

Original Article



Oleanolic Acid Acetate Inhibits Mast Cell Activation in Ovalbumin-Induced Allergic Airway Inflammation

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ABSTRACT

Purpose: Asthma is a complex, heterogeneous chronic inflammatory airway disease with multiple phenotypes. There has been a great progress in managing asthma, but there are still unmet needs for developing uncontrolled asthma treatments. The present study aimed to determine the effectiveness of oleanolic acid acetate (OAA) from *Vigna angularis* against allergic airway inflammation and the underlying mechanism of action with a focus on mast cells.

Methods: To investigate the effect of OAA in allergic airway inflammation, we used the ovalbumin (OVA)-sensitized and challenged mice. To examine allergic airway inflammation associated with immune responses of mast cell activation *in vitro*, various types of mast cells were used. Systemic and cutaneous anaphylaxis models were used for mast cell-mediated hyper-responsiveness *in vivo*.

Results: OAA reduced OVA-induced airway inflammatory responses such as bronchospasm, increase of immune cell infiltration and serum immunoglobulin E and G₁ levels. Especially, OAA decreased the mast cell infiltration, and β -hexosaminidase release as a mast cell activation marker in the bronchoalveolar lavage fluid. OAA inhibited mast cell degranulation in mast cell line (RBL-2H3) and primary cells (rat peritoneal mast cell and mouse bone marrow-derived mast cell). Mechanistically, OAA suppressed intracellular signaling pathways including the phosphorylation of phospholipase C γ and nuclear factor- κ B, resulting from the suppression of intracellular calcium influx and pro-inflammatory cytokine expression. Further, oral administration of OAA attenuated mast cell-mediated systemic and cutaneous anaphylaxis.

Conclusions: Our study showed that OAA can inhibit mast cell-mediated allergic reaction. Consequently, the application of OAA to mast cells for the allergic airway inflammation facilitate a new direction of treating allergic asthma.

Keywords: Allergy, asthma; mast cells; ovalbumin; *Vigna*

INTRODUCTION

Allergic asthma is an airway inflammation characterized by airway constriction, shortness of breath, wheezing, and cough due to various causes including air pollution, dust mite, cigarette smoke, and genetics.¹ This disorder is typically characterized by the increase

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Disclosure

There are no financial or other issues that might lead to conflict of interest.

in CD4⁺ T cells infiltrating the lungs polarized toward a T-helper (Th) 2 cell response, as well as influx of eosinophils. It is mediated by the increased immunoglobulin E (IgE) and altered Th2 cytokines, which leads airway hyperresponsiveness, mucus production, pulmonary inflammation, and remodeling resulting loss of lung functions.² In clinical, many medication such as inhaled steroid and anti-inflammatory medicines are used for treatment of eosinophilic allergic asthma, however, it is limited to symptoms relief.³ Therefore, research of therapeutics for asthma-causing cells instead of eosinophils can suggest a new therapeutic direction for asthma treatment.

Mast cells are related to Th2 responses and play a central role in immediate-type allergic reactions and allergic inflammation. Mast cells release various inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines, and metabolites of arachidonic acid, which act on the vasculature, smooth muscle, mucous glands, and inflammatory cells.⁴ Recent studies showed that mast cells and mast cell-derived mediators were increased on the airway of asthma patients, which can be disease indicator.^{5,6} Mast cells not only play an important role in the early asthmatic reaction, but also the inflammation-inducing leukotriene C4, prostaglandin D2, and inflammatory cytokines synthesized from mast cells activate or recruit surrounding inflammatory cells to maintain persistent allergic inflammation in the airways. It is also known to play a major role in the pathogenesis of intrinsic asthma as well as extrinsic asthma.⁷ Therefore, we believe that the mast cell-focused research is appropriate for the development of anti-allergic airway inflammatory drugs.

Vigna angularis (adzuki bean) is one of the most abundant crops in Asia and has long been used in traditional medicine in Korea, China, and Japan. The water or ethanol (EtOH) extract of *V. angularis* has been found to downregulate adipocyte proliferation and differentiation in obesity,⁸ phosphorylation of the JAK2/STAT3 pathway in arthritis,⁹ and allergic inflammation by inhibiting calcium influx and downstream Lyn/Syk/phospholipase C (PLC) γ signaling in mast cells.¹⁰ *V. angularis* extracts also reduce atopic skin inflammation by regulating Th1 and Th2 responses.¹¹ Oleanolic acid is a bioactive triterpenoid isolated from *V. angularis* and has been studied for its anti-allergic and anti-airway inflammatory properties.^{12,14} Oleanolic acid acetate (OAA) is an improved compound with an acetyl group added to oleanolic acid. We previously reported that OAA could suppress rheumatoid arthritis by regulating T cell immune responses.¹⁵ Based on these reports, we expected that OAA inhibits allergic inflammation involved in mast cell activation. In our study, we verified that OAA not only alleviates allergic airway inflammation, but also has the potential to treatment for allergic asthma through inhibiting mast cell activation.

MATERIALS AND METHODS

Preparation of OAA

OAA was purified from *V. angularis* as previously described.⁹ *V. angularis* was purchased from an herbal medicine store in Jeongeup, Korea. Air-dried and pulverized *V. angularis* (20 kg) was extracted twice with 95% EtOH at 70°C. The residue (100 L) was filtered through a 0.45 μ m filter and evaporated in a rotary evaporator to yield the 95% EtOH extract (2.2 kg). The extract was suspended in distilled water and sequentially fractionated with ethyl acetate, *n*-BuOH, and distilled water. To isolate OAA, the ethyl acetate extract was further chromatographed on a silica gel column (230–400 mesh, 2 kg; Merck, Billerica, MA, USA) using a step gradient of an *n*-hexane-ethyl acetate solvent system (100:1, 80:1, 60:1, 40:1, 20:1, 10:1, and 1:1; each 1 L,

v/v) to yield 5 fractions (H1–H5) by thin-layer chromatography. OAA (1.3 g) was re-crystallized from H-3 in MeOH, and spectroscopic analyses were performed to identify the compound. The purity of the isolated compound used in this study was more than 96%.

Ovalbumin (OVA)-induced allergic airway inflammation

A total of 30 mice were randomly divided into 6 treatment groups with 5 mice in each group as follows: phosphate-buffered saline (PBS) only (negative control); OVA sensitization/challenge only; OVA sensitization/challenge and OAA (2, 10, and 50 mg/kg); and dexamethasone (Dexa) (2 mg/kg). The mice were sensitized on days 0 and 7 by intraperitoneal injection of the OVA mixture (100 µg of OVA and 4 mg of alum adjuvant in 200 µL of PBS). On days 14–16 after sensitization, the mice were challenged for 30 minutes with OVA (3% in PBS). On day 17, the OVA concentration was increased to 5% for the last challenge. All treatments were administered by an aerosol process using an ultrasonic nebulizer (NE-U17; OMRON Corp., Tokyo, Japan). At the time of OVA challenge, the mice were treated with the corresponding drug once daily by oral administration. OAA and Dexa were dissolved in phosphate-buffered saline before use. The dosage of OAA was maintained by previously animal experiments.^{15,47} The control mice were intraperitoneally injected with PBS on days 0 and 7 and challenged with PBS on days 14, 15, and 16. The blood was obtained from the abdominal artery of each mouse after bronchoalveolar lavage fluid (BALF) collection to measure serum Igs.

The BALF was isolated as previously described.¹⁸ At 24 hours after the OVA challenge, the mice were euthanized with CO₂, and bronchoalveolar lavage was performed 3 times through a tracheal cannula with 0.5 mL of autoclaved PBS to obtain the BALF, which was then centrifuged at 400 × *g* for 10 minutes at 4°C. The supernatants were stored at –80°C until analysis, and the pellets were resuspended in PBS (100 µL), centrifuged onto slides, and stained with Diff-Quik staining solution (Sysmex Co., Kobe, Japan). The stained eosinophils, neutrophils, and lymphocytes were counted using a hemocytometer.

β-hexosaminidase release

The release of β-hexosaminidase was used as a marker of mast cell degranulation. The measurement of β-hexosaminidase release in the BALF was performed using only the supernatant. Anti-dinitrophenyl (DNP) IgE (100 ng/mL)-sensitized rat basophilic leukemia (RBL-2H3; 5 × 10⁵ cells/well in 12-well plates) cells, rat peritoneal mast cells (RPMCs; 2 × 10⁴ cells/well in 24-well plates), and mouse bone marrow-derived mast cells (mBMMCs; 5 × 10⁵ cells/well in 12-well plates) were pretreated with OAA for 1 hour after washing 3 times with PBS and stimulated with DNP-human serum albumin (HSA) (100 ng/mL) for 4 hours, 2 hours, and 30 minutes, respectively. After incubation, the cells were separated from the media by centrifugation at 150 × *g* for 5 minutes at 4°C. Then, the supernatant (40 µL) was transferred to 96-well plates and incubated with an equal volume of substrate solution (1 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer, pH 4.5) for 1 hour at 37°C. The cells were lysed with 0.5% Triton X-100 before removing the supernatant to measure the total β-hexosaminidase activity. The reaction was stopped by adding 200 µL of stop solution (0.1 M Na₂CO₃-NaHCO₃, pH 10). The absorbance was measured at 405 nm using a spectrophotometer. The release of β-hexosaminidase in the culture media was determined by combining the absorbance of the culture media and cell lysate.

$$\% \text{ Degranulation} = \text{OD}_{\text{culture media}} / (\text{OD}_{\text{culture media}} + \text{OD}_{\text{cell lysate}}) \times 100$$

Histamine release

To assess mast cell degranulation, the levels of histamines in the culture media and serum were measured. Anti-DNP IgE (100 ng/mL)-sensitized RBL-2H3 cells (5×10^5 cells/well in 12-well plates), RPMCs (2×10^4 cells/well in 24-well plates), and mBMMCs (5×10^5 cells/well in 12-well plates) were pretreated with OAA for 1 hour after washing 3 times with PBS and stimulated with DNP-HSA (100 ng/mL) for 4 hours, 2 hours, and 30 minutes, respectively. The cells were separated from the media by centrifugation at $150 \times g$ for 5 minutes at 4°C . Mouse blood was centrifuged at $400 \times g$ for 15 minutes at 4°C , and the serum was collected. To measure histamine in the separated media and serum, 0.1 N HCl and 60% perchloric acid were added followed by centrifugation. The supernatant was transferred to 1.5 mL tubes, and 5 M NaCl, 5 N NaOH, and *n*-butanol were added and vortexed. Then, the samples were centrifuged. The supernatant was shaken with 0.1 N HCl and *n*-heptane followed by centrifugation. Histamine in the aqueous layer was measured using the *o*-phthaldialdehyde spectrofluorometric procedure as previously described.¹⁹ The fluorescence intensity was detected at an excitation of 380 nm and emission of 440 nm using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Intracellular calcium

The concentration of intracellular calcium was measured using the fluorescence indicator Fluo-3/AM (Invitrogen, Carlsbad, CA, USA). Anti-DNP IgE (100 ng/mL)-sensitized RBL-2H3 cells (2×10^4 cells/well in 96-well plates) were preincubated with Fluo-3/AM (5 μM) for 1 hour at 37°C . The cells were treated with OAA or BAPTA-AM for 1 hour after washing 3 times with PBS and stimulated with DNP-HSA (100 ng/mL). BAPTA-AM, a calcium chelator, was used as a positive control. The fluorescence intensity was measured using a fluorescence plate reader at an excitation of 485 nm and emission of 510 nm. The intracellular calcium level in untreated control cells was assigned a relative absorbance value of 1.

Statistical analysis

Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed with one way analysis of variance, followed by Duncan's multiple range tests. $P < 0.05$ was considered to indicate significance.

RESULTS

Effect of OAA on OVA-induced allergic airway inflammation

The allergic airway inflammation showed the infiltrated immune cells such as eosinophils, neutrophils, and lymphocytes initiate airway smooth muscle hypertrophy and hyperplasia, increased mucus production, edema, thickening of the sub-basement membrane, and fibrosis.^{20,21} To evaluate the effects of OAA, total and differential counts of inflammatory cells were performed on the BALF. **Supplementary Fig. S1A** shows the scheme of animal experiments. We observed increased airway constriction and inflammatory cell infiltration in the lung sections of OVA-induced allergic airway inflammation mice compared with those of control mice. In addition, the number of inflammatory cells such as eosinophils, neutrophils, and lymphocytes in the BALF was significantly increased. However, OAA administrated mice showed a dose-dependent decrease in OVA-induced asthmatic inflammation (**Fig. 1A-C**). In addition, IgG₁ and IgE levels were higher in the serum of OVA-induced mice than in the serum of control mice. OAA administrated mice showed the reduction of IgG₁ and IgE levels in the serum (**Fig. 1D**). Mch-induced pulmonary resistance is a widely used method for investigating

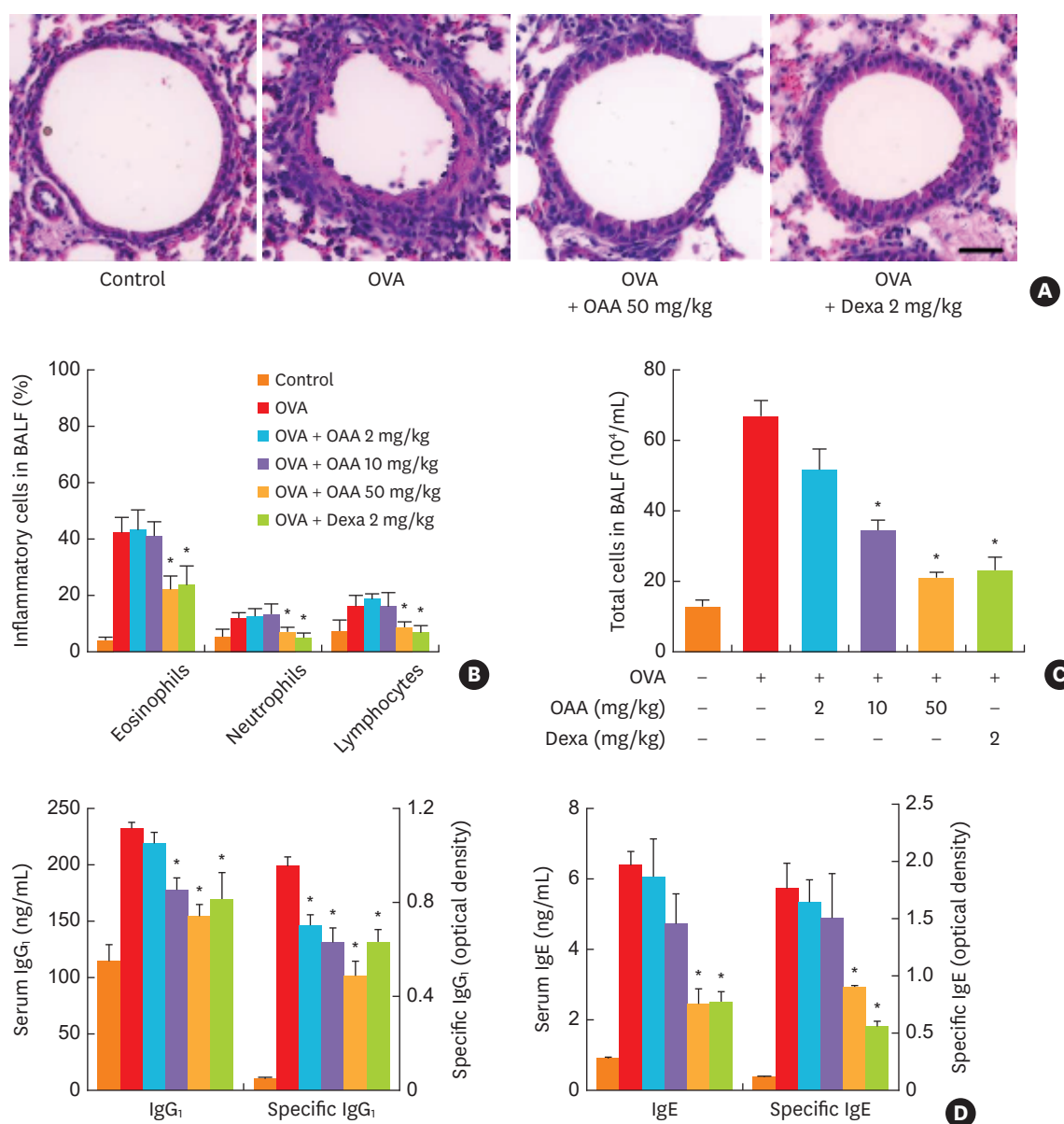


Fig. 1. Effects of OAA on OVA-induced airway inflammation. Mice were sensitized by intraperitoneal injection of an OVA-alum adjuvant mixture on days 0 and 7. All mice were challenged with 3% OVA on days 14–16 and challenged with 5% OVA on day 17 using an ultrasonic nebulizer. At the time of challenge, OAA or Dexa was orally administered for 17 days. At 24 hours after the final challenge, the mice were sacrificed, and the BALF was collected, which was separated into the supernatant and cell pellet. (A) Representative images of lung sections stained with hematoxylin and eosin ($\times 200$ magnification, scale bar: 0.1 mm). (B, C) Counting of Diff-Quik-stained immune cells in the resuspended pellet of the BALF. (D) Blood was obtained from the abdominal artery of each mouse after BALF collection to measure serum immunoglobulins. The collected blood was immediately centrifuged at $400 \times g$ for 10 minutes at 4°C , and the bottom layer was used. Igs were analyzed by enzyme-linked immunosorbent assay. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexa, dexamethasone; BALF, bronchoalveolar lavage fluid; Ig, immunoglobulin.

* $P < 0.05$.

lung dysfunction.²² In our study, OVA-induced allergic airway inflammation showed the increased pulmonary resistance and elastance (**Supplementary Fig. S2A and B**), and decreased compliance (**Supplementary Fig. S2C**), however, OAA administration diminished those effects.

Effect of OAA in mast cell activation on OVA-induced allergic airway inflammation

Previous report showed that the numbers of lung mast cells were increased in OVA-induced airway inflammation mice.²³ To determine whether allergic airway inflammation is related to mast cells, we examined mast cell infiltration into the lungs by toluidine blue staining. In lung sections with OVA-induced airway inflammation, the infiltration of mast cells was markedly increased. However, the infiltration of mast cells was reduced in OAA-pretreated lung sections compared with OVA-induced lung sections (**Fig. 2A and B**). We also measured the level of β -hexosaminidase, a marker of mast cell degranulation, in the BALF.²⁴ β -hexosaminidase level was higher in the BALF of OVA-induced mice than in the BALF of control mice; however, it was decreased by OAA treatment (**Fig. 2C**).

Effect of OAA on mast cell degranulation

To determine the association of the anti-allergic airway inflammatory properties of OAA with the regulation of mast cell activation, we examined the effect of OAA on mast cell degranulation in various types of mast cells. We assessed the effect of OAA on the cell viability of mast cells by MTT assay. RBL-2H3, RPMCs, and mBMMCs were incubated for 24 hours with various concentrations (0.1–100 μ M) of OAA. As shown in **Supplementary Fig. S3**, up to 10 μ M of OAA did not exert cytotoxicity.

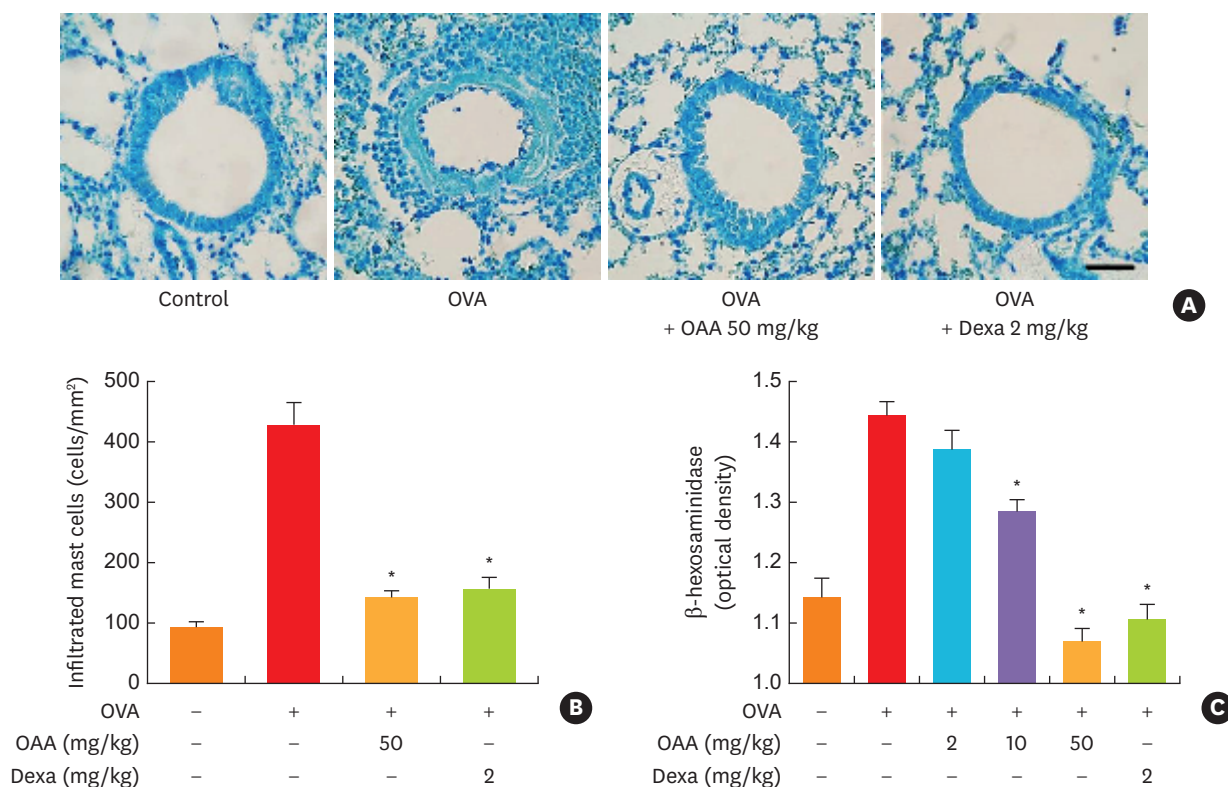


Fig. 2. Effect of OAA in mast cell activation on OVA-induced allergic airway inflammation. (A) The infiltration of mast cells into the lung was observed by toluidine blue-staining of lung sections ($\times 200$ magnification, scale bar: 0.1 mm). (B) Mast cells were counted in the toluidine blue-stained lung sections. (C) To evaluate mast cell degranulation, β -hexosaminidase level in the bronchoalveolar lavage fluid was measured. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexa, dexamethasone.

* $P < 0.05$.

DNP-HSA-challenged RBL-2H3 released high levels of β -hexosaminidase and histamine. Histamine plays an important role in normal human physiology as well as in various pathologies.²⁵ OAA (0.1–10 μ M) reduced β -hexosaminidase and histamine release in DNP-HSA-challenged cells in a concentration-dependent manner (**Fig. 3A**). The inhibitory effect of OAA on degranulation from mast cells was confirmed using mature mast cells (RPMCs; **Fig. 3B**) and mast cell progenitors isolated from the bone marrow (mBMMCs; **Fig. 3C**).

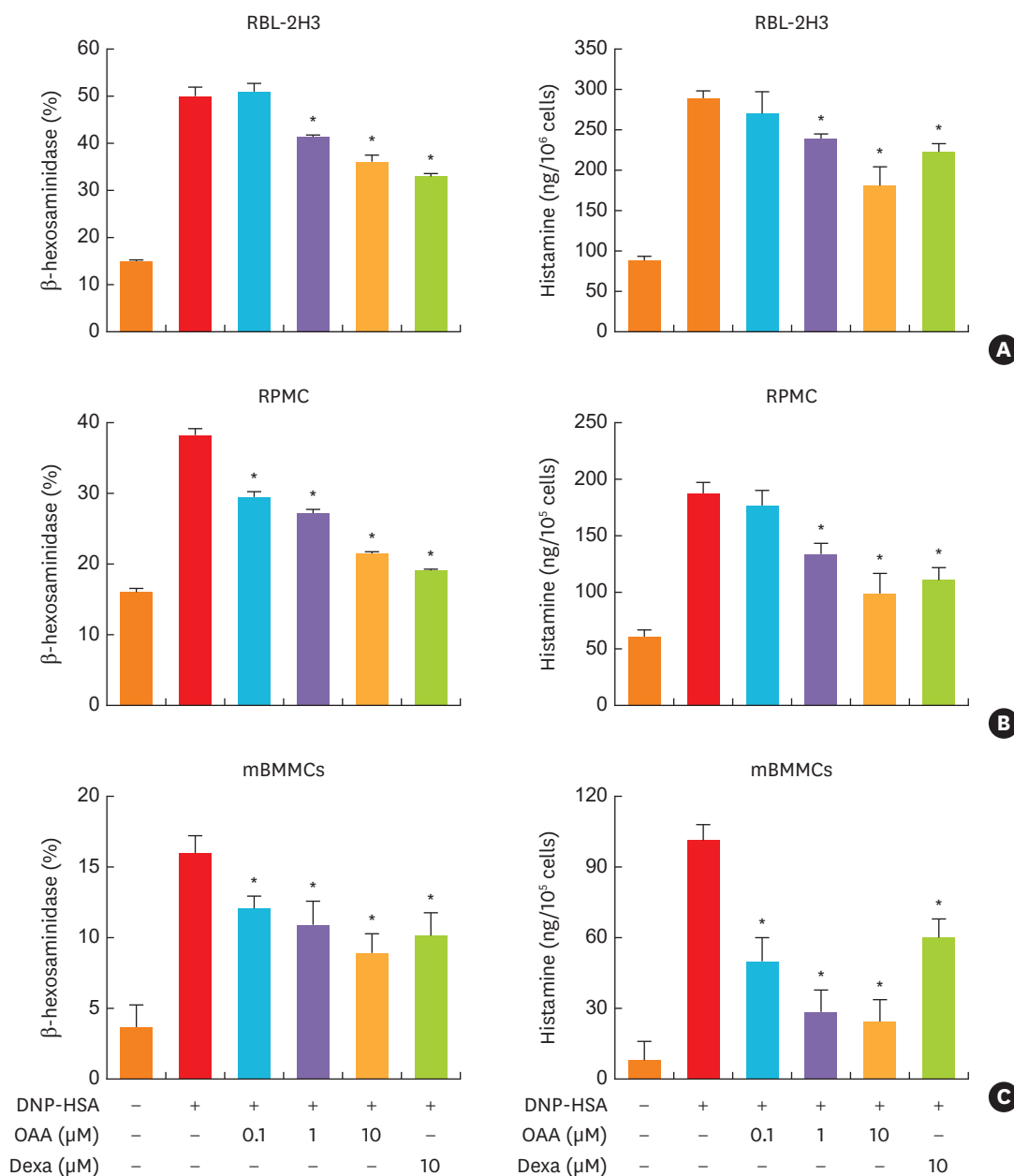


Fig. 3. Effects of OAA on mast cell degranulation. (A) RBL-2H3, (B) RPMCs, and (C) mBMMCs were pretreated with or without OAA and Dexa. β -hexosaminidase levels were determined using a spectrophotometer. Histamine levels were determined using a fluorescence plate reader. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexa, dexamethasone; RBL, rat basophilic leukemia; RPMC, rat peritoneal mast cell; mBMMC, mouse bone marrow-derived mast cell; DNP, dinitrophenyl; HSA, human serum albumin.

* $P < 0.05$.

Effect of OAA on the expression and secretion of pro-inflammatory cytokines

The expression of tumor necrosis factor (TNF)- α and interleukin (IL)-4, major cytokines in the allergic inflammatory reaction, was evaluated. We assessed the effects of OAA on pro-inflammatory cytokine expression in RBL-2H3 by quantitative polymerase chain reaction and enzyme-linked immunosorbent assay. The gene expression of TNF- α and IL-4 was increased following challenge with DNP-HSA (**Fig. 4A**). However, OAA (0.1-10 μ M) inhibited the expression of these cytokines in a concentration-dependent manner. The results were consistent with TNF- α and IL-4 levels in the culture media (**Fig. 4B**).

Effect of OAA on the mast cell signaling pathway

To determine the mechanisms by which OAA inhibits the production of pro-inflammatory cytokines and degranulation, we investigated the effect of OAA on known intracellular signaling molecules. The phosphorylation of Lyn, Syk and PLC γ was increased following challenge with DNP-HSA. The pre-treatment of OAA decreased phosphorylation of PLC γ , but not effect on Lyn and Syk levels (**Fig. 4C**). Moreover, intracellular calcium levels were elevated following challenge with DNP-HSA and were reduced by pretreatment with OAA (**Fig. 4D**).

The phosphorylation of Akt, IKK α/β , degradation of I κ B α , and nuclear translocation of p65 NF- κ B was increased following challenge with DNP-HSA. However, the phosphorylation of these molecules were suppressed by pretreatment with OAA (**Fig. 4E**). To demonstrate the inhibitory effects of OAA on NF- κ B, we performed an NF- κ B-dependent gene reporter assay. RBL-2H3 were transiently transfected with an NF- κ B luciferase reporter construct or empty vector. Sensitization with anti-DNP IgE and exposure of cells to DNP-HSA increased the luciferase activity of cells transfected with the NF- κ B luciferase reporter construct. However, pre-treatment of OAA inhibited luciferase activity (**Fig. 4F**). Inhibitors for Src family protein kinases (PP2) and NF- κ B (PDTC) were used as positive controls.

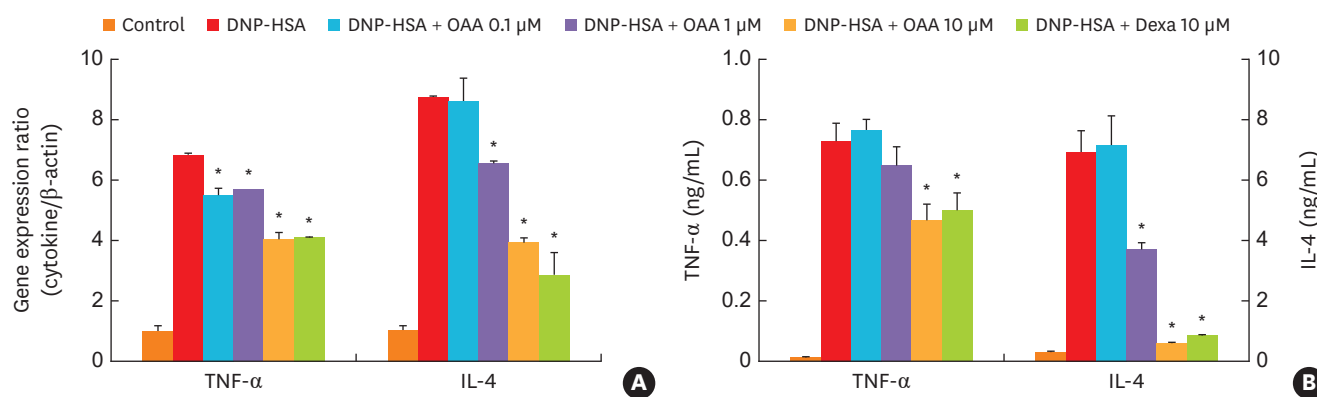


Fig. 4. Effects of OAA on pro-inflammatory cytokine expression and signaling protein activation. (A) The gene expression of pro-inflammatory cytokines was analyzed by quantitative polymerase chain reaction. (B) The secretion of pro-inflammatory cytokines was measured by enzyme-linked immunosorbent assay. (C, E) The protein was separated into nuclear or cytosolic fractions, and Western blotting was performed to analyze protein samples with specific antibodies (cytosolic: Lyn, Syk, PLC γ , Akt, IKK α/β , and I κ B α ; nuclear: NF- κ B). β -actin and lamin B1 were used as loading controls. The band shown is representative of 3 independent experiments. The band intensity of Western blotting was quantified by ImageJ software. The representative band intensity was digitized to the relative intensity. (D) RBL-2H3 were preincubated with Fluo-3/AM. Intracellular calcium was detected using a fluorescence plate reader. BAPTA, a calcium chelator, was used as a positive control. (F) RBL-2H3 cells were transiently transfected with the NF- κ B luciferase reporter construct or empty vector and stimulated with DNP-HSA to perform NF- κ B-dependent transcriptional activity assay. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexa, dexamethasone; RBL, rat basophilic leukemia; RPMC, rat peritoneal mast cell; mBMMC, mouse bone marrow-derived mast cell; DNP, dinitrophenyl; HSA, human serum albumin; N-, nuclear; PLC, phospholipase C; NF, nuclear factor.

* $P < 0.05$.

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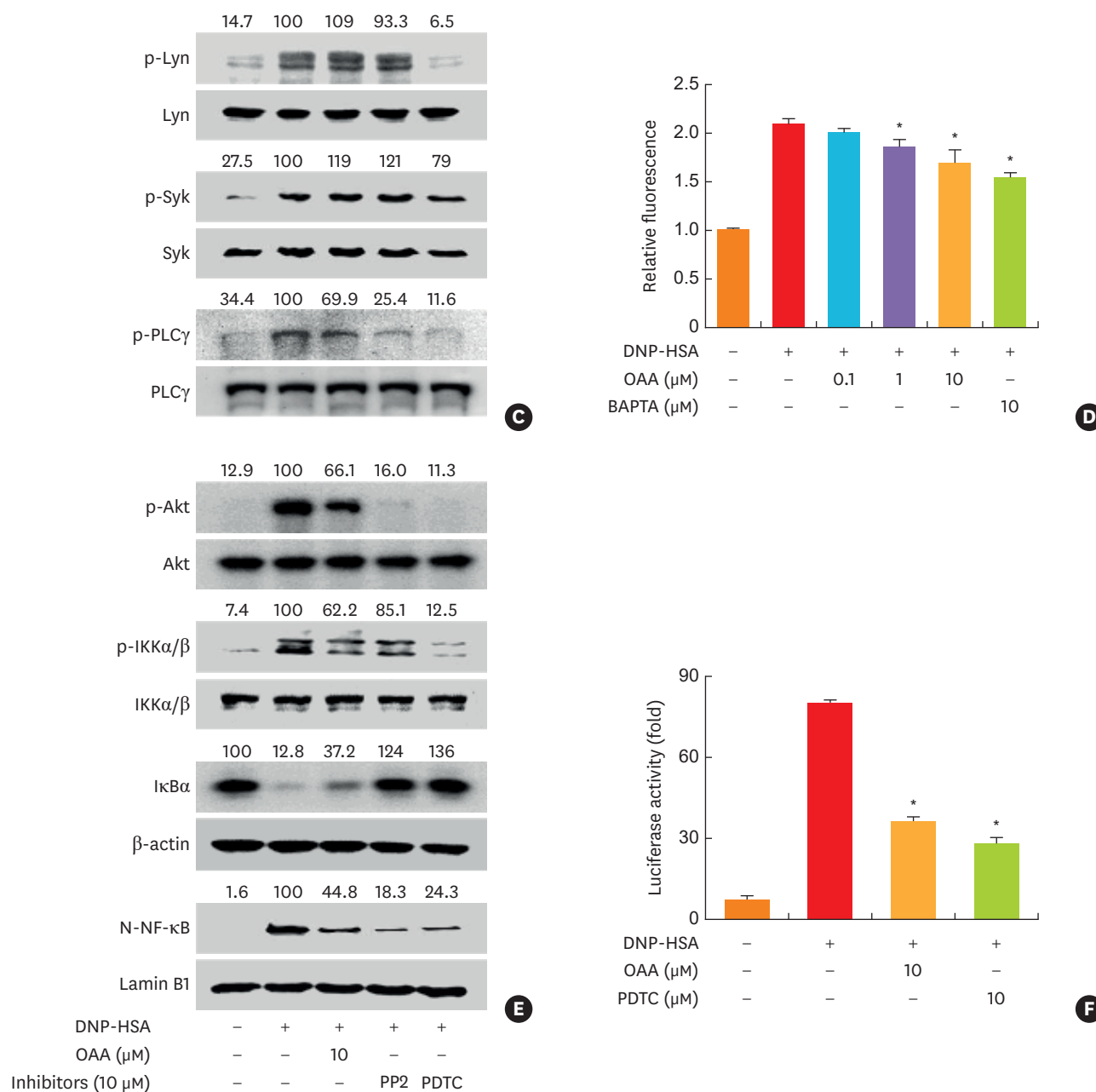


Fig. 4. (Continued) Effects of OAA on pro-inflammatory cytokine expression and signaling protein activation. (A) The gene expression of pro-inflammatory cytokines was analyzed by quantitative polymerase chain reaction. (B) The secretion of pro-inflammatory cytokines was measured by enzyme-linked immunosorbent assay. (C, E) The protein was separated into nuclear or cytosolic fractions, and Western blotting was performed to analyze protein samples with specific antibodies (cytosolic: Lyn, Syk, PLCγ, Akt, IKKα/β, and IκBα; nuclear: NF-κB). β-actin and lamin B1 were used as loading controls. The band shown is representative of 3 independent experiments. The band intensity of Western blotting was quantified by ImageJ software. The representative band intensity was digitized to the relative intensity. (D) RBL-2H3 were preincubated with Fluo-3/AM. Intracellular calcium was detected using a fluorescence plate reader. BAPTA, a calcium chelator, was used as a positive control. (F) RBL-2H3 cells were transiently transfected with the NF-κB luciferase reporter construct or empty vector and stimulated with DNP-HSA to perform NF-κB-dependent transcriptional activity assay. Each data point represents the mean ± standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexa, dexamethasone; RBL, rat basophilic leukemia; RPMC, rat peritoneal mast cell; mBMMC, mouse bone marrow-derived mast cell; DNP, dinitrophenyl; HSA, human serum albumin; N-, nuclear; PLC, phospholipase C; NF, nuclear factor.

* $P < 0.05$.

Effect of OAA on systemic and local anaphylaxis

To determine if the OAA effect is related to mast cell suppression, we used the mast cell-mediated anaphylaxis model. Active systemic anaphylaxis (ASA) model is widely used to investigate allergic response, which is leaded production of serum IgE by OVA expose. After intraperitoneal injection with OVA, the mice were observed for 90 minutes. OVA-challenged mice showed hypothermia compared with control mice. However, hypothermia in OAA-treated mice was suppressed (**Fig. 5A and B**). The serum histamine levels of OVA-challenged mice were elevated compared with those of control mice. In contrast, OAA-treated mice had decreased histamine levels (**Fig. 5C**). IL-4 is a key cytokine responsible for the production of IgE from plasma cells and triggers the mast cell-mediated allergic response. Our results demonstrated that IL-4 and OVA-specific IgE levels were increased after challenge with OVA and were decreased following the administration of OAA (**Fig. 5D and E**).

The passive cutaneous anaphylaxis (PCA) model is a key *in vivo* model of IgE-mediated local allergic reaction mediated by the mast cell activation. After the injection of 4% Evans blue mixed with antigens, the PCA reaction site showed that vascular permeability was markedly increased, as indicated by the amount of Evans blue dye extravasation. When OAA was orally administered to these mice, vascular permeability of the ears was attenuated, as demonstrated by the extent of ear blue staining and intensity of Evans blue extraction of the ears (**Supplementary Fig. S4A and B**). To confirm the effect of OAA on mast cell activation *in vivo*, compound 48/80-induced systemic anaphylaxis model also employed. Compound 48/80 induced an increased mortality rate and serum histamine level; however, OAA-administered mice had a reduced mortality rate (**Supplementary Table S1**) and serum histamine level (**Supplementary Fig. S4C**).

DISCUSSION

Allergic airway inflammation is a complex process beginning with the activation of allergen-specific Th2 cells by antigen-presenting cells. In these immune responses, the activation and infiltration of innate immune cells such as eosinophils, neutrophils and mast cells occur.^{26,27} In particular, mast cells have long been regarded as important cells involved in allergic reactions as well as allergic airway inflammation.² The activated mast cells can release various mediators, including histamine, leukotrienes, prostaglandins, cytokines, chemokines, chymase, and tryptase, which induce bronchoconstriction, mucus secretion, inflammation, and airway remodeling in asthma.^{28,29} For this reason, we suggest that mast cells could be a therapeutic target for asthma.

In clinical study, mast cells infiltrate into the lamina propria of airway mucosa and epithelium, and airway smooth muscle, and mast cell mediators increase in BALF of human asthma patients.⁵ These suggests that mast cells play an important role in the development of asthma, and can be a clinical disease marker.⁶ In terms of targeting mast cell mediators, antihistamines are more effective in patients with antagonist-resistant (e.g., leukotriene antagonist) asthma.³ Therefore, we believe that it would be reasonable to try to develop an asthma treatment that can inhibit the mast cell itself rather than targeting a single mast cell mediator. Previous research has reported that OAA inhibited the eosinophils and mast cell activation as well as Th2 responses and IgE production.¹⁶ Our data showed that the treatment of OAA alleviated OVA-induced airway inflammatory symptoms including not only leukocytes infiltration and IgE production but also mast cell infiltration and activation. Our

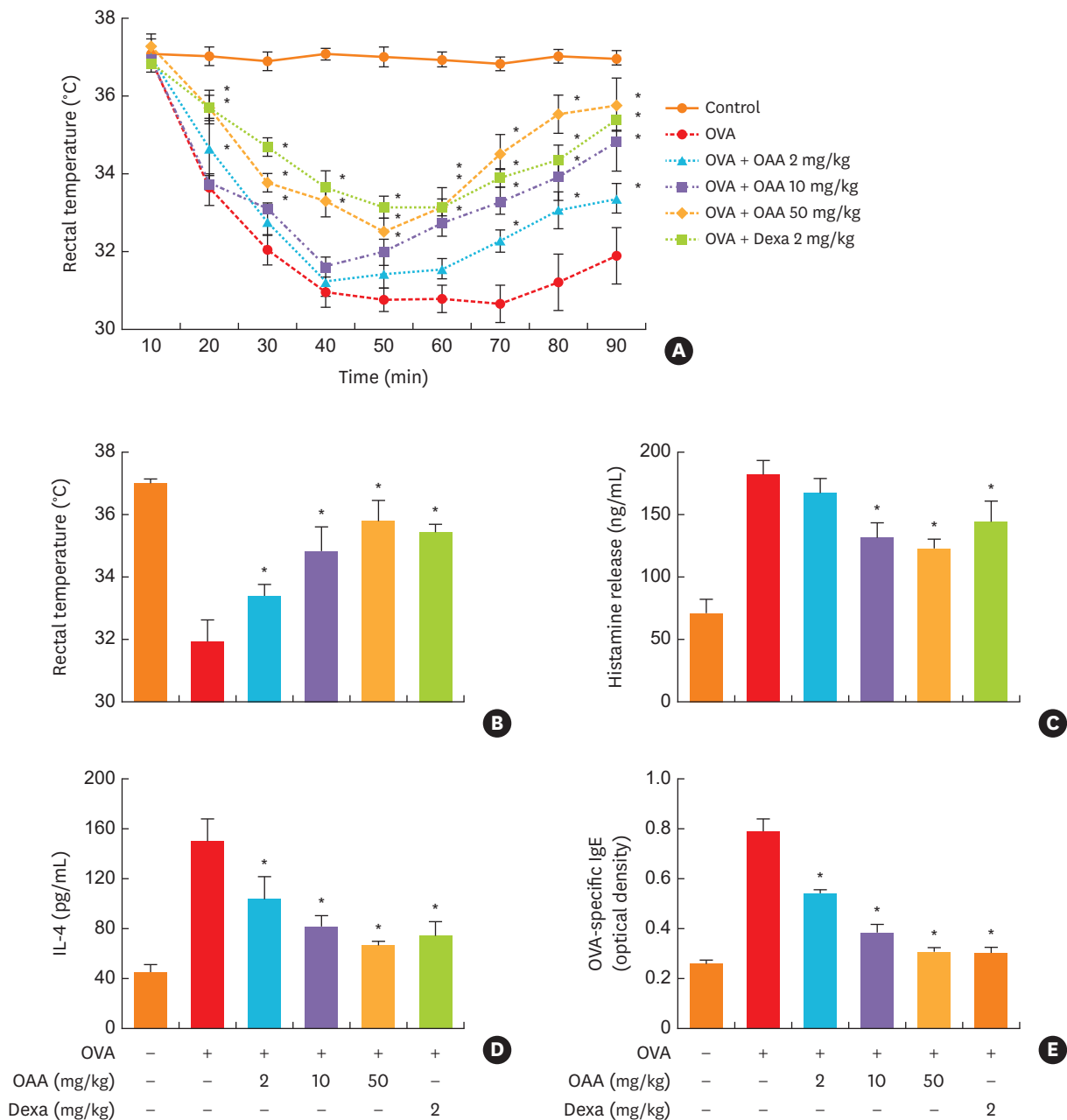


Fig. 5. Effects of OAA on OVA-induced anaphylaxis mice models. (A) Mice were sensitized by intraperitoneal injection of an OVA-alum adjuvant mixture on days 0 and 7. OAA or Dexamethasone was orally administered 3 times once every 2 days after the second sensitization. On day 14, the mice were challenged with OVA, and the rectal temperature was measured every 10 minutes for 90 minutes. (B) The rectal temperature of mice at 60 minutes. (C-E) Blood was obtained from the abdominal artery of each mouse to measure serum histamine, IL-4, and OVA-specific IgE levels. Histamine levels were determined using a fluorescence plate reader. IL-4 and OVA-specific IgE levels were determined by enzyme-linked immunosorbent assay. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

OAA, oleanolic acid acetate; OVA, ovalbumin; Dexamethasone, dexamethasone; IL, interleukin; IgE, immunoglobulin E.

* $P < 0.05$.

study suggests that OAA can be a therapeutic agent for mast cell-mediated allergic airway inflammation and asthma by demonstrating the mechanisms involved in mast cell activation.

Anaphylaxis is induced by the release of allergic mediators from activated mast cells. In particular, histamine is a major factor that induces allergic responses. OVA is an abundant

glycoprotein in egg white and a major allergen.^{30,31} Sensitization with OVA enhances IgE production in the serum, after which re-exposure to OVA initiates an allergic response by the binding of antigens and IgE-receptor complexes on the surface of mast cells.^{32,33} In particular, hypothermia, an allergic response to OVA challenge, is caused by increased serum histamine levels.³⁴ Therefore, the OVA-induced ASA model is an appropriate animal model for mast cell-mediated type I hypersensitivity.³⁵ The IgE-mediated PCA model is another well-characterized animal model of allergic reaction. Local injection of anti-DNP IgE followed by intravenous antigenic challenge induces local plasma extravasation and vascular permeability.³⁶ In both animal models, increased histamine release would result in vasodilation, which eventually causes hypothermia and Evans blue pigmentation. In addition, serum IgE in the OVA-induced ASA model is increased after challenge with OVA. OVA stimulates the production of IgE by B cells, which requires the differentiation of naïve T cell into Th2 cells and is essential for mast cell activation.³⁷ Increased IgE levels have been reported to contribute to systemic anaphylaxis in mice.³⁵ Moreover, compound 48/80 can induce mast cell degranulation, which causes systemic anaphylactic shock.³⁸ In our study, these symptoms were also inhibited by oral administration of OAA. Based on the results, OAA suppress the allergic reaction by direct inhibiting mast cell activation.

We used 3 types of mature mast cells (RBL-2H3, RPMCs, and mBMMCs) to assess the anti-allergic inflammatory effect of OAA. RBL-2H3 is one of the most popular mast cell lines, whereas RPMCs are primary mast cells isolated from the peritoneal cavity. mBMMCs are maturing mast cell progenitors isolated from the bone marrow. As previously described, histamine causes immediate allergic responses including vascular permeability, vasodilation, bronchoconstriction, and hypothermia.²⁵ Various studies have reported that the release of histamine is regulated by intracellular calcium, an important secondary messenger.^{39,40} The binding of antigens to the IgE-FcεRI complex on mast cells initiate phosphorylation of Src family protein kinases such as Lyn and Syk.⁴¹ These kinases lead to the phosphorylation of PLCγ, which catalyzes the hydrolysis of phospholipid phosphatidylinositol 4,5-bisphosphate, resulting in the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3 binding to its receptor in the endoplasmic reticulum (ER) immediately releases calcium from ER stores. Finally, the extrusion of granules via fusion of the granule membrane releases histamine.^{42,43} Our results showed that antigen-mediated histamine release was suppressed by OAA treatment in all types of mast cells. Furthermore, our intracellular signaling experiment showed that OAA suppressed the phosphorylation of PLCγ but not Lyn and Syk, suggesting that OAA inhibits downstream of PLCγ. Previous research has reported that OAA suppressed calcium signaling through inhibiting the PLCγ phosphorylation in osteoclast differentiation,¹⁷ which supports our data. Therefore, OAA is thought to attenuate the degranulation of mast cells by inhibiting intracellular calcium influx due to the specific inhibition of PLCγ. Our results also showed that the phosphorylation of Akt was attenuated by treatment with OAA. Akt activates the IKKα/β complex, which induces IκBα degradation and NF-κB translocation.²⁴ NF-κB is a key transcription factor that induces the expression of various genes involved in inflammatory responses.⁴⁴ Consequently, mast cells become activated and initiate the release of allergic mediators by degranulation. Therefore, similar to PLCγ inhibition, Akt phosphorylation is also an essential target for regulating mast cell-mediated inflammatory responses.^{24,45} In our study, the gene expression of pro-inflammatory cytokines was markedly increased after DNP-HSA challenge, however, inhibited by OAA treatment. A previous study has reported that OAA could suppress the activation of Akt, MAPK, and NF-κB and subsequently attenuate immune responses.¹⁵ Although additional mechanistic studies of OAA on the

signaling pathway between Src kinases and PLC γ are required, it is expected that OAA can inhibit PLC γ specifically.

Oleanolic acid, a precursor of OAA, was assessed for bioavailability by the study of pharmacokinetics.⁴⁶ This study was conducted with different injection route of oleanolic acid, the area under the plasma level-time curve represents bioavailability. The intravenous injection of oleanolic acid at dose of 2 mg/kg showed the 5-fold higher levels than oral administration of oleanolic acid at dose of 50 mg/kg. The low bioavailability of oleanolic acid by oral administration may be due to a poor gastric absorption and hepatic metabolism.⁴⁶ In a previous study, OAA is metabolized to oleanolic acid by hydrolysis *in vivo*. The plasma concentration of OAA showed higher than oleanolic acid through the intravenous injection of same dosage. The 2 mg/kg of OAA injected by intravenous showed a half-life at 1.5 hours, and mean area under curve of plasma level was 1,375 ng·h/mL.⁴⁷ Although the efficacy of OAA by the route of administration has not been verified, the dosage of OAA was decided through the bioavailability of OAA, and our study was sufficient to demonstrate the effect of OAA against allergic airway inflammation and asthma. Through the comparative analysis between OA and OAA, OAA showed more improved effects on recovery of pulmonary resistance, mast cell degranulation and pro-inflammatory cytokine secretion (**Supplementary Fig. S2, S5, and S6**). These results suggest the superior effectiveness of OAA than OA.

In conclusion, our results suggest that the anti-allergic airway inflammatory effect of OAA may be attributed to the suppression of mast cells. Especially, this effect is associated with the inhibition of intracellular calcium influx and pro-inflammatory cytokine expression due to PLC γ and Akt suppression. Since so-called mast cell stabilizers do not have a large effect on preventing symptoms or suppressing underlying allergic airway inflammation, it has been frequently suggested that mast cells cannot be important in asthma pathophysiology. Although several commonly used drugs can inhibit mast cell degranulation, these drugs are ineffective in inhibiting mast cell activation in chronic asthma. Therefore, targeting mast cells in chronic asthma remains a significant challenge and an urgent goal. In our study, we demonstrated that OAA inhibited the activation of mast cells and contributed to the mast cell stabilization in allergic asthma. The identification of precise target molecules for OAA between Src kinases and PLC γ , and plausible mechanism for the pleiotropic effect of OAA still remains. However, based on our results, OAA could be a therapeutic candidate for the treatment of allergic airway inflammatory diseases targeting mast cells.

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SUPPLEMENTARY MATERIALS

Supplementary Information

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Supplementary Table S1

Effects of OAA on compound 48/80-induced systemic anaphylaxis

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Supplementary Fig. S1

Experimental design for animal models. (A) The mice were sensitized with OVA-adjuvant mixture on days 0 and 7. On days 14 until 17, mice were challenged with OVA. At the time of challenge, mice received the OAA. (B) The mice were sensitized with OVA-adjuvant mixture on days 0 and 7. After sensitization, mice received the OAA at 3 times a week. On day 14, mice were challenged with OVA. (C) Mice ear skin was sensitized with anti-DNP-IgE. After 2 days, mice received OAA for 1 hour. Evans blue and DNP-HSA mixture was intravenous injected 30 minutes before the termination of experiment. (D) Mice received OAA for 1 hour before the injection of compound 48/80.

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Supplementary Fig. S2

Mch-induced pulmonary resistance. Pulmonary resistance in OVA-induced allergic airway inflammation mice were detected by forced oscillation technique. The mouse was exposed various concentration of Mch, and respiratory resistance (A), elastance (B), and compliance (C) were measured. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

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Supplementary Fig. S3

Effect of OAA on cell viability. The cytotoxicity of OAA on (A) RBL-2H3, (B) RPMCs, and (C) mBMMCs was measured by MTT assay. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

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Supplementary Fig. S4

Effects of OAA on IgE-mediated passive cutaneous and compound 48/80-induced systemic anaphylaxis mice models. (A) Evans blue pigmentation in the IgE-mediated PCA mice model. The ear skin of mice ($n = 5/\text{group}$) was sensitized by intradermal injection of anti-DNP IgE (0.5 mg/site) for 48 hours. OAA was orally administered at doses of 2, 10, and 50 mg/kg body weight 1 hour before intravenous injection of a DNP-HSA and 4% Evans blue (1:1) mixture. After 30 minutes, the ears were collected to measure dye pigmentation. (B) The dye was extracted as described in the Materials and Methods section and detected using a spectrophotometer. (C) Serum histamine levels in the compound 48/80-induced systemic anaphylaxis mice model. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

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Supplementary Fig. S5

Comparative effects of OA and OAA on OVA-induced allergic airway inflammation. (A) Serum IgE levels and BALF pro-inflammatory cytokines such as TNF- α (B) and IL-4 (C) were measured by enzyme-linked immunosorbent assay. (D) β -hexosaminidase levels in BALF was measured using by spectrophotometer. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

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Supplementary Fig. S6

Comparative effects of OA and OAA on mast cell degranulation and pro-inflammatory cytokine expression. (A) β -hexosaminidase levels were determined using a spectrophotometer. (B) Histamine levels were determined using a fluorescence plate reader. The secretion of pro-inflammatory cytokines such as TNF- α (C) and IL-4 (D) was measured by enzyme-linked immunosorbent assay. Each data point represents the mean $\pm$ standard error of the mean of 3 independent experiments.

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