Heliyon 9 (2023) e22932

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Aromadendrin inhibits PMA-induced cytokine formation/NF-κB activation in A549 cells and ovalbumin-induced bronchial inflammation in mice

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ARTICLE INFO

Keywords:
Asthma
Aromadendrin
Molecules
Eosinophils
NF-κB

ABSTRACT

Hyperproduction of immune cell-derived inflammatory molecules and recruitment of immune cells promote the development of allergic asthma (AA). Aromadendrin (ARO) has various biological properties including anti-inflammatory effects. In this study, we evaluated the ameliorative effects of ARO on the development of AA *in vitro* and *in vivo*. Phorbol 12-myristate 13-acetate (PMA, 100 nM) was used to induce inflammation in A549 airway epithelial cells. The cohesion of A549 and eosinophil EOL-1 cells was studied. Ovalbumin (30 or 60 μg)/Alum (3 mg) mixture was adapted for AA induction in mice. ARO (5 or 10 mg/kg, p. o.) was administered to mice to investigate its ameliorative effect on AA development. Enzyme-linked immunosorbent assay, western blotting, and hematoxylin and eosin/periodic acid Schiff staining were performed to study the ameliorative effect of ARO on bronchial inflammation. In PMA-stimulated A549 cells, the upregulation of cytokines (interleukin [IL]-1β/IL-6/tumor necrosis factor alpha [TNF-α]/monocyte chemoattractant protein [MCP]-1]) and nuclear factor kappa B (NF-κB) activation was effectively reduced by ARO pretreatment. ARO suppressed the adhesion of A549 cells and eosinophils. In ovalbumin-induced AA mice, the levels of cells, such as eosinophils, Th2 cytokines, MCP-1 in bronchoalveolar lavage fluid, IgE in serum, and inducible nitric oxide synthase/cyclooxygenase-2 expression in the lung tissue were upregulated, which were all suppressed by ARO. In addition, the increase in cell inflow and mucus formation in the lungs of AA mice was reversed by ARO as per histological analysis. ARO also modulated NF-κB activation in the lungs of AA mice. Overall, the anti-inflammatory properties of ARO in *vitro/in vivo* studies of AA were notable. Thus, ARO has a modulatory effect on bronchial inflammation and may be a potential adjuvant for AA treatment.

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https://doi.org/10.1016/j.heliyon.2023.e22932
Received 24 July 2023; Received in revised form 19 November 2023; Accepted 22 November 2023
Available online 26 November 2023

1. Introduction

Allergic asthma (AA), caused by inhaled allergens, is the most common asthma phenotype [1]. Inflammatory molecules released by airway epithelial cells promote bronchial inflammation by affecting eosinophil recruitment [2]. Th2 cytokines play pivotal roles in eosinophil influx, B cell activation, immunoglobulin E (IgE) production, and mucus formation [3,4]. A substantial increase in cells, such as eosinophils was confirmed in the bronchoalveolar lavage (BAL) fluid of AA-mice [5,6]. Macrophages are the main source of monocyte chemoattractant protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [7]. MCP-1 is known to affect immune cell recruitment [8–10], and iNOS upregulation is related to mucus formation in AA [11].

Nuclear factor kappa B (NF-κB) activation promotes the expression of cytokines/chemokines/mediators leading to bronchial inflammation [7,12]. Thus, the modulation of NF-κB activation for improving bronchial inflammation has been emphasized in inflammatory lung diseases, including AA [13,14]. Phorbol 12-myristate 13-acetate (PMA) induces cytokine production and NF-κB activation in A549 airway epithelial cells [15]. The utility of ovalbumin (OVA) for AA induction *in vivo* has been confirmed through its ability to induce eosinophil/macrophage inrush, IL-4/IL-5/IL-13/IgE/mucus generation, iNOS/COX-2 upregulation, and phosphorylation of NF-κB [16,17].

Plant phenolic compounds (PPCs) have wide variety of biological effects both *in vitro* and *in vivo* [18–24]. Aromadendrin (ARO), also known as dihydrokaempferol, is a phenolic compound found in *Bauhinia championii* (Benth.) Benth. [25], the pulp of *Euterpe oleracea* Martius. [26], rhizome of *Smilax glabra* [27], and leaves of *Olea europaea* L [28]. Venditti et al. reported anti-inflammatory effects of ARO in activated keratinocytes [28]. Previous observations indicate that ARO suppresses lipopolysaccharide (LPS)-induced iNOS/COX-2 expression and NF-κB activation in RAW 264.7 macrophages [29], and recent observations have shown its inhibitory effect on T-cell-derived cytokines [30]. These results reflect the varied biological properties of ARO. However, to the best of our knowledge, studies reflecting the ameliorative effects of ARO on AA have not been reported. Considering the modulatory effect of ARO on activated macrophages [28] through suppression of iNOS expression, an important factor in AA [11], we aimed to investigated the ameliorative potential of ARO on the inflammatory response in *vitro* and *in vivo* models of AA.

2. Materials and methods

2.1. Reagents and cell culture

ARO was obtained from natural products manufacturer (ChemFaces, Wuhan, China). A549 cells (airway epithelial cell line, ATCC) were grown in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) at 37 °C in a CO_2 incubator. To analyze cell viability, 2×10^4 cells were transferred into a cell culture plate (96-well) and incubated with ARO (10–200 μM). Subsequently, the cells were maintained for 24 h with or without PMA (100 nM). Cell viability was determined using CytoX kit (Daejeon, Korea). To detect molecule formation from A549 cells, 0.5×10^5 cells were transferred to a cell culture plate (96-well), incubated with 10–100 μM ARO for 1 h and were maintained for 20 h with PMA. The secreted levels of cytokines in the culture supernatants were analyzed using the respective ELISA kits (BD Biosciences & R&D Systems, Inc., Minneapolis, MN, USA).

2.2. Adhesion of airway epithelial cells and eosinophils

A549 cells were seeded into 96-well plates at 1×10^4 cells/well, treated with ARO for 1 h, and retained with 100 nM PMA for 5 h. Calcein-AM-stained EOL-1 cells (human eosinophils) were then transferred into culture plates containing EOL-1 cells (0.5×10^5 cells) for 1 h. Subsequently, cells were carefully rinsed with phosphate buffered saline (PBS) and observed under a fluorescence microscope.

2.3. Establishment of OVA-exposed AA mice

The experimental mice (6-week-old female BALB/c) were purchased from Koatech Co., Ltd. (Pyeongtaek, Korea). After 1 week of acclimatization, the experimental groups (each, $n = 6$) were established as follows: (i) normal control (NC); (ii) ovalbumin (OVA), ovalbumin + alum sensitization/ovalbumin inhalation; (iii) dexamethasone (DEX), ovalbumin +1 mg/kg DEX-treatment; (iv) aromadendrin (ARO) 5, ovalbumin +5 mg/kg ARO-treatment; and (v) ARO 10, ovalbumin +10 mg/kg ARO-treatment groups.

The OVA-induced AA was established in the mice as per a previous protocol [5]. Briefly, a mixture of OVA and alum was intraperitoneally injected into mice for 2 times at day 0 and day 7, respectively. Mice were exposed to 1 % OVA aerosols from days 11–13 for 1 h/day. ARO (5 or 10 mg/kg) or DEX (1 mg/kg) was orally administered to mice through days 9–13. ARO and DEX were dissolved in 1 % dimethyl sulfoxide and 1 % Tween-20 in PBS.

2.4. Detection of immune cells and molecules

To detect immune cells and Th2 cytokines in the BALF, mice were anesthetized as per previously reported conditions (intraperitoneal injection of Zoletil and xylazine) [16]. Immune cell morphology was distinguished using Diff-Quik staining, and the number of cells was determined using a light microscope (400× magnification) [31]. The levels of IL-4, 5, 13, and MCP-1 in the BALF supernatant and IgE in the serum were determined using ELISA kits (R&D Systems, Inc., MN, USA).

2.5. Western blot analysis

To estimate the expression levels of phosphorylated (p)-p65/p-IκBα in A549 cells and of iNOS/COX-2/p-p65/p-IκBα in the lungs of mice, lysates from cell culture and lung tissue were prepared using lysis buffer, which included inhibitors of protease and phosphatase. Protein quantification was performed using a bicinchoninic acid (BCA) assay, and proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, each membrane was incubated with blocking solution (5 % skim milk in tris-buffered saline with Tween 20 (TBST)) and primary antibodies (Table 1). Each membrane was retained with the corresponding secondary antibodies.

2.6. Histological analysis

To confirm histological changes in the lungs of mice, lung tissues were isolated from mice, rinsed with PBS, fixed in fixative solution, embedded in paraffin wax and were sectioned using a rotary microtome (4 μm thickness) and stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining solutions [32]. Histopathological scoring was performed using semiquantitative data, by two independent observers [5,33]. Cell existence around the airway epithelium was detected using H&E staining and scored from 0 to 2 according to the degree of existence: 0, no existence; 0.5, minor existence; 1, moderate existence; and 2, extensive existence. Mucus formation in the airway epithelium was detected using PAS staining and scored from 0 to 2 according to the degree of formation: 0, no formation; 0.5, minor formation; 1, moderate formation; and 2, extensive formation.

2.7. Ethics statement

The procedures for the animal experiments were approved by the IACUC of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Chungbuk, Korea; KRIBB-AEC-22074) on March 23, 2022. The procedure was performed in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and the Korean National Laws for Animal Welfare.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was analyzed using a two-tailed Student's t-test for *in vitro* experiments. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) was used to assess the significance of differences among the groups in the *in vivo* experiments using SPSS (version 20.0; IBM Corp., Armonk, NY, USA). All the data met the assumptions of homoscedasticity and normality. One asterisk (*) and two asterisks (**) represent statistical significance at $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Inhibitory activity of ARO against PMA-induced cytokines in A549 cells

As shown in Fig. 1A, notable cytotoxicity was not observed until at least 100 μM ARO was used. Thus, 10, 25, 50, and 100 μM concentrations were selected to determine the anti-inflammatory effects of ARO *in vitro*.

Experimental results from the enzyme-linked immunosorbent assay (ELISA) indicated that the formation of IL-1β was markedly increased in the cell culture supernatant of PMA-stimulated A549 cells, whereas ARO pretreatment mitigated this increase (NC vs. PMA group, $p < 0.01$; PMA group vs. PMA +100 μM ARO group, $p < 0.01$) (Fig. 1B). As shown in Fig. 1C–E, there was a significant increase in IL-6 (Fig. 1C), tumor necrosis factor alpha (TNF-α, Fig. 1D), and MCP-1 levels (Fig. 1E) in the cell culture supernatant of PMA-stimulated A549 cells compared to NC (NC vs. PMA group, $p < 0.01$); however, pretreatment with ARO effectively decreased the levels of these molecules (PMA group vs. PMA +100 μM ARO group, $p < 0.01$). The inhibitory rates of IL-1β, IL-6, TNF-α, and MCP-1 were 83.2 %, 34.4 %, 78.4 %, and 63.3 %, respectively, with 100 μM ARO.

3.2. Inhibitory activity of ARO against PMA-induced NF-κB activation in A549 cells

The inhibitory effects of ARO on cytokines and chemokines were confirmed in PMA-stimulated A549 cells (Fig. 1). Thus, we used

Table 1
List of antibodies.

NO	Primary antibody	Company	Molecular weight	Dilution	Host	Secondary antibody
1	p-p65 (3033 S)	Cell Signaling	65	1:1000	Rabbit	Goat anti rabbit-HRP
2	p65 (sc-8008)	Santa Cruz	65	1:1000	Mouse	Goat anti mouse-HRP
3	p-IκBα (2859 S)	Cell Signaling	40	1:1000	Rabbit	Goat anti rabbit-HRP
4	iNOS (ab136918)	Abcam	130	1:1000	Rabbit	Goat anti rabbit-HRP
5	COX-2 (sc-376,861)	Santa Cruz	70–72	1:1000	Mouse	Goat anti mouse-HRP
6	β-action (sc-47778)	Santa Cruz	43	1:2000	Mouse	Goat anti mouse-HRP

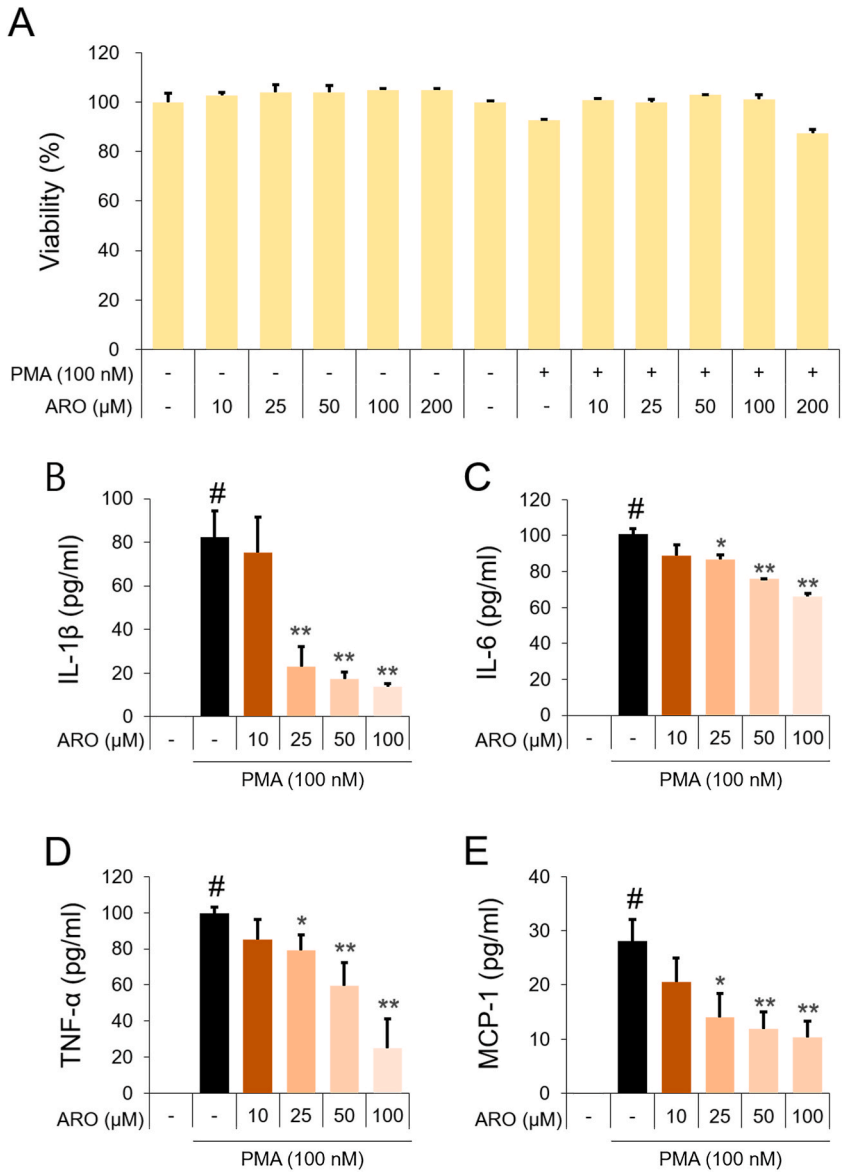

Fig. 1. Inhibition of PMA-induced cytokines with ARO pretreatment, in A549 cells. A549 cells were incubated with ARO for 1 h and maintained with PMA for 20 h. (A) Cell viability was analyzed using CytoX assay. (B–E) The production of IL-1β, IL-6, TNF-α, and MCP-1 in the cell culture supernatant was analyzed using ELISA kits. Data are expressed as the mean ± SD ($^{\#}p < 0.01$ for comparison with control; $^{*}p < 0.05$, $^{**}p < 0.01$ for comparison with PMA).

western blotting to investigate whether ARO suppresses NF-κB activation in PMA-stimulated A549 cells. As shown in Fig. 2, prominent increase in NF-κB p65/IκBα activation was confirmed in cell culture lysates of PMA-stimulated A549 (NC vs. PMA group, $p < 0.01$) (Fig. 2A–C). ARO modulated NF-κB activation (Fig. 2A and B) as well as IκBα activation (Fig. 2A and C) in PMA-stimulated A549 cells (PMA group vs. PMA +100 μM ARO group; $p < 0.05$). These results indicate that ARO may exert anti-inflammatory effects by modulating NF-κB activation.

3.3. Inhibitory activity of ARO against airway cell-eosinophil adhesion

Based on the inhibitory effects of ARO on airway cell-derived cytokines and chemokines (Fig. 1), we examined whether ARO improved eosinophil adhesion to airway cells. As shown in Fig. 3, the high fluorescence, which reflects the increased adhesion of airway cells (A549 cells) and eosinophils (EOL-1 cells) in the PMA-treated group, was mitigated by ARO pretreatment.

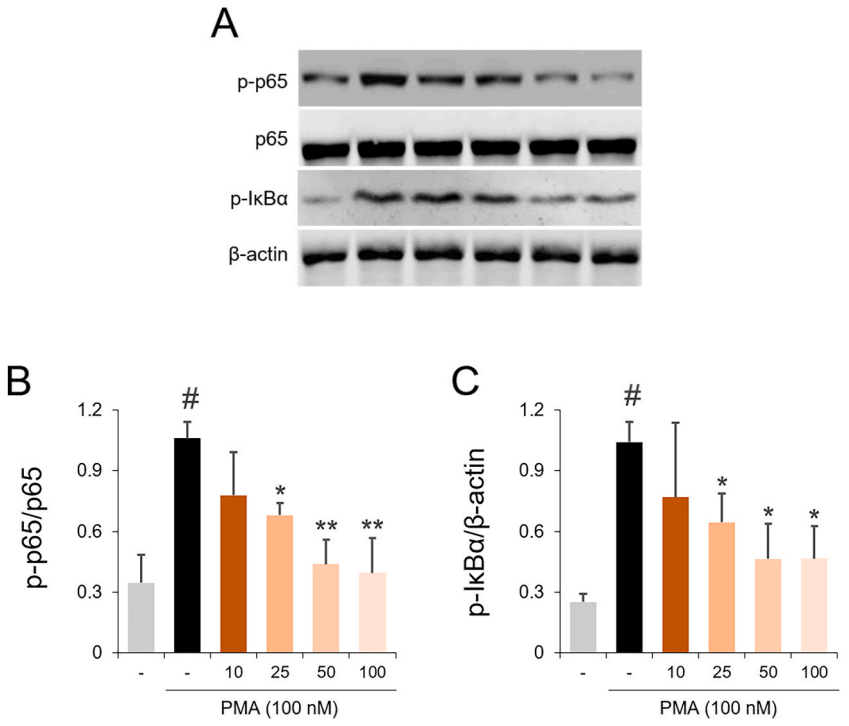

Fig. 2. Inhibition of PMA-induced NF-κB activation with ARO pretreatment, in A549 cells. A549 cells were incubated with ARO for 1 h and maintained with PMA for 1 h. (A) The expression of phosphorylated (p)–NF–κB p65 and p-IκBα in whole cell lysates was analyzed using western blotting. (B–C) Quantitative analysis was performed using ImageJ software. Data are expressed as the mean ± SD ($^{\#}p < 0.01$ for comparison with control; $^{*}p < 0.05$, $^{**}p < 0.01$ for comparison with PMA).

3.4. Inhibitory activity of ARO against immune cell recruitment and inflammatory molecule generation in AA mice

We evaluated whether ARO inhibits cell recruitment in an experimental mouse model of AA (Fig. 4). As shown in Fig. 5A–D, the number of immune cells such as eosinophils (Fig. 5A and B), and macrophages (Fig. 5A and C) was sharply increased in the BALF of OVA-induced AA mice compared to the NC group (NC vs. ovalbumin group, $p < 0.01$). Oral administration of ARO (5 or 10 mg/kg) or dexamethasone (DEX positive control: 1 mg/kg) inhibited the increase in eosinophils/macrophages in the BALF of OVA-induced AA mice (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +5 mg/kg ARO group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$) (Fig. 5A–D). The inhibitory rates of eosinophils were 91.7 % (DEX, 1 mg/kg), 79.8 % (ARO, 5 mg/kg), and 86.3 % (ARO, 10 mg/kg). The inhibitory rates of macrophages were 73.2 % (DEX, 1 mg/kg), 49.3 % (ARO,

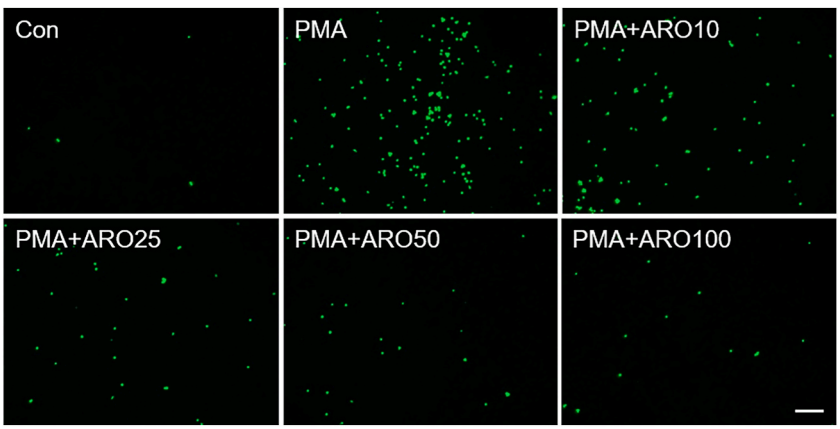

Fig. 3. Inhibition of airway cell-eosinophil adhesion by ARO pretreatment. A549 cells were incubated with ARO for 1 h and maintained with PMA for 5 h. Next, stained EOL-1 cells were transferred to a cell culture plate that contained A549 cells and were incubated for 1 h. Subsequently, the cells were observed using a fluorescence microscope (magnification × 100, scale bar 100 μm).

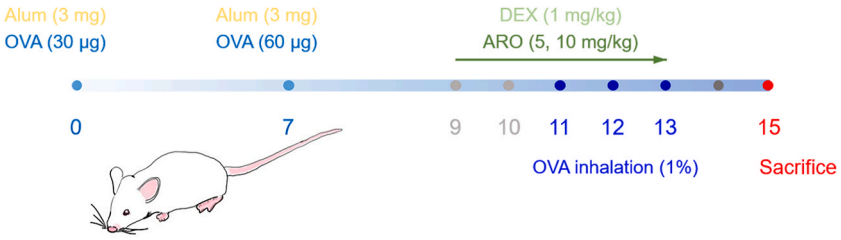

Fig. 4. Animal model establishment and ARO treatment. To induce bronchial inflammation in BALB/c mice, i. p. injection of the OVA/alum mixture was performed on days 0 and 7, and OVA inhalation was performed on days 11–13. To evaluate the ameliorative effects of ARO and DEX, these were orally administered on days 9–13. The mice were euthanized on day 15, and BALF, serum, and lung tissues were collected from each animal for analysis.

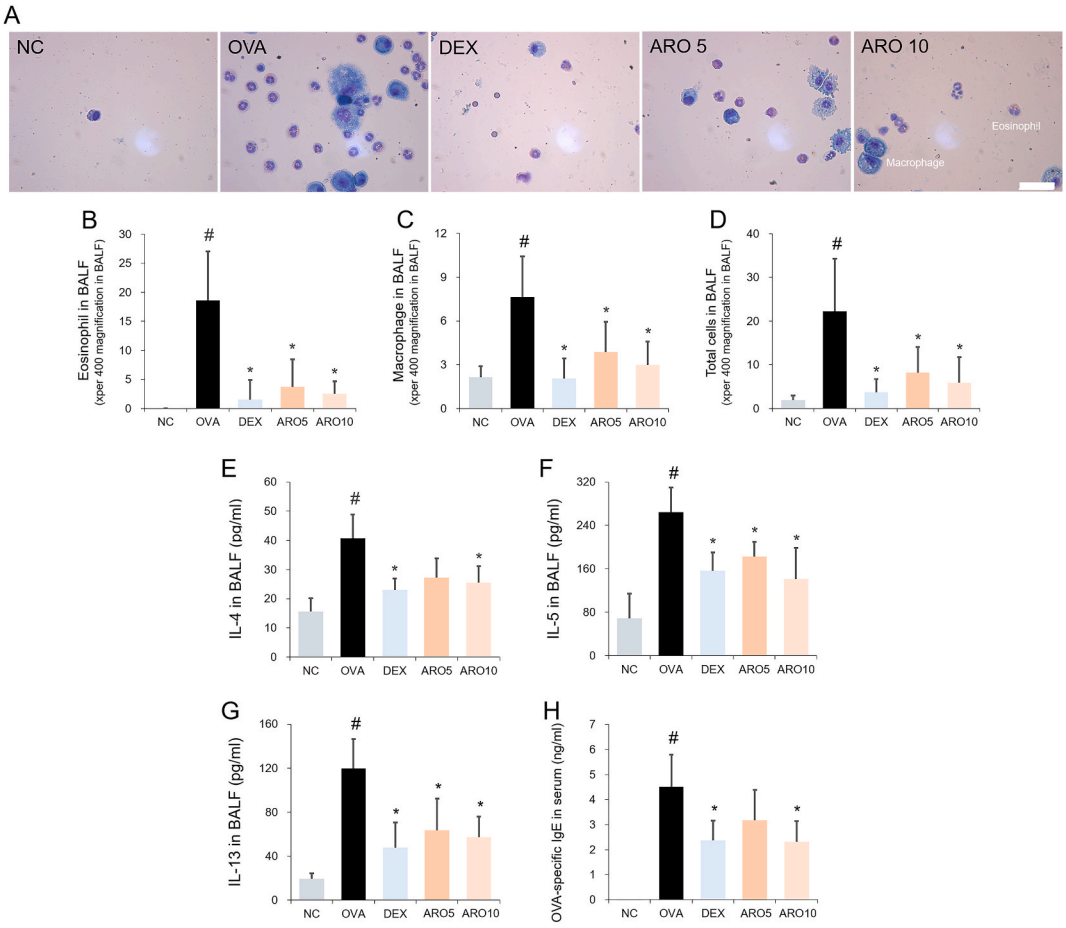

Fig. 5. Inhibition of immune cell recruitment and cytokine secretion in AA mice by ARO. (A) Immune cell images. (B–D) Counts of immune cells in BALF (magnification × 400). (E–G) Levels of Th2 cytokines in BALF were examined using ELISA. (H) The level of IgE in serum was detected using ELISA. Data are expressed as the mean ± SD ($^{\#}p < 0.05$ for comparison with normal control; $^{*}p < 0.05$ for comparison with OVA group). NC: normal control mice; OVA: OVA sensitized mice; DEX: 1 mg/kg DEX-treated OVA mice; ARO 5: 5 mg/kg aromadendrin (ARO)-treated OVA mice, and ARO 10: 10 mg/kg ARO-treated OVA mice.

5 mg/kg), and 60.7 % (ARO, 10 mg/kg).

In this study, IL-4 production (Fig. 5E)/-5 (Fig. 5F)/-13 (Fig. 5G) was found to be upregulated in the BALF of the AA mice (NC vs. ovalbumin group, $p < 0.01$), whereas this upregulation was decreased with ARO or DEX treatments (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$) (Fig. 5E–G). The IL-4 inhibitory rates were 43.5 % (DEX, 1 mg/kg), 33.0 % (ARO, 5 mg/kg), and 37.3 % (ARO, 10 mg/kg). The IL-5 inhibitory rates were 40.9 % (DEX, 1 mg/kg), 30.8 % (ARO, 5 mg/kg), and 46.5 % (ARO, 10 mg/kg). The IL-13 inhibitory rates were 60.2 % (DEX, 1 mg/kg), 46.9 % (ARO, 5 mg/kg), and

52.3 % (ARO, 10 mg/kg). Furthermore, ARO and DEX reduced the serum IgE levels (Fig. 5H) in AA mice (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$) (Fig. 5H). The IgE inhibitory rates were 47.4 % (DEX, 1 mg/kg), 29.6 % (ARO, 5 mg/kg), and 48.6 % (ARO, 10 mg/kg). In general, 10 mg/kg ARO had a notable effect on immune cell recruitment and Th2 cytokine and IgE generation, and its effect was comparable to that of 1 mg/kg DEX. No significant difference was observed between the DEX and ARO groups ($p < 0.453$). No symptoms, such as dyspnea or vomiting, were observed at the set concentrations of ARO or DEX.

3.5. Inhibitory activity of ARO against iNOS/COX-2 expression in AA mice

In the present study, we used western blotting to confirm upregulated expression of iNOS (Fig. 6A and B) and COX-2 (Fig. 6A and C) in the lungs of the AA group (NC vs. ovalbumin group, $p < 0.01$) (Fig. 6A–C). Interestingly, ARO suppressed the expression of these molecules (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +5 mg/kg ARO group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$).

3.6. Inhibitory activity of ARO against inflammatory cell influx in AA mice

H&E staining (Fig. 7A) and quantitative analysis (Fig. 7B) indicated the presence of inflammatory cells around the airway epithelium in the lungs of AA mice (Fig. 7A, blue arrowheads; Fig. 7B, NC vs. ovalbumin groups, $p < 0.05$). However, this characteristic was reversed in the AA groups treated with ARO or DEX (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$).

Next, we investigated whether ARO had an inhibitory effect on MCP-1 secretion *in vivo*. Interestingly, ARO suppressed MCP-1 expression (Fig. 7C) in the BALF of the AA animal models (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$) (Fig. 7C). The MCP-1 inhibitory rates were 44.7 % (DEX, 1 mg/kg), 24.7 % (ARO, 5 mg/kg), and 50.5 % (ARO, 10 mg/kg).

3.7. Inhibitory activity of ARO against mucus formation in AA mice

In the *in vivo* study, we confirmed the inhibitory effects of ARO on IL-13 generation and iNOS expression (Figs. 5 and 6). Thus, ARO was expected to affect mucus formation. PAS staining was performed to detect mucus formation. As shown in Fig. 8A and B, mucus was notably formed in the airway epithelium of the AA mice (Fig. 8A, blue arrowheads; Fig. 8B, NC vs. ovalbumin group, $p < 0.05$), which were blocked by ARO and DEX. (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group, $p < 0.05$).

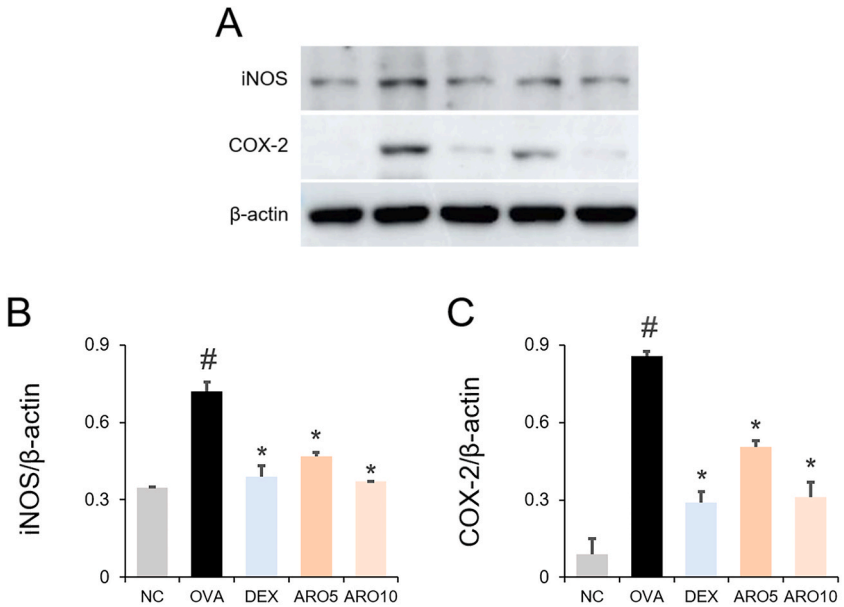

Fig. 6. Inhibition of iNOS/COX expression in AA mice by ARO. (A) The expression levels of iNOS and COX-2 in the lung tissue of AA mice were detected using western blotting. (B–C) Quantitative analysis was performed using ImageJ software. Data are expressed as the mean ± SD ($^{\#}p < 0.05$ for comparison with normal control; $^{*}p < 0.05$ for comparison with OVA group).

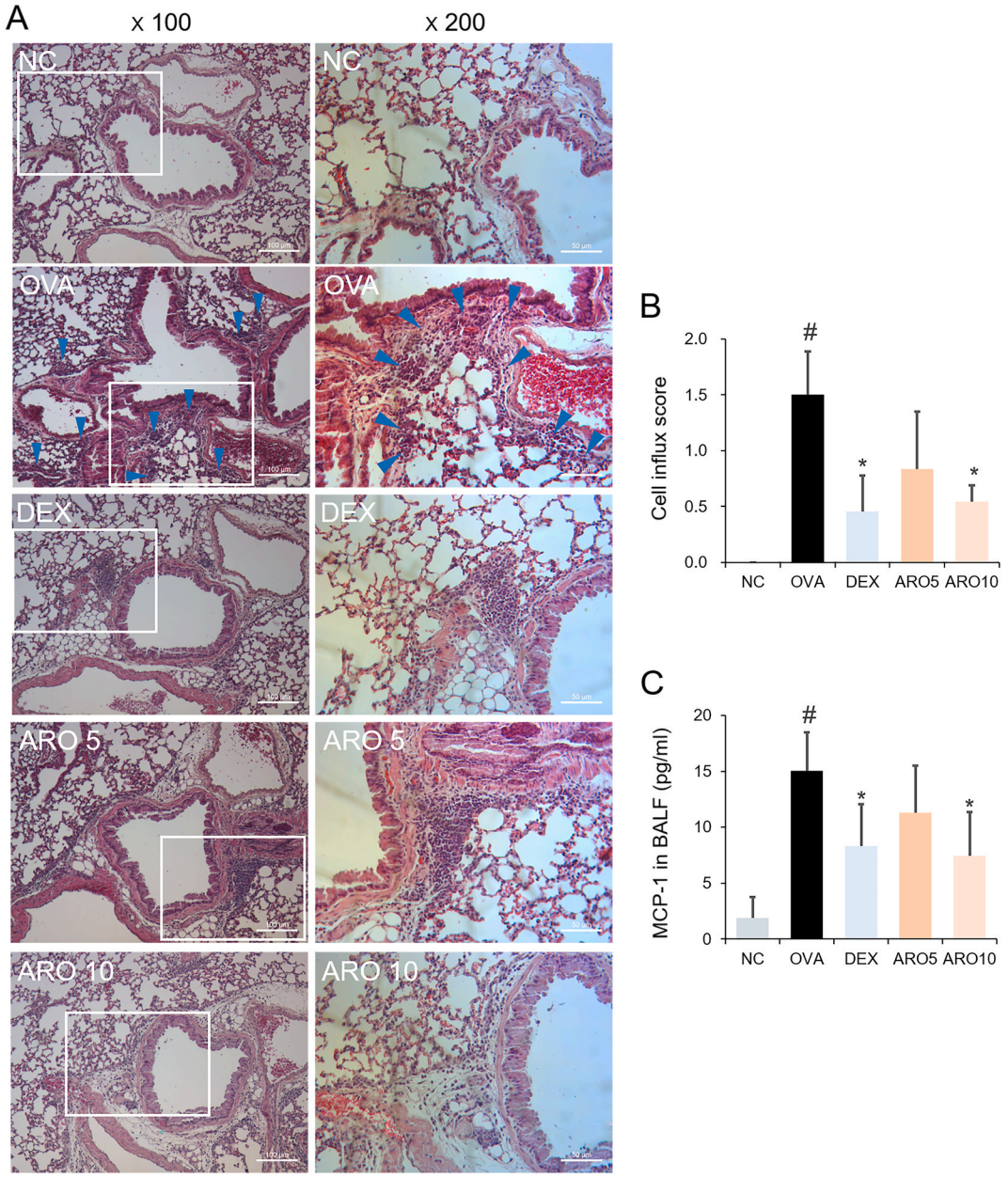

Fig. 7. Inhibition of inflammatory cell influx in AA mice by ARO. (A) The histological changes that reflect the presence of inflammatory cells in the lungs of AA mice were confirmed using H&E staining (left, magnification × 100, scale bar 100 μm; right, magnification × 200, scale bar 50 μm). A high influx of cells was detected around the airway epithelium in the lungs (blue arrowheads). This was ameliorated by ARO administration. (B) The scoring of cell influx was determined by semi-quantitative analysis. (C) The level of MCP-1 in the BALF of AA mice was detected using ELISA. Data are expressed as the mean ± SD ($^{\#}p < 0.05$ for comparison with normal control; $^{*}p < 0.05$ for comparison with OVA group).

3.8. Inhibitory activity of ARO against NF-κB activation in AA mice

The upregulation of NF-κB (Fig. 9A and B) and IκBα (Fig. 9A and C) was confirmed in the lung tissue lysates of AA mice (NC vs. OVA group, $p < 0.05$) (Fig. 9A–C). However, ARO and DEX exerted an inhibitory effect on, not only the activation of NF-κB p65 but also the activation of IκB, an NF-κB inhibitor, in the lungs of AA mice (ovalbumin group vs. OVA +1 mg/kg DEX group, $p < 0.05$; ovalbumin group vs. OVA +5 mg/kg ARO group, $p < 0.05$; ovalbumin group vs. OVA +10 mg/kg ARO group).

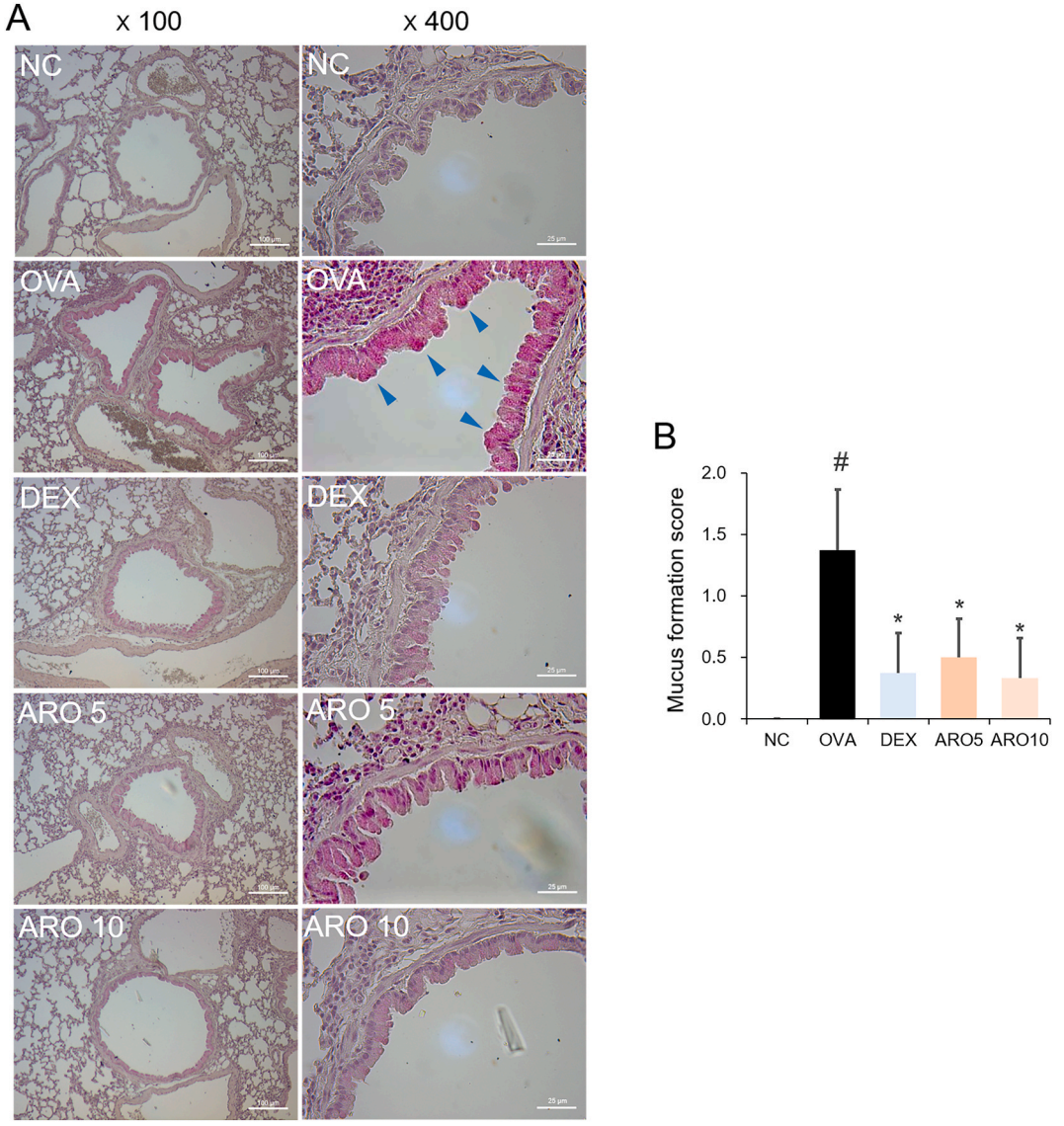

Fig. 8. Inhibition of mucus formation in AA mice by ARO. (A) The histological changes that reflect notable formation of mucus in the lungs of AA mice were confirmed using PAS staining (left, magnification × 100, scale bar 100 μm: right, magnification × 400; scale bar, 25 μm). A high formation of mucus was detected on the airway epithelium in the lungs (blue arrowheads). This was suppressed by ARO administration. (B) The scoring of mucus formation was determined by semi-quantitative analysis. Data are expressed as the mean ± SD ($^{\#}p < 0.05$ for comparison with normal control; $^{*}p < 0.05$ for comparison with OVA group).

4. Discussion

In this study, we confirmed that ARO exhibits anti-asthmatic effect through suppression of inflammatory cytokines and NF-κB activation, in PMA-stimulated A549 cells and in OVA-exposed AA mice.

Eosinophil adhesion to airway epithelium is an important event in AA [34]. Recent studies have revealed that the combination of airway epithelial cells and eosinophils is weakened by the suppression of airway epithelial cell-derived cytokines such as IL-6 and MCP-1 [15]. Compounds that can suppress airway epithelial cell-derived cytokines and airway cell-eosinophil adhesion exert ameliorative effects against bronchial inflammation in experimental models of AA [16,35,36]. In our *in vitro* study, ARO inhibited not only IL-1β, IL-6, TNF-α, and MCP-1 production in A549 cells but also airway cell-eosinophil cohesion. These results suggest that the inhibitory effect of ARO on airway cell-derived cytokines may suppress airway cell-eosinophil adhesion. To further assess this aspect, the ameliorative effects of ARO on bronchial inflammation were examined *in vivo* in AA mice. As expected, ARO ameliorated OVA-induced bronchial inflammation by suppressing eosinophil and macrophage recruitment and Th2 cytokine (IL-4, -5, and -13), IgE, and MCP-1 generation in AA mice. In general, this effect was significant and was comparable to that of DEX. Therefore, we believe

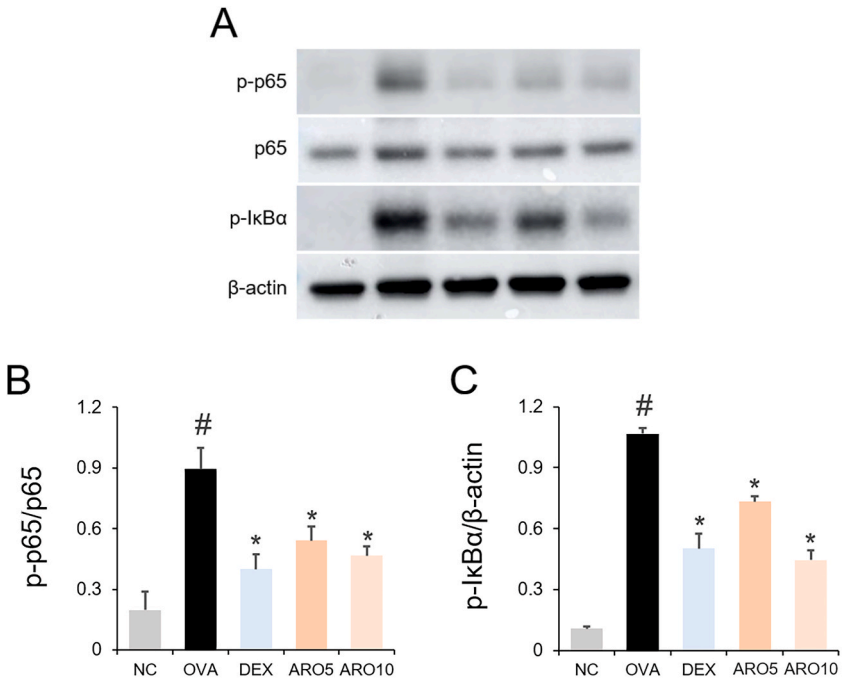

Fig. 9. Inhibition of NF-κB activation in AA mice by ARO. (A) The expression levels of p–NF–κB p65 and p-IκBα in the lungs of AA mice were detected using western blotting. (B–C) Quantitative analysis was performed using ImageJ software. Data are expressed as the mean ± SD ($^{\#}p < 0.05$ for comparison with normal control; $^{*}p < 0.05$ for comparison with OVA group).

that ARO may be a valuable adjuvant for AA treatment.

Th2 cytokines (IL-4, -5, -13) are closely associated with the promotion of AA. It has been shown that the upregulation of Th2 cytokines (IL-4/-5/-13) is induced in OVA-induced AA mice [37,38]. IL-4 induces B cell activation [39] and IL-5 causes eosinophil activation and influx in AA [40]. IL-4 and -5 affect MCP-1 secretion by epithelial cells, leading to macrophage influx [41,42]. IL-13 has been implicated in B cell and mast cell activation and IgE and mucus formation in AA [43–45]. Considering the inhibitory effect of ARO on Th2 cytokines, observed in our *in vivo* experiments, it is expected that the ARO-induced reduction in IL-4/-5/-13 may affect the downregulation of macrophage/eosinophil influx and MCP-1/IgE production. In addition, the inhibition of IL-13 by ARO is expected to reduce mucus formation.

iNOS participates in mucus formation [11]. Elevated COX-2 levels have been reported in asthmatic lungs [46], and the usefulness of COX-2 inhibition has been reported in asthma studies [47,48]. Inam et al. reported that iNOS was upregulated in asthmatic mice [16, 49]. Lee et al. demonstrated the inhibitory effect of ARO on iNOS and COX-2 expression *in vitro* [29]. Thus, we investigated whether ARO suppresses the expression of these molecules in AA *in vivo*. Similar to the model established by Inam et al. iNOS upregulation was also found in AA mice, in our study. In addition, significant upregulation of COX-2 expression was observed in the lungs of the AA mice. Interestingly, ARO inhibited the expression of these molecules in AA mice. These results indicate that the inhibitory effect of ARO on iNOS expression may affect mucus hyperformation associated with airflow obstruction [50], suggesting that ARO contributes to the amelioration of airway obstruction.

Increase in NF-κB phosphorylation causes hyperinflammation by inducing the expression of molecules, such as cytokines (IL-1β/IL-6/TNF-α), chemokines (MCP-1), and mediators (iNOS/COX-2) [12]. Thus, the therapeutic approach for inflammatory lung diseases focuses on the regulation of NF-κB activation. In the present study, the ability of ARO to inactivate NF-κB was confirmed, with its suppressive effect on the formation of IL-1β/IL-6/TNF-α/MCP-1 (*in vitro*) and the expression of iNOS/COX-2 (*in vivo*); in particular, its effect was comparable with the positive control, DEX, in the *in vivo* study. This result implied that NF-κB inactivation induced by ARO may be associated with the amelioration of bronchial inflammation.

Previous studies indicated that compounds from natural products can ameliorate bronchial inflammation by suppressing inflammatory molecules, NF-κB activation and immune cell influx in experimental models of AA [2,16,51]. The results of the present study showed the inhibitory effect of ARO on cytokine secretion, iNOS/COX-2 expression, immune cell influx, and mucus formation. These effects were confirmed with its inhibitory activity on NF-κB phosphorylation. Therefore, these findings indicate that ARO may modulate NF-κB activation in AA therapy.

Our study has some limitations. In this study, A549 cells, a human lung epithelial cell line, were used; primary human cells were not used. Despite the continuous use of A549 cells in *vitro* models of AA for studying the effects and molecular mechanisms of active compounds [2,9,10], the use of primary bronchial epithelial cells from patients with asthma will reinforce the evidence for the anti-asthmatic effect of compounds such as ARO. In addition, considering the reductive effect of ARO on Th2 cytokines *in vivo*, further investigation of ARO modulation of STAT and GATA3 transcription factor activation may be needed. These transcription factors are

known to promote Th2 responses; *in vitro* (primary T cells or T cell lines) and *in vivo* studies will help further establish the effect and mechanism of ARO.

5. Conclusion

In summary, our results from *in vitro* and *in vivo* studies indicate an ameliorative effect of ARO against the PMA-induced inflammatory response in A549 cells, A549 cell-EOL-1 adhesion, and OVA-induced bronchial inflammation in mice. The underlying molecular mechanism for the ameliorative effect of ARO may be associated with modulating NF-κB activation. Thus, our results indicate that ARO has a modulatory effect on bronchial inflammation and may be a potent adjuvant for AA treatment. Pharmacokinetic or bioavailability studies of ARO may help in considering its practical application in AA treatment.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Jin-Mi Park: Data curation, Validation, Visualization, Writing – original draft. **Ji-Won Park:** Data curation, Investigation, Validation, Visualization, Writing – original draft. **Juhyun Lee:** Data curation, Validation, Visualization. **Seung-Ho Kim:** Data curation, Validation. **Da-Yun Seo:** Data curation, Visualization. **Kyung-Seop Ahn:** Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Sang-Bae Han:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Jae-Won Lee:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This research was supported by grants from the KRIBB Research Initiative Program (Grant No. KGM5522322), Korea Drug Development Fund funded by Ministry of Science and ICT, Ministry of Trade, Industry, and Energy, and Ministry of Health and Welfare (grant no. HN21C0996), the Bio & Medical Technology Development Program of the National Research Foundation (NRF) and the Korean government (MSIT) (Grant. No. NRF–2020R1A2C2101228), and the Honam National Institute of Biological Resources (HNIBR), funded by the Ministry of Environment (MOE)(Grant No. HNIBR202302113) of the Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22932.

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