai* (Hua et al., 2008, 2009). Recently, an AK antiseptic action (Yang et al., 2009), and topical anti-inflammatory and antipruritic effects (Choi et al., 2009), have been reported. However, there have been no *in vivo* studies of the anti-asthmatic properties of AK. Therefore, in the present study, we employed a bronchial asthma model to examine the ability of AK to control Th2-type cytokines, IgE, oxidative stress, eosinophil infiltration, and other factors that play important roles in allergic inflammation. Our data suggest that AK may be of therapeutic value as a novel treatment of asthma.

2. Materials and methods

2.1. Preparation of *A. katsumadai* seeds ethanolic extract (AK)

Seeds of *A. katsumadai* were purchased in October 2008 from HMAX (Chungbuk, Korea). These materials were confirmed taxonomically by professor Je-Hyun Lee of Dongguk University, Gyeongju, Republic of Korea. A voucher specimen has been deposited at the Korea Institute of Oriental Medicine (KIOM), Daejeon, Korea. Dried seeds of *A. katsumadai* (200 g) were suspended in 70% (v/v) ethanol (2 L) and extracted with sonication for 1 h. This process was repeated three times. The extract was then filtered through filter paper and allowed to evaporate to dryness, resulting in 14.92 g of product. The concentrated AK was freshly dissolved in phosphate-buffered saline (PBS) before use.

2.2. Animals

Specific pathogen-free (SPF) inbred female BALB/c mice (6–7 weeks of age), routinely screened serologically for relevant respiratory pathogens, were purchased from the Daehan Biolink Co. Ltd. (Seoul, Korea). Mice were maintained in conventional animal housing facilities for 1 week before use and were given access to food and water *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and all handling of animals followed the dictates of the National Animal Welfare Law of Korea.

2.3. Sensitization and airway challenge

Mice were received ($n = 7$ or 8) one of the following treatments: (1) sham-sensitization plus challenge with PBS (ip/Neb); (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St. Louis, MO) (ip/Neb); or, (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and AK (100 or 200 mg/kg) p.o. Briefly, mice were sensitized by intraperitoneal (ip) injection of either PBS or 20 μ g OVA emulsified in 2 mg aluminum hydroxide in 200 μ L PBS buffer (pH 7.4), on days 0 and 14. Each mouse was subsequently challenged through the airway with either PBS or OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (Neb) (NE-U12 instrument; Omron Co., Tokyo, Japan) on days 21, 22, and 23 after initial sensitization. To characterize the suppressive effects of AK, mice were sacrificed 48 h after the last challenge (i.e., on day 25). A schematic diagram of this treatment schedule is shown in Fig. 1.

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

Mice were sacrificed with an overdose of 50 mg/kg pentobarbital 48 h after the last challenge, and tracheotomies were performed. After ice-cold PBS (0.6 mL) was instilled into the lungs, BALF was obtained by three aspirations (total volume 1.5 mL) via tracheal cannulation. The volume of each BALF was centrifuged, and the supernatant collected and stored at -70°C . Cell pellets were resuspended in 1.0 mL PBS and 100 μ L aliquots placed onto slides and centrifuged ($200\times g$, 4°C , 10 min) in a cytospin machine (Hanil Science Industrial, Seoul, Korea). After slides were dried, cells were fixed and stained using Diff-Quik[®] Stain reagents (B4132-1A; Dade Behring Inc., Deerfield, IL) according to the manufacturer's instructions. The total number of inflammatory cells was determined by counting cells in at least five squares of a hemocytometer. Dead cells, identified with trypan blue stain, were excluded.

2.5. 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 the highly fluorescent DCF by cellular peroxides including hydrogen peroxide. In brief, cells from BALF were washed with PBS and next treated with 25 μ M DCF-DA for

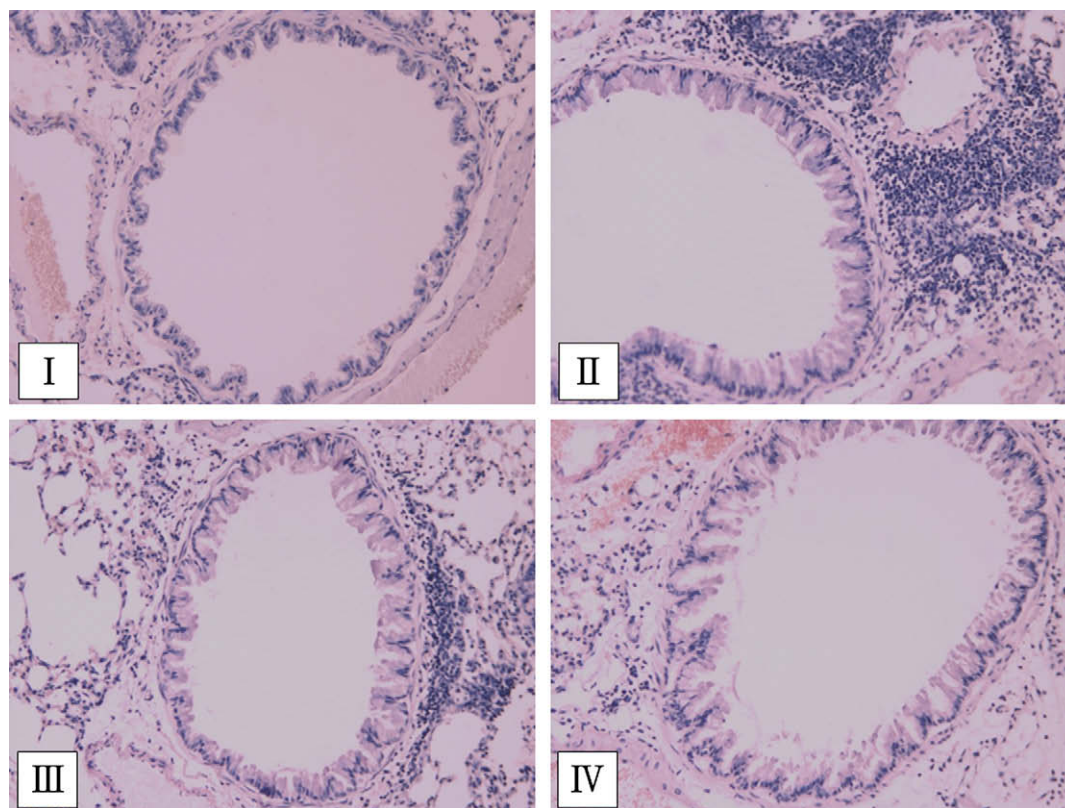


Fig. 3. Effect of AK on the recruitment of leukocytes in lung tissue. Histological examination of lung tissue was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (magnification $\times 200$). I, negative control (PBS only); II, OVA-sensitized/challenged mice; III, AK (100 mg/kg) + OVA-sensitized/challenged mice; IV, AK (200 mg/kg) + OVA-sensitized/challenged mice.

10 min at 37 °C. To measure intracellular ROS activity, fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Waltham, MA).

2.6. Measurement of total IgE and IgG2a in BALF and serum

Total IgE and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with anti-IgE or -IgG2a antibodies (anti-mouse IgE; 10 µg/mL; Serotec, Oxford, UK; or anti-mouse IgG2a conjugated to HRP; Bethyl Laboratories, TX, USA) in PBS-Tween 20. After washing four times, 200 µL of *o*-phenylenediamine dihydrochloride (Sigma) was added to each well. The plate was incubated for 10 min in the dark before absorbance was measured at 450 nm.

2.7. Measurement of Th2 cytokines in BALF

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

2.8. Semi-quantitative RT-PCR

RT-PCR was performed to measure the relative quantities of mRNA encoding IL-4, IL-5, IL-13, and eotaxin in the lung. Total RNA, extracted from lung tissue using TRIzol reagent as described in the manufacturer's instructions, was incubated at 37 °C for 90 min using the first-strand cDNA synthesis kit (Promega, Madison, WI) to synthesize cDNA. The primer sequences used were as follows: mouse IL-4, 5'-TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C-3' and 5'-TGC ATG ATG CTC TTT AGG CTT TCC-3'; mouse IL-5, 5'-ATG ACT GTG CCT CTG TGC CTG GAG C-3' and 5'-CTG TTT TTC CTG GAG TAA ACT GGG G-3'. PCR was carried out with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. β -Actin was used as an internal control for each PCR reaction. The final PCR products were separated on 1% (w/v) agarose gels and visualized by ethidium bromide staining. Densitometric analysis was performed using Quantity One software (Bio-rad Laboratories, Hercules, CA).

2.9. 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 next embedded in paraffin, cut into sections of 4 µm thickness, and stained with H and E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and periodic acid-Schiff (PAS) stain (IMEB Inc., San Marcos, CA). Tissues were subsequently mounted under cover slips using Dako-mounting medium (Dakocytomation, Denmark, CA), and mucus production determined.

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

ALT and AST levels were determined to assess liver function using commercial kits (Beckman Coulter, Inc., Fullerton, CA). The levels of AST and ALT were determined using a Beckman CX4 auto-analyzer (Beckman Coulter, Inc.).

2.11. Statistical analysis

Data are expressed as means $\pm$ SDs (standard deviations of the mean) and differences were considered significant at $P < 0.05$. Statistical significance was determined using Student's two-tailed *t*-test to compare independent means, or a two-way ANOVA test.

3. Results

3.1. Effect of AK on OVA-induced eosinophilia in BALF

To evaluate the suppression of eosinophilia by AK in OVA-challenged mice, cells recruited to the BALF were counted 48 h after the last challenge. As shown in Fig. 2, OVA caused a marked influx of leukocytes into the BALF, as compared with the negative control (naïve mice). OVA-sensitized/challenged mice had a 18-fold increase in total cells recovered in BALF compared with negative control mice. Pretreatment with AK significantly reduced the total

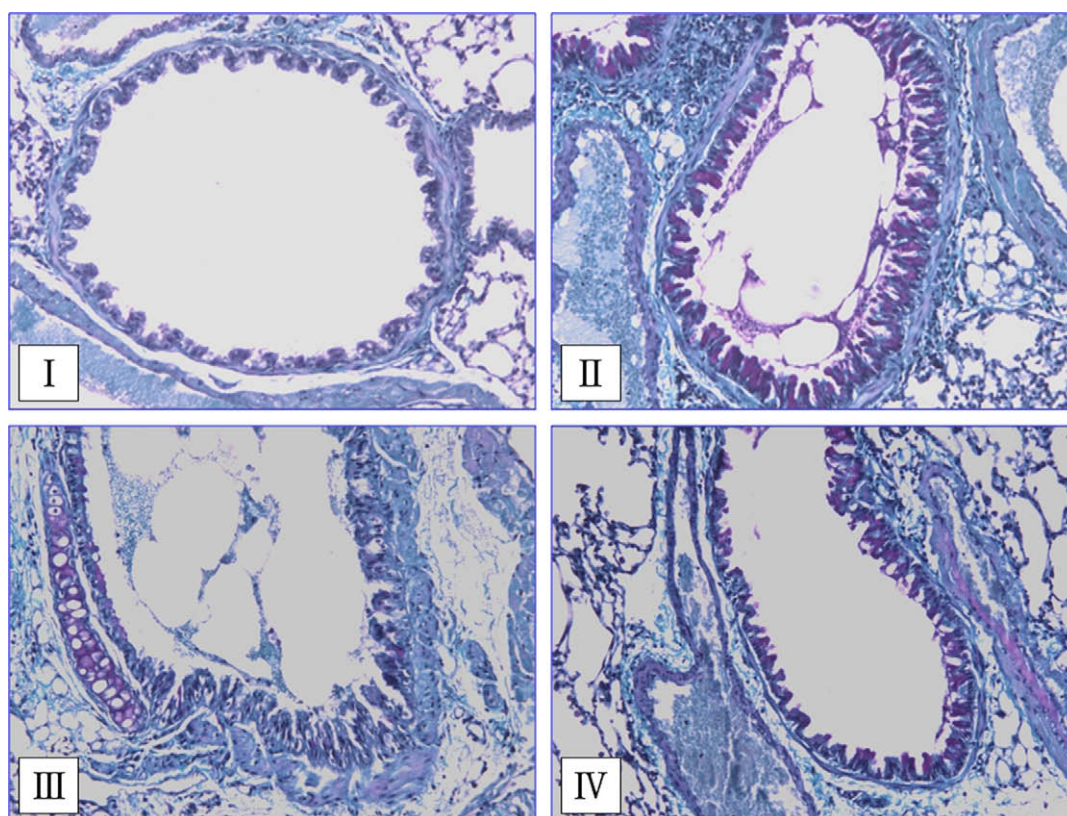


Fig. 4. Effect of AK on mucus production. Histological examination of mucus secretion in lung tissue was measured 48 h after the last OVA challenge in mice. Lung tissues were fixed, sectioned at 4 µm thickness, and stained with PAS reagent to assess mucus production. (magnification $\times 200$). I, negative control (PBS only); II, OVA-sensitized/challenged mice; III, AK (100 mg/kg) + OVA-sensitized/challenged mice; IV, AK (200 mg/kg) + OVA-sensitized/challenged mice.

number of cells, by 49%, in BALF of OVA-sensitized/challenged mice ($P < 0.05$, OVA vs. AK group), in a dose-dependent manner. In addition, the number of eosinophils in BALF from OVA-sensitized/challenged mice showed the most significant proportional increase compared with negative control animals ($P < 0.001$, negative control vs. OVA). Pretreatment with AK significantly reduced eosinophil infiltration into BALF, in a dose-dependent manner.

3.2. Effect of AK on OVA-induced ROS generation in BALF

As illustrated in Fig. 2B, OVA-sensitized/challenged mice markedly increased ROS levels in BALF. However, pre-administration of AK significantly decreased the levels of ROS generation in BALF, in a dose-dependent manner.

3.3. Effect of AK on OVA-induced eosinophilia in lung tissue

To estimate the anti-inflammatory or anti-remodeling effects of AK, lung tissues were collected 48 h after the final OVA challenge (day 25). Light-microscopic analysis revealed that inflammatory cells were present in the peribronchiolar and perivascular tissues of OVA-challenged mice. The number of infiltrating cells, especially eosinophils, found around the airways and blood vessels, was significantly increased in OVA-sensitized/challenged mice, but significantly lower in mice given 100 or 200 mg/kg of AK. No inflammatory cells were observed in control mice (Fig. 3).

3.4. Effect of AK on airway goblet-cell hyperplasia and mucus production

Mucus hypersecretion is a characteristic of airway remodeling. To evaluate the possible suppression by AK of mucus overproduc-

tion caused by goblet-cell hyperplasia, we stained lung sections with PAS, and scored percentages of stained areas in each experimental group. In OVA-treated mice, overproduction of mucus was clearly observed as a violet color in the bronchial airways. In contrast, mucus production was markedly diminished in mice receiving 100 or 200 mg/kg of AK, after OVA challenge (Fig. 4). Negative control mice did not exhibit overproduction of mucus. These observations indicate that AK significantly reduced goblet-cell hyperplasia and mucus hypersecretion in the airway remodeling process.

3.5. Effect of AK on the release of total IgE and IgG2a into BALF and serum

Total IgE and IgG2a levels for each experimental group were determined by ELISA 48 h after the final OVA challenge. OVA-treated mice demonstrated dramatically elevated levels of IgE and IgG2a in BALF and serum as compared with concentrations seen in control mice. When compared with the OVA-challenged

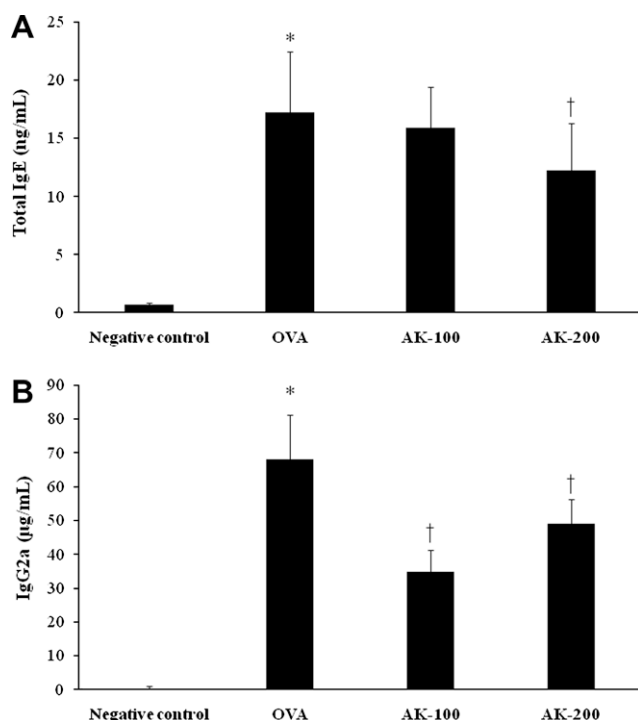


Fig. 5. Effect of AK on total IgE in BALF and IgG2a levels in serum. BALF and serum were collected 48 h after the last OVA challenge of mice. Each sample was analyzed by ELISA. (A) Total IgE level in BALF; (B) IgG2a level in serum. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AK-1, AK (100 mg/kg) + OVA-sensitized/challenged mice; AK-2, AK (200 mg/kg) + OVA-sensitized/challenged mice. Significant difference from NC, $P < 0.05$, and †significant difference from OVA, $P < 0.05$.

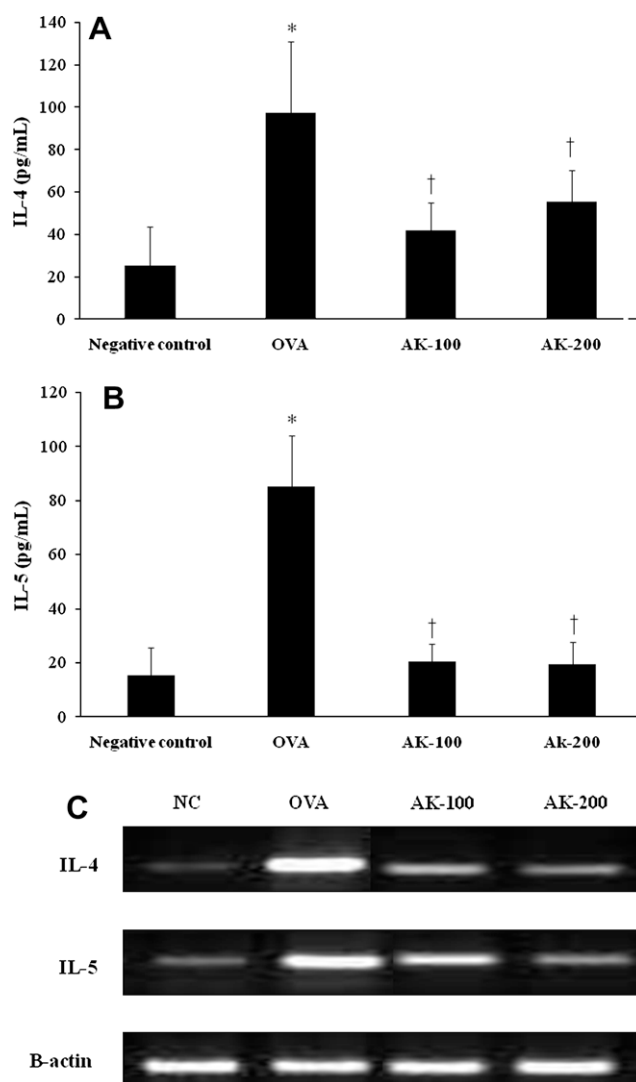


Fig. 6. Effect of AK on cytokine levels in BALF and lung tissue. BALF was collected 48 h after the last OVA challenge of mice. Each sample was analyzed by ELISA. (A) IL-4 level in BALF; (B) IL-5 level in BALF; (C) mRNA levels encoding interleukin IL-4 and IL-5 in lung tissue. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; AK-1, AK (100 mg/kg) + OVA-sensitized/challenged mice; AK-2, AK (200 mg/kg) + OVA-sensitized/challenged mice. Significant difference from NC, $P < 0.05$, and †significant difference from OVA, $P < 0.05$.

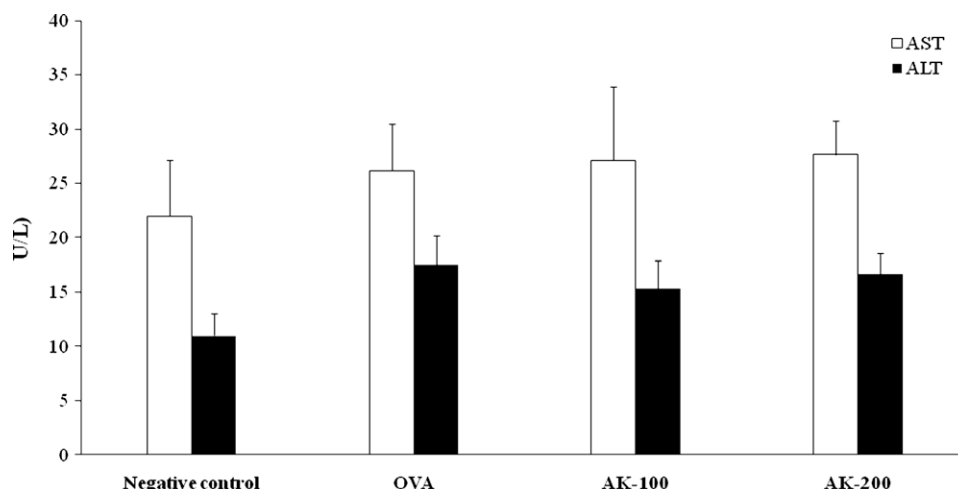


Fig. 7. Effect of AK on AST and ALT levels in serum. Serum was collected 48 h after the last OVA challenge of mice. Each sample was analyzed by using commercial kits (Beckman Coulter, Inc., Fullerton, CA).

group, mice that received AK exhibited reduced total IgE and IgG2a levels in BALF and serum (Fig. 5A and B).

3.6. Effect of AK on cytokine levels in BALF

To determine the effect of AK on cytokine release in OVA-induced asthmatic mice, the levels of cytokines (IL-4 and IL-5) in BALF were measured by ELISA 48 h after the final challenge. As AK treatment reduced the number of inflammatory cells in BALF, we measured the levels of mRNA encoding relevant molecules. Fig. 6A and B show that, following asthma induction, AK significantly inhibited elevation of IL-4 and IL-5.

3.7. Effect of AK on cytokine expression in lung tissues

Since AK reduced the number of inflammatory cells in the BAL fluid, we next investigated both the mRNA and protein expression of the associated molecules. As shown in Fig. 6C, AK significantly inhibited the elevated mRNA expression of IL-4 and IL-5 following asthma induction.

3.8. The toxicity of AK in serum

To confirm the toxicity of AK, we investigated the alteration of ALT and AST levels in serum. ALT and AST levels were determined to assess liver function. The levels of ALT and AST were not altered by AK (Fig. 7).

4. Discussion

Inflammatory diseases of the lung such as asthma and chronic obstructive pulmonary disease (COPD) represent a major worldwide health problem. Although potent anti-inflammatory drugs, such as glucocorticoids, are available to treat asthma, these drugs produce unwanted side effects and exhibit limited efficacy in treatment. The mouse model of allergen-induced asthma is commonly used to investigate the link between airway hyperresponsiveness, inflammation, and remodeling. In most asthma phenotypes, there is an increase in eosinophils in the tissues, blood, and bronchoalveolar lavage fluid (BALF). Moreover, airway inflammation is initiated by the infiltration of eosinophils and other inflammatory cells such as Th2 cells, and their secreted products.

In the present study, we demonstrate that AK treatment in the OVA-induced mouse model resulted in an anti-asthmatic effect, by suppression of elevation in the levels of Immunoglobulin (Ig)E, IgG2a, IL-4 and IL-5, and reduction of inflammatory cell infiltration into BALF and serum. AK substantially inhibited both OVA-induced eosinophilia in lung tissue and mucus hypersecretion by airway goblet cells.

The Th2 cytokines IL-4, IL-5, and IL-13, play particularly important roles in the lungs of asthmatic subjects because all enhance IgE production, IL-5 increases eosinophil accumulation, and IL-13 directly stimulates mucus hypersecretion and airway hyper-reactivity (AHR). AK-treated mice in the OVA-induced mouse model had significantly reduced levels of IgE, IgG2a, IL-4, and IL-5 in both plasma and BALF. AK most probably acts through reduction of levels of Th2 cytokines responsible for eosinophil recruitment into the asthmatic lung. IgE, together with IgG1, has been closely associated with Th2-type responses, whereas IgG2a has been allied with Th1-type responses. Oral administration of AK to OVA-sensitized/challenged mice significantly decreased levels of both IgE and IgG2a in serum, but had no effect on overall IgG levels. The maintenance of total IgG levels may be through increased production of the Th1-type cytokines IL-12 and IFN- γ and/or inhibition of Th2-type IL-5 and IL-13 synthesis. A significant reduction in goblet-cell hyperplasia and mucus production was also seen in AK-treated OVA-induced mice. The significant reduction in mucus synthesis observed in IL-4-receptor- α -deficient mice or following IL-4-receptor treatment suggested that IL-4 and IL-13 played roles in mucus production (Grunig et al., 1998). Eosinophils infiltrating the airway also have an effect on mucus secretion by epithelial goblet cells (Shimizu et al., 2003). Our findings imply that the protective effects of AK on IL-4 production and eosinophilia in BALF, and mRNA expression of IL-4 in lung tissue, attenuated mucus hypersecretion. In addition, histological analysis showed that AK inhibited inflammatory cell infiltration into lung tissue. At sites of inflammation, inflammatory cells, including eosinophils, neutrophils, and macrophages, are capable of generating ROS, which can participate in the development of a variety of diseases, including allergic asthma (Conner and Grisham, 1996; Leusen et al., 1996; Babior, 1999). Such ROS may contribute to tissue injury and inflammatory reactions. Airway epithelial cells are known to produce ROS in response to many stimuli, including weed pollens and viruses (Boldogh et al., 2005; Liu et al., 2004). These ROS oxi-

dize cell membrane phospholipids and generate lipid hydroperoxides which impair membrane functions such as transport, and cause dysfunction of channel proteins and the loss of fluidity leading to tissue injury (Cienciewicki et al., 2008). Therefore, the regulation of intracellular ROS during inflammation may have potential advantages with regard to the treatment of lung inflammatory diseases. Consistent with these findings, our results demonstrate potent anti-oxidant effects of AK in OVA-induced murine asthma model as measured by DCF fluorescence, which consists mainly of recruited inflammatory cells, was significantly increased in OVA-induced mice. AK treatment, however, was able to reduce ROS levels in BALF. Inflammatory cells recruited to asthmatic airways are known to have an exceptional ability to produce ROS. In the present study, AK treatment of OVA-induced mice resulted in a significant reduction in goblet-cell hyperplasia and mucus production. These results suggest that AK can prevent ROS-mediated airway inflammation in an asthmatic mouse model.

In summary, we determined that AK effectively suppressed elevated IgE, IgG2a, and cytokine levels; eosinophilia; and mucus overproduction, in an OVA-induced mouse model of asthma. Our results suggest that AK treatment may induce pharmacological effects that control asthma. We further suggest that AK could be a potential herbal medicine for use in the treatment of asthma and other inflammatory diseases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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