BIOMOLECULES
& THERAPEUTICS

Anti-Allergic Effect of Oroxylin A from *Oroxylum indicum* Using *in vivo* and *in vitro* Experiments

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Abstract

Oroxylum indicum has long been used in Asian traditional medicine to prevent and treat respiratory diseases, diabetes, diarrhea and other conditions. Oroxylin A is a flavone that is present in *Oroxylum indicum* and in *Scutellaria baicalensis*. Because the root extracts of both plants have been shown to have anti-allergic effects, the authors investigated whether oroxylin A is likely to have beneficial effects on allergic asthma using female Balb/c mice and rat RBL-2H3 mast cells. Antigen-induced degranulation was measured *in vitro* by measuring β-hexosaminidase activity. A murine ovalbumin-induced allergic asthma model was used to test the *in vivo* efficacy of oroxylin A. Sensitization and challenge of ovalbumin induced allergic asthma responses, the accumulations of eosinophils and Th2 cytokine levels in bronchoalveolar lavage fluid and lung tissues. Oroxylin A administration decreased numbers of inflammatory cells, especially eosinophils, and reduced the expression and secretion of Th2 cytokines, including IL-4 and IL-13, in lung tissues and bronchoalveolar lavage fluid. Histologic studies showed oroxylin A reduced inflammatory signs and mucin production in lungs. These findings provide evidence that oroxylin A has potential use as an anti-allergic therapeutic.

Key Words: Oroxylin A, Anti-allergy, Anti-asthma, Degranulation, Mast cell, Oroxylum indicum

INTRODUCTION

Oroxylum indicum has been used for centuries as a traditional medicine in Asia for the prevention and treatment of several diseases, such as, respiratory diseases, arthritis, rheumatism, diabetes, and diarrhea (Dinda *et al*., 2015), and oroxylin A, a major flavonoid in the stems and root bark of *Oroxylum indicum*, has been reported to inhibit the growth of lung, breast, colon, glioma, and hepatocarcinoma cells (Dai *et al*., 2013; Qiao *et al*., 2015; Wei *et al*., 2015; Zou *et al*., 2015; Wei *et al*., 2016). In addition, this flavone has also been found in roots of *Scutellaria baicalensis*, which is also used in traditional medicine (Shah *et al*., 1936; Li and Chen, 2005; Li *et al*., 2011). In mouse macrophages treated with lipopolysaccharide, oroxylin A (Fig. 1A) showed inhibitory activity by up-regulating nuclear factor erythroid 2-related factor 2 (Nrf2) and suppressing the activation of nuclear factor-κB (NF-κB) (Chen *et al*., 2000; Ye *et al*., 2014). In addition, oroxylin A has also been reported to inhibit vascular endothelial growth factor- and lipopolysaccharide-induced angiogenesis (Gao *et al*., 2010; Song *et al*., 2012).

Because extracts of *Oroxylum indicum* and *Scutellaria baicalensis* have been shown to have anti-allergic and anti-asthma effects (Jung *et al*., 2012; Dinda *et al*., 2015), we sought to determine whether oroxylin A is responsible for the anti-allergic effects of *Oroxylum indicum* and *Scutellaria baicalensis*.

MATERIALS AND METHODS

Materials

Oroxylin A were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China) and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Five-week-old female Balb/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a laboratory animal facility in Pusan National University, and provided unrestricted amounts of food and water. The animal protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care Committee

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Table 1. Primers and conditions

Gene	Primer Sequence (5'→ 3')	Size (bp)	Annealing temperature (°C)	Cycles
mIL-4	F: 5'-CTA GTT GTC ATC CTG CTC TTC TTT-3'	360	49	32
	R: 5'-CTT TAG GCT TTC AG GAA GTC TTT-3'			
mIL-5	F: 5'-AGA ATC AAA CTG TCC GTG GG-3'	105	53	33
	R: 5'-GTC TCT CCT CGC CAC ACT TC-3'			
mIL-13	F: 5'-CAG CAT GGT ATG GAG TGT GG-3'	168	49	33
	R: 5'-TGG GCT ACT TCG ATT TTG GT-3'			
mIFN-γ	F: 5'-ACT GGC AAA AGG ATG GTG AC-3'	237	53	33
	R: 5'-TGA GCT CAT TGA ATG CTT GG-3'			
mIL-2	F: 5'- AAC CTG AAA CTC CCC AGG AT-3'	254	53	34
	R: 5'-TCC ACC ACA GTT GCT GAC TC-3'			
mGADH	F: 5'-TTC ACC ACC ATG GAG AAG GC-3'	237	60	27
	R: 5'-GGC ATG GAC TGT GGT CAT GA-3'			

(PNU-IACUC) with respect to ethicality and scientific care (Approval Number ED-PNU2012-0068).

Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), cultured at 37°C in a 5% CO_2 humidified incubator, and maintained in high glucose DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (Lee *et al*., 2013).

Measurement of degranulation

Degranulation was estimated by measuring β-hexosaminidase release (Lian *et al*., 2015). Briefly, RBL-2H3 cells ($2×10^5$ cells/well in 24-well plates) were sensitized with 0.2 μg/ml of monoclonal anti-dinitrophenyl specific mouse IgE (DNP-IgE, Sigma-Aldrich, D8406) overnight at 37°C in a 5% CO_2 incubator. To remove DNP-IgE before stimulation, cells were washed twice with PIPES buffer (pH 7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 0.1% BSA, and 1 mM $CaCl_2$. Cells were then incubated in 400 μl PIPES buffer with different concentrations of oroxylin A and incubated at 37°C for 30 min. Human dinitrophenyl albumin (1 μg/ml; DNP-hAb, Sigma-Aldrich, A6661) was then added, and cells were incubated for another 15 min at 37°C to induce degranulation. Aliquots (25 μl) of medium were then transferred to a 96-well microplate and incubated for 110 min with 25 μl of 5 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, N9376) in 0.1 M citrate buffer (pH 4.5). The reaction was terminated by adding 200 μl of 0.05 M sodium carbonate buffer (pH 10.0; 0.05 MNa_2CO_3/0.05 M $NaHCO_3$). Absorbance (OD) at 405 nm was measured using a microplate reader.

Induction of asthma in BALB/c mice and oroxylin A administration

Six-week old BALB/c mice (22 g) were divided into four groups (n=5), that is, a phosphate-buffered saline (PBS)-injected control group, an OVA-injected asthma group, and OVA-injected plus oroxylin A-treated asthma groups (1 mg/kg and 5 mg/kg). Asthma was induced by the intraperitoneal injection of 50 μg OVA (Sigma-Aldrich, A5503) and 1 mg aluminum hydroxide (Sigma-Aldrich, 239186) administered on days

0 and day 14. From day 28, mice were exposed to nebulized OVA for 20 min for three consecutive days (Aoki *et al*., 2010). Oroxylin A was administered 30 min before OVA challenge by intraperitoneal injection. Two days later (day 32), bronchoalveolar lavage fluid (BALF) was collected from lungs and immune cells in BALF were stained and counted (Lee *et al*., 2013).

On day 32, lungs were excised and fixed with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Tissues were paraffin embedded, sectioned at 4 μm, and sections were thaw-mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at -20°C until further processed.

Histologic analysis of the lung and cell counting in BALF

Lung sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) (Aoki *et al*., 2014). All five animals in each group were analyzed. For PAS staining, sections were deparaffinized, hydrated to water, and then placed in periodic acid solution for 15 min at room temperature. After rinsing in water, sections were stained in Schiff's regent (Sigma-Aldrich, 3952) for 15 min, rinsed in running tap water, and stained in hematoxylin for 90 sec. rinsed, dehydrated, and coverslipped. For H&E staining, sections were washed in lukewarm tap water for 5 min, counterstained with hematoxylin solution (Gill No. 3, Sigma-Aldrich, GHS-3) for 90 sec, washed in running tap water, dehydrated, and coversliped with Permount (Fisher Scientific Inc, SP-15, Waltham, MA, USA).

Lung inflammation was scored using these coverslipped sections by a treatment-blind observer. Degree of lung inflammation was evaluated on a subjective scale of 0-3, as previously described (Tournoy *et al*., 2000; Kwak *et al*., 2003), as follows, 0 was assigned when no inflammation was detected, 1 when occasional cuffing and inflammatory cells were observed, 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and 3 when most bronchi or vessels were surrounded by a thick layer (>five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of lung inflammation score. Five lung sections per mouse were scored and inflammation scores are expressed as averages (Braber *et al*., 2010). Mucin-secreting cells of airways were stained with PAS, and

stained cells were counted in large-caliber preterminal bronchi in at least two lung sections per animal. Lengths of basal lamina of bronchi were measured using Image J software (National Institute of Health, MD, USA). Results are expressed as the mean number of PAS positive cells in bronchi per millimeter of basement membrane (Aoki *et al*., 2013).

Cells in BALF were attached to slide glass using a Cell-spin (5 min, 500 rpm, Hanil Electric, Seoul, Korea), fixed in methanol for 30 sec, processed with May-Grunwald solution (Sigma-Aldrich, 32856) for 8 min, and then with Giemsa solution (Sigma-Aldrich, 32884) for 12 min. Slides were then rinsed and covered.

Reverse transcriptase-PCR

To determine the expressions of asthmatic markers in Th2 cells by RT-PCR, first strand cDNA was first synthesized from total RNA isolated using Trizol reagent (Invitrogen, Waltham, MA, USA). Total RNAs were isolated from all five animals in each group. Synthesized cDNA products, primers for each gene, and Promega Go-Taq DNA polymerase (Madison, WI, USA) were used for PCR. Specific primers and PCR conditions are detailed in Table 1, and primer sequences and PCR conditions are listed in Table 1. PCR was performed over amplification 27 cycles of denaturation at 95°C for 30 s, annealing at 49-53°C for 30 s, and elongation at 72°C for 30 s in an Eppendorf Mastcycler PCR machine (Hamburg, Germany) (Youn *et al*., 2013). Aliquots (7 μL) of the PCR products so obtained were electrophoresed in 1.2% agarose gels and stained with ethidium bromide (Kang *et al*., 2014).

Measurement of cytokines (IL-4 and IL-13)

BALFs from five animals per group were analyzed for IL-4 and IL-13 using eBioscience ELISA kits. Briefly, 96-well plates (NUNC, Penfield, NY, USA) were coated overnight at 4°C with capture antibody for IL-4 (cat. 14-7041-68; eBioscience, San Diego, CA, USA) or IL-13 (cat. 14-7043-68; eBioscience). Following washing, plates were blocked for 1 h at room temperature with blocking buffer. Standard dilutions of cytokines were prepared and added to wells with supernatants of BALF. Plates were incubated for 2 h at room temperature with shaking and washed five times. Biotinylated detection antibody for IL-4 (cat. 33-7042-68C; eBioscience) or for IL-13 (cat. 33-7135-68B; eBioscience) was then added and incubated for 1 h at room temperature with shaking. Plates were then washed five times and avidin-HRP was added for 30 min at room temperature with shaking. Plates were then washed fourteen times and incubated with substrate solution for 15 min at room temperature. Stop solution was then added and absorbance was read at 450 nm (Vo *et al*., 2014).

Statistics

Results are expressed as the means ± SEs of the numbers of indicated determinations. Statistical significances of differences were determined by analysis of variance (ANOVA) with turkey's post hoc test, and statistical significance was accepted for *p*-values <0.05. The analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).
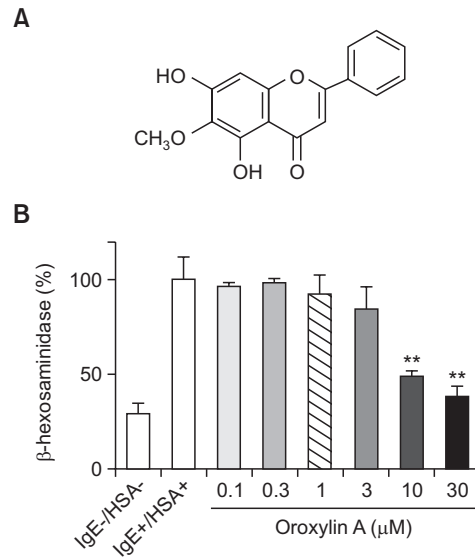

Fig. 1. Structure and effect of oroxylin A on antigen-induced β-hexosaminidase release in RBL-2H3 mast cells. (A) The structure of oroxylin A. (B) RBL-2H3 cells were sensitized for 18 hr with anti DNP-IgE and then challenged with DNP human serum albumin. Oroxylin A was administered at the indicated concentrations 30 min before antigen challenge. Results are presented as means ± SEs of three independent experiments. Statistical significance: **p<0.01 vs. the HSA-treated group.

RESULTS

Oroxylin A inhibited antigen-induced degranulation in RBL-2H3 mast cells

Mast cells function during the initiation and development of allergic response. FcεRI on the membranes of mast cells traps circulating IgE. This antigen exposure induces the cross-linking of cell-bound IgE and causes degranulation (Lane and Lee, 1996), and the degranulations of histamine, leukotrienes, and prostaglandins from antigen-exposed mast cells is a key step in allergic response. Rat basophilic RBL-2H3 leukemia cells, a tumor analogue of mast cells with high FcεRI surface expression, were used to determine whether oroxylin A inhibits antigen-induced degranulation. Degranulation was assessed by measuring β-hexosaminidase activity in media after antigen exposure, as previously described (Lee *et al*., 2015). Oroxylin A inhibited the antigen-induced release of β-hexosaminidase in a concentration-dependent manner (Fig. 1B). Significant inhibition was observed at concentrations above 10 μM (Fig. 1B).

Oroxylin A inhibited eosinophil accumulation in BALF of OVA-induced mice

The inhibitory effect of oroxylin A *in vitro* on antigen-induced degranulation in RBL-2H3 mast cells was confirmed in an OVA-induced asthma mouse model using oroxylin A doses of 1 or 5 mg/kg. Cell numbers and population distributions of cells in BALF were analyzed. As shown in Fig. 2, total cell numbers in BALF increased to 205% in the OVA-induced asthma group versus PBS-treated controls (Fig. 2B). This increase in total cells in the OVA group was inhibited by 1 and 5 mg/kg of oroxylin A by 51% and 84%, respectively (Fig. 2B). Further analyses of cell populations in BALF supported this observa-
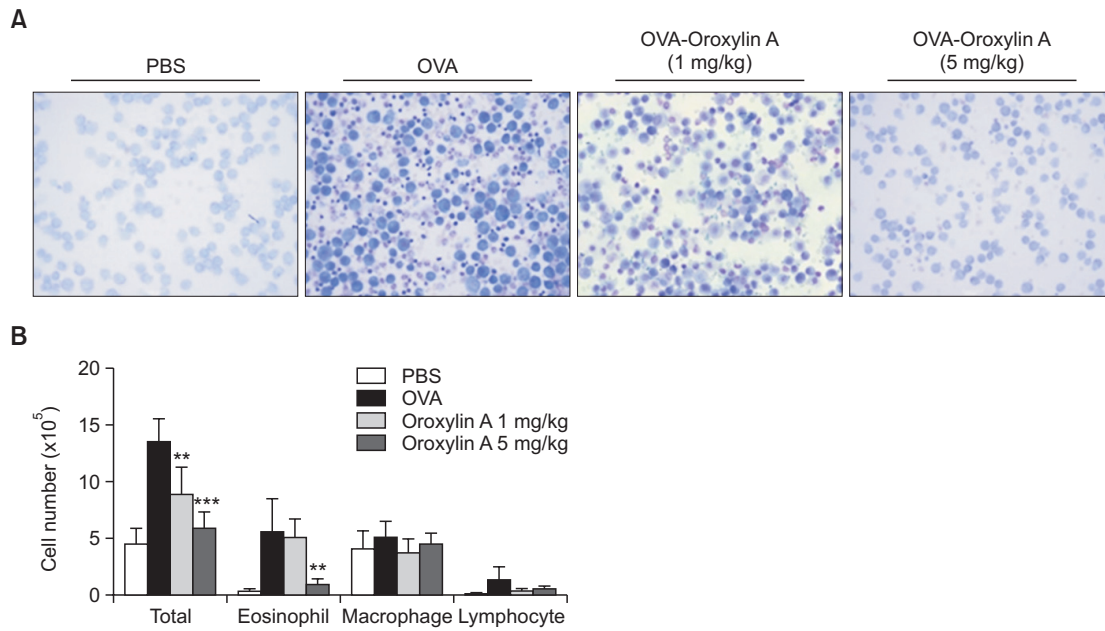
Fig. 2. Effect of oroxylin A on immune cell accumlation in BALF of OVA-induced asthmatic mice. Mice (5 per group) were sensitized with OVA twice by intraperitoneal injection (day 0 and 14) and later challenged with nebulized OVA on days 28, 29, and 30. Oroxylin A was administered at 1 or 5 mg/kg i.p. on these days 30 min before OVA challenge. Cells in BALF were stained using May-Grunwald stain and counted. (A) Total cell counts in the BALF of PBS control mice, OVA-induced asthmatic mice (OVA), and the oroxylin A-treated OVA groups (1 or 5 mg/kg). (B) Cells counts of eosinophils, macrophages, and lymphocytes in the BALF samples obtained from these four groups. The values shown are means ± SEs (n=5). Statistical significance: **p<0.01, and ***p<0.001 vs. the OVA-treated group.

tion (Fig. 2B). In particular, eosinophil numbers in BALF were 1900% in the OVA group versus the PBS controls. In the OVA +5 mg/kg of oroxylin A group, this increase in eosinophil count was inhibited by 89% (Fig. 2B). On the other hand, BALF macrophage and lymphocyte counts were not significantly increased in the OVA group and were not changed by oroxylin A treatment (Fig. 2B).

Oroxylin A inhibited inflammatory signs in the lungs of OVA-induced asthma model mice

Histologic analyses were performed of lung samples from mice 2 days after final antigen challenge. Fig. 3A shows representative H&E staining of lungs in the PBS, OVA, OVA+1 mg/kg oroxylin A, and OVA+5 mg/kg oroxylin A groups. In the figure, eosinophils appear as small black dots, as indicated by arrows. In the PBS group, eosinophils were rarely observed (Fig. 3A). However, in the OVA group, eosinophils densely surrounded bronchioles (Fig. 3A). Treatment with oroxylin A (1 or 5 mg/kg) reduced this eosinophil infiltration in a dose-dependent manner (Fig. 3A). Degrees of lung inflammation were also semi-quantitatively analyzed using a subjective scale of 0-3, as previously described (Tournoy *et al*., 2000; Kwak *et al*., 2003; Choi *et al*., 2016). The inflammation score in the OVA-treated group was nearly 3 and treatment with oroxylin A was found to reduce the inflammation score in a dose-dependent manner (Fig. 3B).

Oroxylin A inhibited mucin secretion in the lungs of OVA-induced asthma model mice

Fig. 4A shows representative PAS staining of the lung samples. PAS staining detected mucous glycoproteins (mucins), which are produced by goblet cells. In the PAS stain-

ing, secreted or stored mucins are stained as purple color. As shown in Fig. 4A, dark stained cells surrounded bronchioles in the OVA group, but not in the PBS group. In contrast, the OVA+oroxylin A groups shows fewer stained cells than the OVA group, indicating inhibition of mucin production by oroxylin A (Fig. 4A). The degree of mucin production was semi-quantitatively analyzed by counting PAS-positive cells on 1 mm lengths of bronchioles (Fig. 4B) (Aoki *et al*., 2013). There were about 100 PAS-positive cells per mm in OVA-treated group, but stained were rare in the PBS group, and oroxylin A reduced PAS-positive cell counts per mm in a dose-dependent manner (Fig. 4B).

Oroxylin A inhibited the production of Th2 cytokines in lung tissues

The cytokines of Th2 cells play key roles in the pathogenesis of asthma (Fish *et al*., 2005; Locksley, 2010). In particular, IL-4, IL-5, and IL-13 are related to eosinophil recruitment and activation, goblet cell metaplasia, and mucus hyper-secretion in epithelial cells, and the proliferation of smooth muscle cells (Fish *et al*., 2005; Locksley, 2010). Therefore, we measured changes in the mRNA levels of IL-4, IL-5, and IL-13 in lung tissues and those of Th1 cytokines, such as, INF-γ and IL-2. As shown in Fig. 5A, 5B, the mRNA levels of IL-4, IL-5, and IL-13 were elevated by 864%, 230%, and 544%, respectively in lung tissues in the OVA group, but these elevations were inhibited by oroxylin A in a dose-dependent manner. The inhibition degrees in the OVA+5 mg/kg oroxylin A group were 69% for IL-4, 92% for IL-5, and 60% for IL-13 (Fig. 5B), and as shown in Fig. 5C, 5D, the mRNA levels of INF-γ and IL-2 were elevated by 33% and 126%, respectively, in lung tissues of the OVA group, that is, they were elevated less than Th2
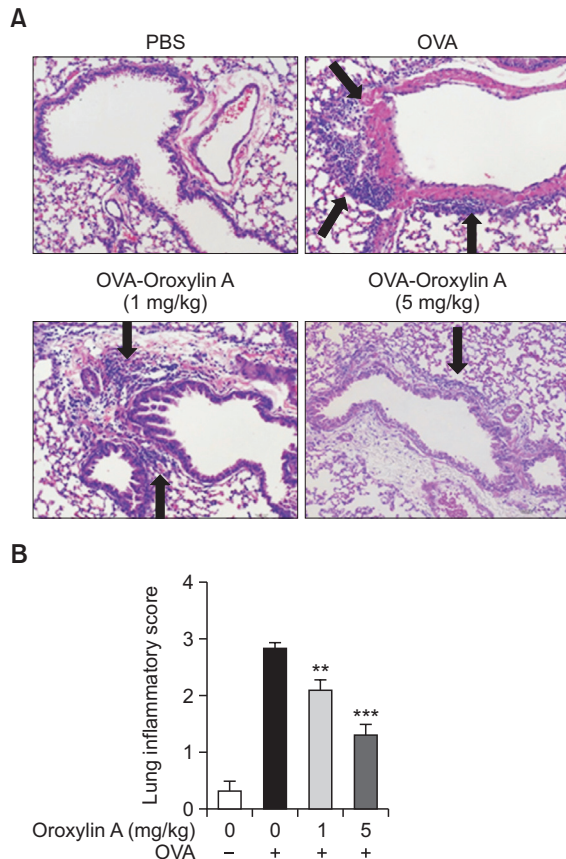
A

PBS   OVA

OVA-Oroxylin A (1 mg/kg)   OVA-Oroxylin A (5 mg/kg)

B

Fig. 3. Protective effect of oroxylin A on airway inflammation. (A) Panels show H&E-stained sections of lung tissues from the PBS group, the OVA group, and the oroxylin A-treated OVA groups (1 or 5 mg/kg). H&E stained sections in the OVA group and oroxylin A-treated OVA groups showed accumulations of eosinophils (small black dots) around bronchioles, whereas in the PBS group eosinophils were rarely observed. More specifically, in the OVA group, eosinophils densely accumulated around bronchioles (arrowed), but in the OVA+1 or 5 mg/kg oroxylin A groups eosinophil accumulations were obviously less than in the OVA group, which suggests oroxylin A improved airway inflammation. (B) Degrees of lung inflammation were evaluated by semi-quantitatively assessing H&E staining; histological findings are reported as scores as described in Materials and Methods and by Tournoy *et al.* (2000) and Kwak *et al.* (2003). The values shown are means ± SEs (n=5). Statistical significance: **$p<0.01$, and ***$p<0.001$ vs. the OVA-treated group.

A

PBS   OVA

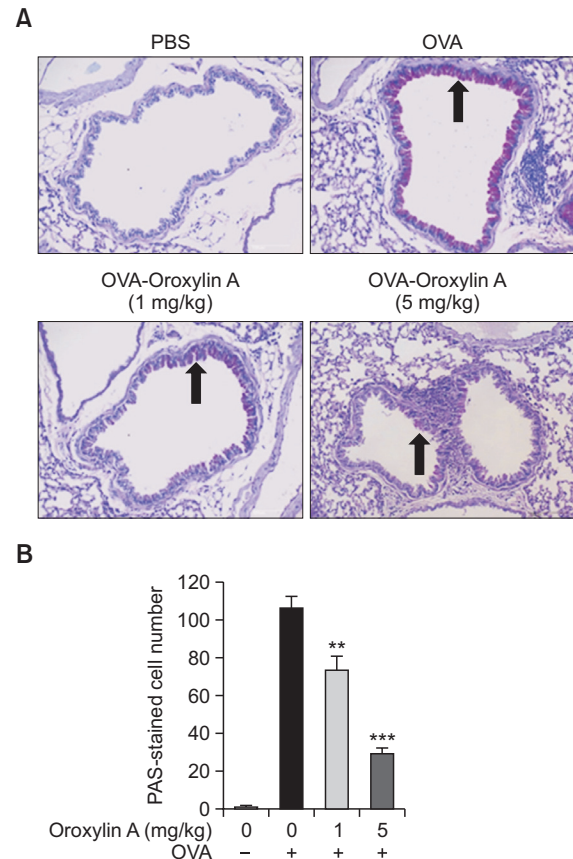OVA-Oroxylin A (1 mg/kg)   OVA-Oroxylin A (5 mg/kg)

B

Fig. 4. Inhibitory effect of oroxylin A on mucous production. (A) Panels show PAS-stained sections of lung tissues from the PBS, OVA, and oroxylin A-treated OVA groups (1 or 5 mg/kg). In the OVA group, but not in the PBS control group, dark purple PAS-staining was observed surrounding bronchioles. In the oroxylin A +OVA groups, staining was markedly less than that observed in the OVA group. (B) Degrees of mucous production were evaluated by counting numbers of PAS-positive cells per mm of bronchiole length. Five mice were used per group. Bar=50 μm. Statistical significance: **$p<0.01$, and ***$p<0.001$ vs. the OVA-treated group.

cytokines. However, these elevations of INF-γ and IL-2 were completely inhibited in the lung tissues of the OVA+ 5 mg/kg oroxylin A group (Fig. 5D).

In order to confirm the effect of oroxylin A on the expressions of Th2 cytokines, the protein levels of IL-4 and IL-13 in BALF were measured by ELISA. As shown in Fig. 6, IL-4 and IL-13 levels were increased by 91% and 164%, respectively, in the OVA group versus the PBS control group (Fig. 6), and these increases were also completely inhibited by oroxylin A at 1 and 5 mg/kg (Fig. 6).

DISCUSSION

Oroxylum indicum and *Scutellaria baicalensis* have been used for centuries as traditional medicines in Asia to treat allergies and asthma (Dinda *et al.*, 2015). Oroxylin A, a flavone present in both plants, has been reported to inhibit the growths of a variety of cancer cells and to inhibit angiogenesis (Gao *et al.*, 2010; Song *et al.*, 2012; Dai *et al.*, 2013; Qiao *et al.*, 2015; Wei *et al.*, 2015; Zou *et al.*, 2015; Wei *et al.*, 2016). In previous studies, oroxylin A was found to exhibit anti-inflammatory effects in RAW264.7 macrophages (Chen *et al.*, 2000; Gao *et al.*, 2010; Song *et al.*, 2012; Ye *et al.*, 2014), and in another pretreatment with oroxylin A was reported to inhibit the lipopolysaccharide (LPS)-induced expressions of COX-2 and iNOS by blocking the binding and transcriptional activation of nuclear factor-κB (NF-κB) (Chen *et al.*, 2000). Subsequently, its anti-inflammatory response was found to be partially mediated by the increased expression of Nrf2 (Ye *et al.*, 2014). Oroxylin A was also found to inhibit *Propionibacterium acnes*-induced productions of pro-inflammatory cytokines, such as, IL-8 and IL-1β in human monocytic THP-1 cells *in vitro* and to have a suppressive effect on *P. acnes*-induced skin inflammation *in vivo* (Tsai *et al.*, 2015). However, the anti-allergic
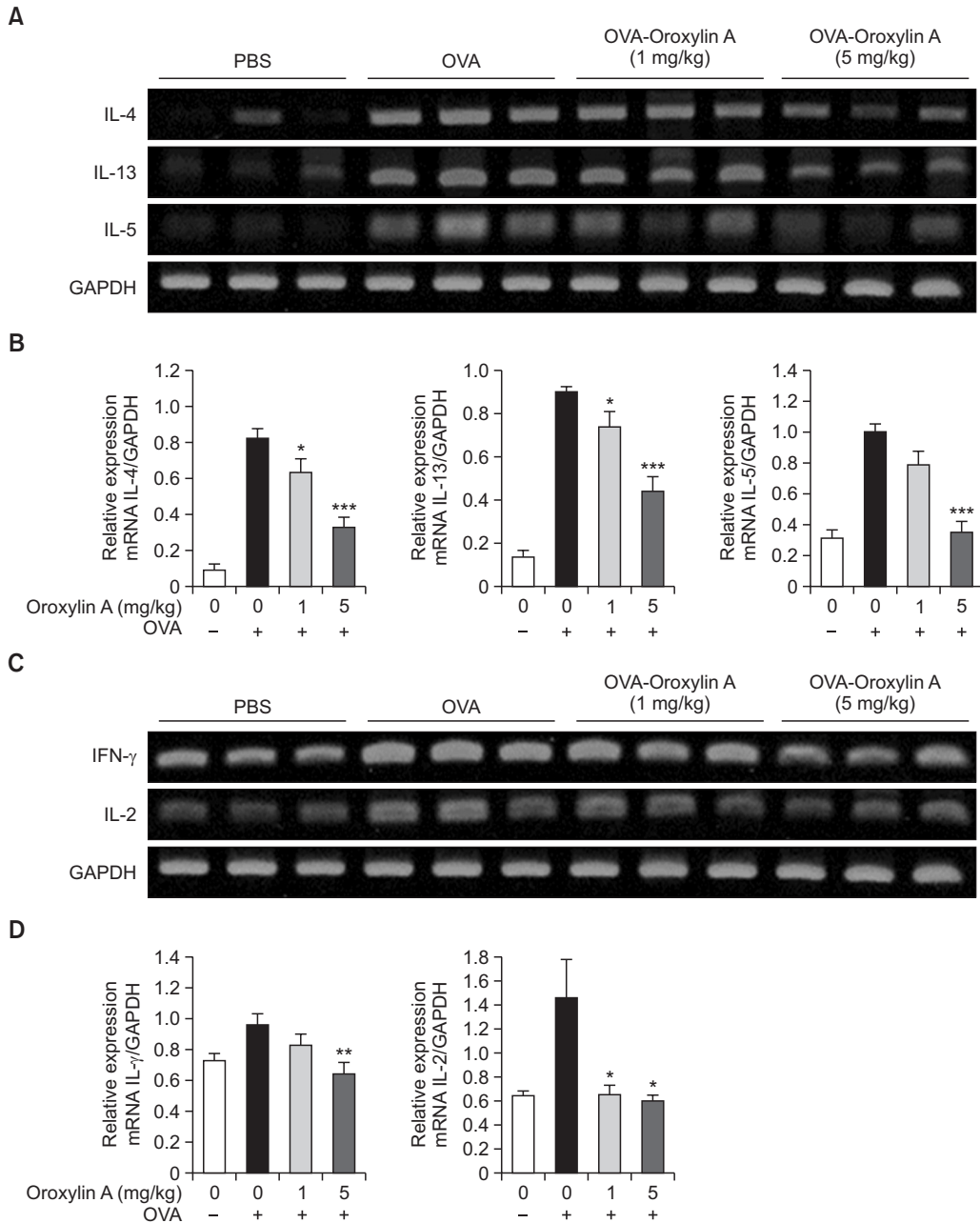
Fig. 5. Oroxylin A inhibited Th2 cytokine expressions at the mRNA level in the lung tissues of the OVA-induced mouse model of asthma. (A) Expressions of IL-4, IL-5, and IL-13 mRNAs in the lung tissues of the OVA-induced model. (B) mRNA levels of IL-4, IL-5, and IL-13 were quantified as ratios versus GAPDH mRNA levels. (C) Expressions of IFN-γ and IL-2 mRNAs in lung tissues. (D) mRNA levels of IFN-γ, and IL-2 were quantified as ratios versus GAPDH mRNA. Each lane represents one of five different mice. The values shown are means ± SEs (n=5). Statistical significance: *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs. OVA-treated mice group. Five mice were used per group.

and anti-asthma effects of oroxylin A have not been previously studied.

In experiments on human fibroblasts, oroxylin A inhibited the productions of IL-4 and TNF-α-induced eotaxin (Nakajima *et al.*, 2001). Because eotaxin is specifically associated with the recruitment of eosinophils to sites of allergic inflammation, it was proposed oroxylin A and baicalein were responsible for the pharmacological efficacy of Scutellaria Root for the treatment of bronchial asthma (Nakajima *et al.*, 2001). In the present study, oroxylin A showed anti-allergic effects *in vitro* and *in vivo*. Furthermore, oroxylin A inhibited antigen-induced degranulation in RBL-2H3 mast cells. This is the first report that oroxylin A inhibits the degranulation of IgE-induced RBL 2H3 cells. IL-4, IL-5, and IL-13 are important Th2 cytokines and contribute to the pathogeneses of allergic diseases (Fish *et al.*, 2005; Locksley, 2010). In the present study, oroxylin A inhibited the inductions of not only Th2 cytokines, which are responsible for IgE production, eosinophil accumulation, and
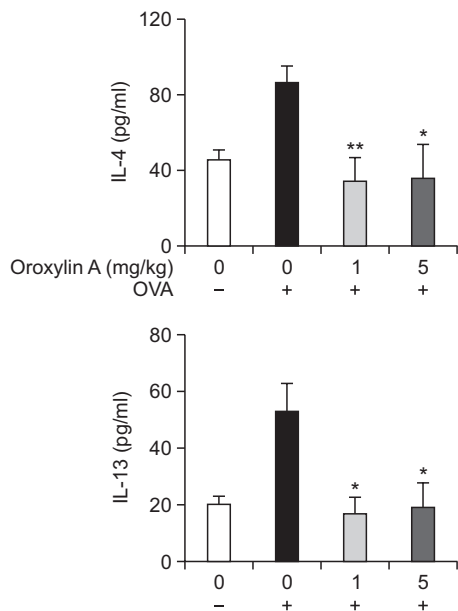
Fig. 6. Effects of oroxylin A on IL-4 and IL-13 levels in BALF. ELISA results for IL-4 and IL-13 were obtained using BALF samples from five mice per group. Values shown are means ± SEs (n=5). Statistical significance: *p<0.05, and **p<0.01 vs. OVA-treated mice.

mucus hypersecretion, but also those of Th1 cytokines, such as, IL-2 and IFN-γ (Jiang *et al.*, 2000) in our OVA-induced mouse model. Furthermore, oroxylin A inhibited IL-4 and IL-13 protein levels in BALF. In addition, histologic studies of lung tissues showed oroxylin A reduced eosinophil accumulation surrounding bronchioles and mucin secretion by goblet cells, which suggests that it may suppress histopathologic changes in lungs, by inhibiting IL-4 and IL-13 levels. These findings indicate that oroxylin A should be viewed as a basis for the developments of anti-allergic and anti-asthmatic treatments.

Recently, Zhou *et al* in online reported similar results with oroxylin A using the same murine OVA-induced allergic asthma model (Zhou *et al.*, 2016). Their results basically support our conclusion of oroxylin A anti-allergic effect. We included *in vitro* experiment, PAS staining, and RT-PCR analysis of Th1 and Th2 cytokines in addition to their experiment. Because they administrated oroxylin A by oral gavage, higher doses of oroxylin A (15, 30, and 60 mg/kg) were used than doses of our i.p. injection (1 and 5 mg/kg). In their results, airway hypersensitivity was measured and inhibition of NF-κB was proposed as a possible mechanism of oroxylin A action (Zhou *et al.*, 2016). However, in our *in vitro* experiment using RBL-2H3 cells, oroxylin A inhibited allergen-induced degranulation. Therefore, oroxylin A may protect allergic asthma not only inhibition of NF-κB but also inhibition of degranulation.

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