**Abstract.** *Sanguisorba officinalis* L. is known to have anti-inflammatory properties. However, the potential effects of *S. officinalis* against asthma have not been reported. In the present study, we investigated the protective effects and underlying mechanisms of *S. officinalis* in a murine ovalbumin (OVA)-induced asthma model. Mice were sensitized and challenged by OVA inhalation to induce airway inflammation and remodeling. *S. officinalis* ethanolic extract (SOEE) markedly decreased the number of infiltrated inflammatory cells, together with a reduction in the levels of T-helper type 2 cytokines and immunoglobulin E levels. Histopathological studies showed that inflammatory cell infiltration and mucus hypersecretion were inhibited by SOEE. In addition, OVA-induced increases in reactive oxygen species were attenuated by SOEE. All these effects were correlated with heme oxygenase-1 (HO-1) induction by SOEE. These results indicate that SOEE has therapeutic potential against bronchial asthma associated with allergic diseases that is due, at least in part, to HO-1 upregulation.

**Introduction**

Bronchial asthma is a chronic respiratory disease of the airway associated with variable airflow obstruction and airway hypersensitiveness (AHR) and inflammation (1-3). It has been recognized that T-helper type 2 (Th2) cells and their cytokines, interleukin (IL)-4, IL-5 and IL-13 are responsible for the initiation and maintenance of allergic disorders (4-6). IL-4 is essential for B-cell maturation and immunoglobulin E (IgE) synthesis, and participates in the initiation of Th2 inflammatory responses. IL-5 is pivotal for growth, differentiation, recruitment and survival of eosinophils. IL-13 plays a significant role in the effector phase of Th2 responses, characterized by eosinophilic inflammation, mucus secretion and AHR (7). These Th2 cytokines induce, prolong and amplify the inflammatory response by increasing the production of allergic-specific IgE, thereby enhancing recruitment, growth and differentiation of eosinophils, and directly causing AHR (8,9).

The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall by mast cells, lymphocytes and eosinophils, and is associated with the increased expression of several inflammatory proteins, including cytokines, enzymes and adhesion molecules, within the airways (10,11). Oxidative stress is induced by a large variety of oxygen free radicals, including reactive oxygen species (ROS). Accumulating clinical and experimental evidence indicates that ROS plays an essential role in the pathogenesis of airway inflammation (12-14). Eosinophils are known to act as the primary effector cells in the pathogenesis of asthma through the release of ROS as well as specific granule proteins (15).

Heme oxygenase (HO) is the rate-limiting enzyme that degrades heme into carbon monoxide, ferritin and biliverdin (16). The inducible form, HO-1, is expressed in response to various stimuli, such as hydrogen peroxide, heat, heavy metal ions, hyperoxide, endotoxin and inflammatory cytokines. Recently, HO-1 has been shown to have cytoprotective properties as well as anti-inflammatory, anti-oxidant, anti-apoptotic and possible immunomodulatory functions (17-19).

*Sanguisorba officinalis* L. has hemostatic, analgesic and astringent properties, and has been used in traditional oriental medicine for the treatment of diarrhea, chronic intestinal infections, duodenal ulcers and bleeding (20,21). It has also been reported to have antiseptic, anti-inflammatory, anti-allergic and anxiolytic activity (22-25).

Until now, no studies have been reported on the actions of *S. officinalis* ethanolic extract (SOEE) on airway inflammation. Therefore, the aim of the present study was to examine whether administration of SOEE protects against murine airway inflammation, and if so, whether HO-1 is involved in its action. Specifically, we used a murine ovalbumin (OVA)-induced model of bronchial asthma to clarify the anti-allergic and anti-inflammatory properties of SOEE.
Materials and methods

Preparation of S. officinalis L. ethanolic extract (SOEE). S. officinalis L. roots were purchased in October 2008 from HMAX (Chungbuk, Korea). A voucher specimen was deposited at the Korea Institute of Oriental Medicine (KIOM), Daejeon Korea. Fresh S. officinalis L. 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 before extraction. Dried roots of S. officinalis (200 g) were extracted three times with 2 l of 70% EtOH by sonication for 1 h. The extract solution was filtered through filter paper, evaporated to dryness and weighed. The yield of dried extract (24.30 g) was 12.15% of the starting crude material.

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

Sensitization and airway challenge. Mice were divided into groups (n=6 or 7 mice/group) that received the following treatments: (i) sham sensitization plus challenge with phosphate-buffered saline (PBS), (ii) sensitization plus challenge with OVA (100 or 200 mg/kg) or montelukast (30 mg/kg) administered orally. 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, and then 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. Animals were sacrificed 48 h after the final challenge (day 25) to characterize the suppressive effects of SOEE. A schematic diagram of the treatment schedule is shown in Fig. 1.

Inflammatory cell counts in bronchoalveolar lavage fluid (BALF). Mice were sacrificed by overdose (50 mg/kg) of pentobarbital 48 h after the last challenge, and a tracheostomy was performed. Following instillation of ice-cold PBS (0.6 ml) into the lungs, BALF was obtained with three aspirations (total volume 1.8 ml) via tracheal cannulation. BALF cells were collected by centrifuging at 3,000 rpm for 10 min, and the supernatants were collected and stored at -70°C. Cell pellets were suspended in 0.5 ml PBS, and 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. A 100-μl aliquot of resuspended BALF cells was pipetted onto a slide and centrifuged (200 x g, 4°C, 10 min) using a cytospin machine (Hanil Science Industrial, Seoul, Korea). After slides were dried, cells were fixed and stained using Diff-Quik® staining reagents (B4132-1A; Dade Behring Inc., Deerfield, IL, USA), according to the manufacturer’s instructions. Collected cells not used immediately were stored at -70°C for subsequent assays.

Fluorescence assay of ROS with 2',7'-dichlorofluorescein. Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. After incubating BALF with 25 μM DCF-DA for 10 min at 37°C, intracellular ROS activity was measured fluorimetrically by exciting at 488 nm excitation and measuring emission at 525 nm using a fluorescence plate reader (Perkin-Elmer, Waltham, MA, USA).

Measurement of IgE levels in BALF. Total and OVA-specific IgE levels in thawed aliquots of frozen cells were measured using enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with 100 μl/well of a 10 μg/ml IgE solution (Serotec, Oxford, UK) in PBS-Tween-20. Antibodies in BALF were detected using isotype-specific secondary antibodies (anti-mouse IgE; Serotec). After washing four times, 200 μl o-phenylenediamine dihydrochloride (Sigma) was added to each well. The plate was incubated for 10 min in the dark, and absorbance was measured at 450 nm. Total and OVA-specific IgE concentrations were calculated from a standard curve generated using recombinant IgE (Serotec).
Membranes were subsequently washed with TBST, and BioTechnology, CA, USA) for 1 h at room temperature. Secondary antibody (1:2,000 dilution; Santa Cruz incubated with horseradish peroxidase (HRP)-conjugated washed three times with TBST at room temperature and primary antibody (1:1,000 dilution; Abcam Inc., MA, USA). by overnight incubation at 4˚C with a mouse anti-HO-1 150 mM NaCl, 0.1% Tween-20) overnight at 4˚C, followed non-fat dry milk dissolved in TBST (10 mM Tris-HCl pH 7.5, and separated proteins were transferred to polyvinylidene and centrifuged for 15 min at 18,800 x g. HO activity was fluorescence (PVDF) membranes (Amersham Biosciences, were extracted from lung tissue using Trizol reagent, according to the manufacturer's instructions. cDNA was prepared by incubating total RNA at 37˚C for 90 min using a first-strand cDNA synthesis kit (Promega, Madison, WI, USA). Target mRNA was amplified using the following primers, mouse IL-4, 5'-TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C-3' and 5'-TGC ATG ATG CTC TTT AGG CCT TCC-3'; mouse IL-5, 5'-ATG ACT GTG CCT TGG TGC CGT GAG C-3' and 5'-CTG TTT TTC CTG GAG TAA TAC GGG G-3'. ACT cycling conditions were 94˚C for 5 min (initial denaturation), followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 60˚C (IL-4, IL-5 and β-actin), extension at 72˚C for 1 min, and a final extension at 72˚C for 10 min. β-Actin was used as the internal control for individual amplification reactions. The final PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining. Densitometric analyses were performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

**HO enzymatic activity.** HO enzymatic activity in the mouse lung was quantified using a modification of an established technique. Briefly, lungs were homogenized on ice in one volume of 100 mM phosphate buffer containing 2 mM MgCl2, and centrifuged for 15 min at 18,800 x g. HO activity was measured in supernatants using ELISA assays, performed according to the manufacturer's instructions. Activity values were expressed on a per-mg-protein basis. The HO-1 levels in lung tissue were measured using a specific mouse HO-1 ELISA kit (Takara, Japan), as described by the manufacturer.

**Immunoblotting.** 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 μg/ml PMSF, 10 μg/ml pepstatin A, 100 μM Na3VO4). Homogenates were centrifuged at 12,000 x g for 25 min at 48˚C, and the protein concentrations in supernatants were determined using the Bradford reagent (Bio-Rad). Proteins (25 μg) were separated by SDS-PAGE on 10% gels, and separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 5% non-fat dry milk dissolved in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) overnight at 4˚C, followed by overnight incubation at 4˚C with a mouse anti-HO-1 primary antibody (1:1,000 dilution; Abcam Inc., MA, USA). After removal of the primary antibody, membranes were washed three times with TBST at room temperature and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. Membranes were subsequently washed with TBST, and immunoreactive bands were visualized using ECL reagents (Amersham Biosciences).

**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-μm 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, USA) to measure mucus production. Tissues were subsequently coverslip-mounted using Dako mounting medium (Dako Cytomation, Denmark, CA, USA). For immunohistochemistry, paraffin sections were deparaffinized, dehydrated and washed in PBS with 0.3% Triton X-100. Slides were preincubated for 10 min at room temperature with 10% goat serum to block non-specific staining, and then incubated with mouse anti-rabbit HO-1 primary antibody (1/200; Abcam) overnight at 4˚C. After removal of primary antibody, slides were washed and incubated with biotinylated secondary antibody at 37˚C for 1 h, and then with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Excess secondary antibody was removed, and sections were washed with PBS and incubated with 0.05% diaminobenzidine (1:200; Millipore, Billerica, MA) for 10 min. Sections were counterstained, rinsed in PBS to terminate the reaction, and coverslipped for microscopic examination.

**Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum.** Liver function was assessed by measuring ALT and AST levels using commercial kits (Beckman Coulter, Inc., Fullerton, CA, USA) and an auto-analyzer (Beckman CX4).

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

**Statistical analysis.** Data are expressed as means ± standard deviations (SD). One-way ANOVAs were used to compare mean responses among treatments. A statistical probability of P<0.05 was considered significant.

**Results**

**Effects of SOEE on OVA-induced eosinophilia and ROS generation in BALF.** To determine whether SOEE treatment exerted anti-inflammatory effects in the OVA-induced airway inflammation model, we first assessed the number of total cells and eosinophils in BALF after SOEE treatment. OVA challenge induced a marked increase in both total cell and eosinophil numbers compared with the negative control group. These OVA-induced increases in total cell numbers and eosinophils were markedly reduced by SOEE treatment (Fig. 2A). As shown in Fig. 2B, ROS levels in BALF were also markedly increased in OVA-challenged mice. Similar to its effects on OVA-induced eosinophilia, SOEE administration significantly and dose-dependently decreased OVA-induced ROS levels in...
BALF. The effects of SOEE on the recruitment of inflammatory cells and ROS generation in BALF were similar to those of montelukast (positive control).

**Effects of SOEE on cytokine levels in BALF.** To further evaluate the effects of SOEE on the levels of secreted Th2 cytokines, we measured IL-4 and IL-5 levels in BALF by ELISA. OVA challenge led to significant increases in levels of IL-4 and IL-5 in BALF compared with the negative control group; SOEE administration significantly attenuated these increases (Fig. 3A and B). These changes in cytokine protein determined by ELISA were paralleled by changes at the mRNA level. OVA induced marked increases in IL-4 and IL-5 mRNA expression compared with the negative control group, and these increases were blunted by SOEE treatment (Fig. 3C). Our results clearly demonstrate that SOEE exerts effects similar to those of montelukast in this model, suppressing IL-4 and IL-5 expression both in BALF and lung tissue of asthmatic mice.

**Effects of SOEE on airway inflammation and mucus production in lung tissue.** Airway inflammation is characterized by bronchiolar and perivascular inflammatory cell infiltrates. To investigate the extent and anatomical localization of inflammation, we performed histopathological analyses of lung tissues. A comparison with the negative control group demonstrated that eosinophil-rich leukocytes infiltrated peribronchiolar and perivascular connective tissue in OVA-challenged lungs (Fig. 4A; OVA). This infiltration of eosinophil-rich leukocytes in lung tissues was significantly attenuated by treatment with 100 or 200 mg/kg SOEE (Fig. 4A).

To assess the suppressive effect of SOEE on mucus overproduction caused by goblet cell hyperplasia, we stained lung tissue sections with PAS. In OVA-challenged mice, mucus overproduction compared with the negative control group was clearly evident as a violet color in the bronchial airways (Fig. 4B). This OVA-induced increase in mucus production was reduced in a dose-dependent manner by SOEE, as evidenced by a marked decrease in the degree of mucus staining in the lungs of SOEE-treated mice (Fig. 4B).
Effects of SOEE on the release of total and OVA-specific IgE into BALF. To evaluate the effects of SOEE on the release of immunoglobulins, we measured the levels of total and OVA-specific IgE in BALF by ELISA. Total and OVA-specific IgE levels in BALF were markedly increased in OVA-challenged mice compared with negative control mice. The increases in both total (Fig. 5A) and OVA-specific IgE (Fig. 5B) induced by OVA were significantly suppressed by SOEE treatment (200 mg/kg).

Effects of SOEE on HO-1 expression and HO activity. To determine whether the inhibitory effects of SOEE on airway inflammation were related to HO-1 induction, we evaluated
HO-1 expression by Western blotting, immunohistochemistry and ELISA analyses, and determined HO activity. Western blot analysis showed that SOEE treatment induced a dose-dependent increase in lung HO-1 protein levels (Fig. 6A). Consistent with this, immunohistochemistry analysis revealed dose-dependent increases in HO-1 staining in sections from the lungs of SOEE-treated mice compared with those of negative controls (Fig. 6B). This increase in the levels of HO-1 protein was accompanied by a trend toward increased HO activity at a high dose (200 mg/kg) of SOEE, measured by ELISA (Fig. 6C), although this difference did not reach statistical significance.

Cytotoxicity of SOEE in a murine model of allergic asthma. AST and ALT levels, commonly used indicators of hepatotoxicity, were not changed by SOEE treatment, indicating normal liver function (Fig. 7).

Discussion

S. officinalis L. has been reported to have antiseptic, anti-inflammatory, anti-allergic and anxiolytic activities (22-25). However, the effects of S. officinalis on airway inflammation and the possible involvement of HO-1 have not been reported. In the present study, we examined the effects of SOEE in an OVA-induced asthmatic mouse model, demonstrating that SOEE exerts protective effects against airway inflammation by suppressing OVA-induced eosinophilia, oxidative stress, increased IL-4, IL-5 and IgE levels, and mucus overproduction. We further show that HO-1 induction may be at least partly responsible for the action of SOEE. In addition, no liver function abnormalities were detected following treatment. Collectively, our results therefore indicate that SOEE is a safe and effective agent for suppressing the early stages of allergic asthma.

Airway inflammation in asthma is a multicellular process involving mainly eosinophils, neutrophils, CD4+ T lymphocytes and mast cells, with eosinophilic infiltration being the most striking feature (26). A prominent cell in the inflammation of allergic asthma is the eosinophil leukocyte, which is present not only in the airway wall but also in the sputum and BALF of uncontrolled asthma (26,27). Using mice devoid of eosinophils, Lee et al. demonstrated that both AHR and mucus accumulation are eosinophil-dependent (28). Our results show that the increased levels of eosinophils and other inflammatory cells in BALF induced by OVA challenge returned to normal levels by SOEE treatment, indicating that SOEE is an efficacious eosinophil-depleting agent. These results are supported by our histopathological findings, which include the demonstration that SOEE significantly reduced OVA-induced leukocyte infiltration, goblet-cell hyperplasia and mucus overproduction.
Oxidative stress has been suggested to play an important role in the pathophysiology of asthma (29-31). Many substances, including allergens, gaseous pollutants, chemicals, drugs, bacteria and viruses, can cause recruitment and activation of inflammatory cells in asthmatic airways. The activated inflammatory cells generate ROS and release them into surrounding cells. When ROS levels overwhelm host antioxidant defenses, oxidative stress causes many detrimental effects on airway functions, including airway smooth muscle contraction, induction of AHR, mucus hypersecretion, epithelial shedding and vascular exudation (29). Our findings demonstrate that SOEE attenuated OVA-induced production of ROS in a dose-dependent manner, suggesting that the protective effect of SOEE is related to its reduction of oxidative stress.

IL-4 is the Th2 cytokine most important in inducing isotype switching to IgE in B lymphocytes (32,33). It is also involved in the host response to both parasitic infection and allergens (34). IL-4-deficient mice display markedly attenuated disease development and exhibit no significant AHR (35). Similarly, IL-5 plays an important role in airway hyperresponsiveness and is a central factor in mediating eosinophil expansion, priming, recruitment and prolonged tissue survival in response to allergic stimuli (36). IL-4 and IL-5 are thus key regulators of airway inflammation and hyperreactivity in asthma. We show here that SOEE administration opposed the effects of OVA challenge on IL-4 and IL-5, reducing both the levels of secreted IL-4 and IL-5 in BALF, and the levels of IL-4 and IL-5 mRNA in lung tissues. These results indicate that SOEE provides relief against AHR by reducing the levels of the Th2 cytokines IL-4 and IL-5.

IgE levels in BALF are dependent on IL-4, IL-5 and IL-13, and may be considered an additional index of Th2 cytokine secretion in the pathogenesis of asthma. Cross-linking of allergen-specific IgEs on the mast cell surface upon allergen challenge is an important initiating event in the early asthmatic reaction. Therefore, interfering with the action of IgE represents a potential therapeutic approach for treating asthma and other allergic respiratory diseases. Indeed, the IgE antibody has been viewed as a target for novel immunological drug development in asthma (37). Our data show that serum levels of total OVA-specific IgE in BALF were significantly reduced by SOEE pretreatment. These results support the conclusion that SOEE suppressed the generation of a Th2-type immune response, including IgE elaboration, in this animal model of asthma.

HO-1 is induced in a mouse model of asthma, and its deficiency leads to an increase in chronic inflammation and leukocyte recruitment (38,39). Several studies have now shown that the induction of HO-1 helps to ameliorate tissue injury and inflammation in a variety of experimental animal models and in humans (40-44), suggesting that HO-1 induction serves to protect cells from injury or inflammation. This interpretation is also consistent with the observation that HO-1 overexpression reduces ocular inflammation by downregulating proinflammatory cytokines (45). On the basis of these previous reports, we examined whether HO-1 induction might play a role in SOEE-mediated attenuation of airway inflammation. Our finding that HO-1 protein was induced by SOEE administration in association with protection against allergic airway inflammation, ROS generation and Th2 cytokine secretion suggests that the beneficial effects of SOEE are mediated, at least in part, by HO-1 upregulation.

In conclusion, our data provide evidence that induction of HO-1 by SOEE might be responsible for the protective effects of this agent against airway inflammation. Given the safety profile and efficacy demonstrated in this model, SOEE might prove to be a novel and effective phytomedicine for asthma.

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