

Oleuropein Curtails Pulmonary Inflammation and Tissue Destruction in Models of Experimental Asthma and Emphysema

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ABSTRACT: Airway inflammation has been implicated in evoking progressive pulmonary disorders including chronic obstructive pulmonary disease (COPD) and asthma as a result of exposure to inhaled irritants, characterized by airway fibrosis, mucus hypersecretion, and loss of alveolar integrity. The current study examined whether oleuropein, a phenylethanoid found in olive leaves, inhibited pulmonary inflammation in experimental models of interleukin (IL)-4-exposed bronchial BEAS-2B epithelial cells and ovalbumin (OVA)- or cigarette smoke (CS)-exposed BALB/c mice. Nontoxic oleuropein at 1–20 μ M diminished eotaxin-1-mediated induction of α -smooth muscle actin and mucin 5AC in epithelial cells stimulated by IL-4 at the transcriptional levels. Oral supplementation of 10–20 mg/kg oleuropein reduced the airway influx of eosinophils and lymphocytes as well as IL-4 secretion in lung promoted by OVA inhalation or CS. In addition, oleuropein suppressed infiltration of macrophages and neutrophils through blocking OVA inhalation- and CS-promoted induction of ICAM-1, F4/80, CD68, and CD11b in airways. OVA-exposed pulmonary fibrosis was detected, while alveolar emphysema was evident in CS-exposed mouse lungs. In alveolar epithelial A549 cells exposed to CS extracts, oleuropein attenuated apoptotic cell loss. Collectively, oleuropein inhibited pulmonary inflammation leading to asthmatic fibrosis and alveolar emphysema driven by influx of inflammatory cells in airways exposed OVA or CS. Therefore, oleuropein may be a promising anti-inflammatory agent for treating asthma and COPD.

KEYWORDS: asthma, chronic obstructive pulmonary disease, cigarette smoke, oleuropein, ovalbumin inhalation, pulmonary inflammation

INTRODUCTION

Proinflammatory responses of the airways have been implicated in evoking chronic obstructive pulmonary disease (COPD) and asthma.^{1–3} Airway narrowing, mucus hypersecretion, and smaller airway stenosis are ascribed to a chronic pulmonary inflammation.^{2,4} These alterations in airways lead to a loss of alveolar integrity through further activating aberrant inflammatory pathways.^{4,5} Airway inflammation is prompted by diverse mediators including reactive oxygen species (ROS) and cell-derived chemotactic factors owing to the presence of environmental toxins.^{6,7} Although bronchial epithelial cells form the interface between the airway environment and the internal milieu, they can induce an airway-specific immune microenvironment and direct immune and inflammatory responses against lung tissue damage.^{8,9} Bronchiolar epithelium-derived monocyte chemoattractant protein-1 and interleukin (IL)-8 can be ascribed to the chemotactic activity of neutrophils.⁶ Additionally, pulmonary mesenchymal cells such as airway smooth muscle cells and lung fibroblasts react with various mediators and produce autocrine responses.¹⁰ In COPD, neutrophils, macrophages, and lymphocytes are prominent, while eosinophils are salient cells in asthmatic airways.^{6,11,12} However, the underlying mechanisms for airway inflammation of asthma and COPD remain unsolved. Underlying mechanisms of small airway destruction and structural changes may reveal potential therapeutic targets in pulmonary diseases.¹³

Several studies are ongoing to pinpoint diverse molecular targets in the airway process to develop novel therapeutic agents.^{14–16} The primary therapeutic option for chronic airway diseases is to combat airway inflammation.^{14,15} In addition, the immunomodulatory therapies in airways may effectively alleviate pulmonary diseases.¹⁵ Inhaled corticosteroids and β -adrenergic receptor agonists, phosphodiesterase antagonists, and statins in clinical applications can influence COPD-associated mechanisms underlying inflammation, mucin overproduction, and tissue destruction.^{16–18} However, the long-term use of these drugs results in undesirable consequences such as risk of pneumonia in patients.¹⁹ Macrolides with antimicrobial, immunomodulatory, and antiviral activities stand for a potential therapeutic remedy for asthma characterized by airway hyperresponsiveness.¹⁵ Currently, the use of macrolides in chronic asthma or acute exacerbations is not yet validated. Therefore, new therapeutic strategies with natural agents that have mostly proven to be safe need to be ripened for pulmonary inflammation to relieve exacerbating rates and all-cause mortality.¹⁴ Numerous studies reveal potential roles for several prevailing natural compounds for the treatment of inflammatory lung diseases.^{20–22} Anti-inflammatory resveratrol, quercetin, mangiferin rich in mango

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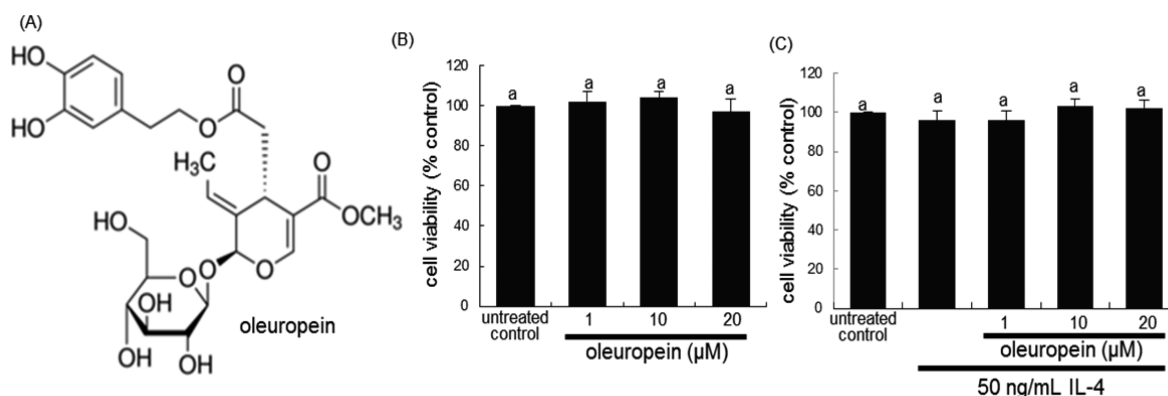


Figure 1. Chemical structure (A) and oleuropein cytotoxicity (B), and oleuropein viability in interleukin (IL)-4-exposed BEAS-2B cells (C). Bronchial epithelial cells were incubated in media containing 50 ng/mL IL-4 in the absence and presence of 1–20 μM oleuropein for 24 h. BEAS-2B cell viability (mean ± SEM, $n = 5$) was measured by using MTT assay and expressed as percent cell survival relative to untreated controls (B, C). Values in bar graphs not sharing a same lower case indicate significant different at $P < 0.05$.

leaf extract, and dihydroquercetin rich in grape leaf extract suppress bleomycin-induced lung damage and pulmonary fibrosis.²² Our new studies reveal that astragalin combated epithelial apoptosis, allergic inflammation, and airway thickening in ovalbumin (OVA)-challenged mice.^{23,24}

It has been well documented that anti-inflammatory resveratrol protects lung diseases such as acute lung injury, asthma, COPD, and lung fibrosis.²⁵ Oleuropein (Figure 1A) is one of phenolic compounds rich in olives and has antioxidant, antimicrobial, and antiobesity properties.^{26–28} However, the beneficial effects of oleuropein on asthma and smoking-induced emphysema have not been reported. On the basis of the evidence that polyphenols abundant in various fruits and vegetables may inhibit inflammatory diseases in airways,^{22,29} this study investigated that oleuropein had the protective properties in asthma model characteristic of eosinophilic inflammation caused by IL-4 and OVA administration. In contrast to asthma, inflammation in COPD by cigarette smoke (CS) leads to progressive remodeling of lung tissues;³⁰ therefore, this study explored the beneficial effects of oleuropein on neutrophilic inflammation in CS-challenged mouse models. The current study demonstrated that oleuropein suppressed OVA- or CS-induced recruitment of eosinophils, macrophages, neutrophils, and lymphocytes and that this compound inhibited alveolar destruction causing emphysema in CS-challenged mice.

MATERIALS AND METHODS

Chemicals. M199, human epidermal growth factor (EGF), hydrocortisone, gelatin, human insulin, apo-transferrin, oleuropein, chicken egg white albumin, and thrombin were provided from the Sigma-Aldrich Chemical (St. Louis, MO) unless specifically described elsewhere. Fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were supplied by the Lonza (Walkersville, MD). Human proteins of IL-4 and eotaxin-1 (CCL11), and goat polyclonal eotaxin-1 antibody were supplied from R&D systems (Minneapolis, MN). Rabbit polyclonal antibodies of intracellular adhesion molecule (ICAM)-1 and CD68 were provided from the Santa Cruz Biotechnology (Dallas, TX). Rabbit polyclonal antibodies of α -smooth muscle actin (α -SMA) and CCR3, mouse monoclonal mucin 5AC (MUC5AC) antibody, rat monoclonal F4/80 antibody (clone A3–1, IgG2b), goat polyclonal CD11b antibody, and rabbit monoclonal proteinase-activated receptor (PAR)-2 (clone EPRI3675, IgG) were purchased from Abcam (Cambridge, UK). Rabbit monoclonal phospho-STAT6 (Tyr641) antibody (Clone 46HIL12, IgG) was obtained from Thermo Fisher Scientific

(Rockford, IL). Horseradish peroxidase (HRP)-conjugated goat antirabbit IgG, donkey antigoat IgG, and goat antimouse IgG were obtained from Jackson Immuno-Research Laboratories (West Grove, PA). Mouse monoclonal β -actin antibody (clone AC-74, IgG2a) was obtained from Sigma-Aldrich Chemicals. Essential fatty acid free bovine serum albumin (BSA) and skim milk were supplied by Becton Dickinson Company (Sparks, MD). 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Santa Cruz Biotechnology. Oleuropein was dissolved in dimethyl sulfoxide (DMSO) for cell culture and animal gavage; a final concentration of DMSO was <0.5%.

Preparation of Cigarette Smoke Extracts (CSE). Research cigarettes (3R4F, 11 mg tar and 0.7 mg nicotine per cigarette) were obtained from University of Kentucky (Lexington, KY). The cigarettes were consecutively smoked through an experimental apparatus with a constant airflow driven by an air vacuum pump. The collected cigarette smoke was bubbled in 10 mL of phosphate buffered saline (PBS). The resulting smoke suspension was filtered through a 0.22-μm pore filter to eliminate bacteria and large particles. The filtrates referred to a 100% cigarette smoke extracts (CSE).

Cell Culture. Human bronchial airway epithelial cell line (BEAS-2B cells, American Type Culture Collection, Manassas, VA) was incubated in 25 mM HEPES-buffered M199 with 10% FBS containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin supplemented with 2.5 μg/mL insulin, 361 ng/mL hydrocortisone, 2.5 μg/mL apotransferrin, and 20 ng/mL EGF. BEAS-2B cells were maintained in 90–95% confluence at 37 °C in an atmosphere of 5% CO₂. BEAS-2B cells were treated with 50 ng/mL IL-4 in the absence and presence of 1–20 μM oleuropein for up to 72 h.

Human alveolar basal epithelial cells (A549 cells, American Type Culture Collection) were cultured in RPMI1680 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. A549 cells were cultured in 90–95% confluence at 37 °C in an atmosphere of 5% CO₂. A549 cells were stimulated with 0.1–20% CSE with and without 1–20 μM oleuropein for up to 72 h.

The cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT, Duchefa Biochemie, Haarlem, Netherlands) assay. The resulted purple formazan product was dissolved in 0.5 mL of 2-propanol with gentle shaking. Absorbance of formazan dye was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm. The cell viability was not influenced when 1–20 μM oleuropein was solely treated to BEAS-2B cells (Figure 1B).

Animal Experiments. Six week-old male BALB/c mice were employed in the current study (Hallym University Breeding Center for Laboratory Animals). Mice were left on a 12 h light/12 h dark cycle at 23 ± 1 °C with 50 ± 10% relative humidity under restricted pathogen-free circumstance, fed a nonpurified diet, and supplied with

water ad libitum at the animal facility of Hallym University. Mice were admitted to acclimatize for 1 week before the experiments began.

For the asthma experiments, mice were divided into four subgroups ($n = 9$ for each subgroup). Chronic allergic asthma was induced in murine models as described in previous studies.^{23,24} Mice were sensitized with 20 μ g of OVA and 50 μ L of Imject Alum (Thermo Fisher Scientific) by a subcutaneous injection twice on day 0 and day 14. Next, 0.1 mL of oleuropein (containing 10 or 20 mg/kg BW) was administered via oral gavage to OVA-sensitized mice 1 h before challenge. Once per week, 5% OVA inhalation to mice was conducted for 20 min in a plastic chamber linked to an ultrasonic nebulizer. Control mice were sensitized and challenged with PBS as the OVA vehicle. For the passive smoking models, mice were also divided into four subgroups ($n = 9$ for each subgroup). Oleuropein (10 or 20 mg/kg BW) was administered to mice via oral gavage once a day (5 days/week) for 8 weeks. An hour later, mice were exposed to smoke of research cigarettes (11 mg tar and 0.7 mg nicotine/cigarette) for 30 min in a specially designed chamber.

All the mice were killed in the presence of an anesthetic. The trachea was cannulated, and both lungs and airways were rinsed in PBS to collect bronchoalveolar lavage fluid (BALF). The numbers of inflammatory cells including neutrophils and eosinophils in BALF were determined using a Hemavet HV950 Multispecies Hematologic Analyzer (Drew Scientific, Oxford, CT). The right lungs were kept at -80°C for Western blotting. Left lungs were preserved and fixed in 4% paraformaldehyde and then used for immunohistochemical analyses. Airway tissue levels of IL-4, IL-1 β , and IL-6 were measured by using commercial ELISA kits (R&D systems).

The current study was approved by the Hallym University Institutional Review Board and Committee on Animal Experimentation (Hallym 2017–36). This study was carried out in compliance with the University's Guidelines for the Care and Use of Laboratory Animals. No dead mice and no evident signs of lethargy were observed during the experimental period.

Western Blot Analysis. BALB/c lung tissue extracts and whole cell lysates were prepared in 1 mM Tris-HCl (pH 6.8) lysis buffer containing 10% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail. Tissue extracts or cell lysates containing equal amounts of proteins were electrophoresed on 6–15% SDS-PAGE and transferred onto a nitrocellulose membrane. Blocking a nonspecific binding was accomplished by using either 3% fatty acid-free BSA or 5% nonfat dry skim milk for 3 h. The membrane was incubated overnight at 4°C with a specific primary antibody of target proteins. The membrane was then incubated with a secondary antibody conjugated to HRP for 1 h. Following washing, the target protein was detected using the Immobilon Western Chemiluminescent HRP substrate (Millipore Corp., Billerica, MA) and the Agfa medical X-ray film blue. Incubation with β -actin antibody was done for the comparative control.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was extracted from lysed BEAS-2B epithelial cells and BALB/c lung tissue extracts with use of a commercial Trizol reagent kit (Molecular Probes Inc., Cincinnati, OH). The complementary DNA was synthesized using 5 μ g of total RNA with 200 units of reverse transcriptase (Promega Corp. Madison, WI) and 0.5 mg/mL oligo-(dT)15 primer (Bioneer, Daejeon, Korea). The PCR was carried out using mRNA transcripts of eotaxin-1, α -SMA, MUC5AC, GAPDH, and β -actin by supplying 25 μ L of 10 mM Tris-HCl buffer (pH 9.0) containing 10 mM dNTP and 5 units of Taq DNA polymerase (Takara Bio Inc., Minamikusatsu, Japan). The sequences of PCR primer sets were listed in Table 1. The PCR conditions of individual target genes were sequentially established as predenaturation at 94°C for 5 min, and 30 cycles of 94°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 45 s, and the final extension was terminated at 72°C for 10 min. After thermocycling, electrophoresis of the final PCR products was done on 1% agarose gel containing 0.1% ethidium bromide, each band was visualized by UV transilluminator, and gel photographs were obtained.

Staining with Hematoxylin and Eosin (HE). For the histological analyses, specimens of small airways and alveoli collected at the end of the experiments were fixed in 10% paraformaldehyde.

Table 1. Sequences of PCR Primer Sets Used for Gene Expression Analysis

identifier		sequence	size (bp)
human eotaxin-1	sense	5'-TAC CCC TTC AGC GAC TAG AGA-3'	446
	antisense	5'-CAC GTG ACA GGG GAT CGA AT-3'	
human α -SMA	sense	5'-CTA TGA GGG CTA TGC CTT GCC-3'	122
	antisense	5'-GCT CAG CAG TAG TAA CGA AGG A-3'	
mouse MUC5AC	sense	5'-CAG GAC TCT CTG AAA TCG TAC CA-3'	128
	antisense	5'-AAG GCT CGT ACC ACA GGG A-3'	
mouse GAPDH	sense	5'-AAC TTT GGC ATT GTG GAA GGG-3'	224
	antisense	5'-GAC ACA TTG GGG GTA GGA ACA C-3'	
human β -actin	sense	5'-GAC TAC CTC ATG AAG ATC-3'	500
	antisense	5'-GAT CCA CAT CTG CTG GAA-3'	

The paraffin-embedded specimens were sectioned at 5 μ m thickness, deparaffinized and stained with HE for 2 min, and promptly dehydrated in 95% absolute alcohol. The HE-stained tissue sections were detected using an optical microscope Axioimager system equipped for fluorescence illumination (Zeiss, Gottingen, Germany). Five images were taken from each section.

Immunohistochemical Staining. For the immunofluorescent immunohistochemical analyses, paraffin-embedded tissue sections of lungs were deparaffinized and hydrated. The sections were preincubated in a boiling sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. The tissues were specifically blocked with 5% BSA in PBS for 1 h. A specific primary antibody against F4/80, CD68, CD11b, or PAR-2 was incubated with the tissue sections overnight. Next, the tissue sections were treated for 1 h with FITC-conjugated IgG or Cy3-conjugated IgG (Rockland, Pottstown, PA). For the counter-staining of nuclei, the nucleic acid dye DAPI was given for 10 min. Stained tissues were mounted on slides, and images of each slide were taken with an optical microscope.

Staining of Alcian Blue and Periodic Acid Schiff (PAS). To examine acid and neutral mucins produced by goblet cells, the paraffin-embedded lung specimens were sectioned at 5 μ m thickness, deparaffinized, stained with alcian blue (Sigma-Aldrich Chemical) for 15 min, and well-washed in running tap water shortly. Subsequently, the lung tissue sections were PAS-stained and dehydrated. The stained tissues were observed using an optical microscope and images obtained from each section.

Hoechst 33258 Staining. After the fixation of A549 cells with ice-cold 4% formaldehyde for 1 h on a glass-chamber slide, the nuclear stain Hoechst 33258 (Promega Co., Madison, WI) was added at a final concentration of 10 μ g/mL for 15 min to concede uptake and equilibration prior to microscopic observation. Cells containing fragmented or condensed nuclei were considered apoptotic. Images of each slide for nuclear morphology were taken with an optical microscope.

Statistical Analysis. The resulted data were expressed as mean $\pm$ SEM for each experimental group. Statistical analyses were carried out by using Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was verified by one-way analysis of variance, followed by Duncan range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

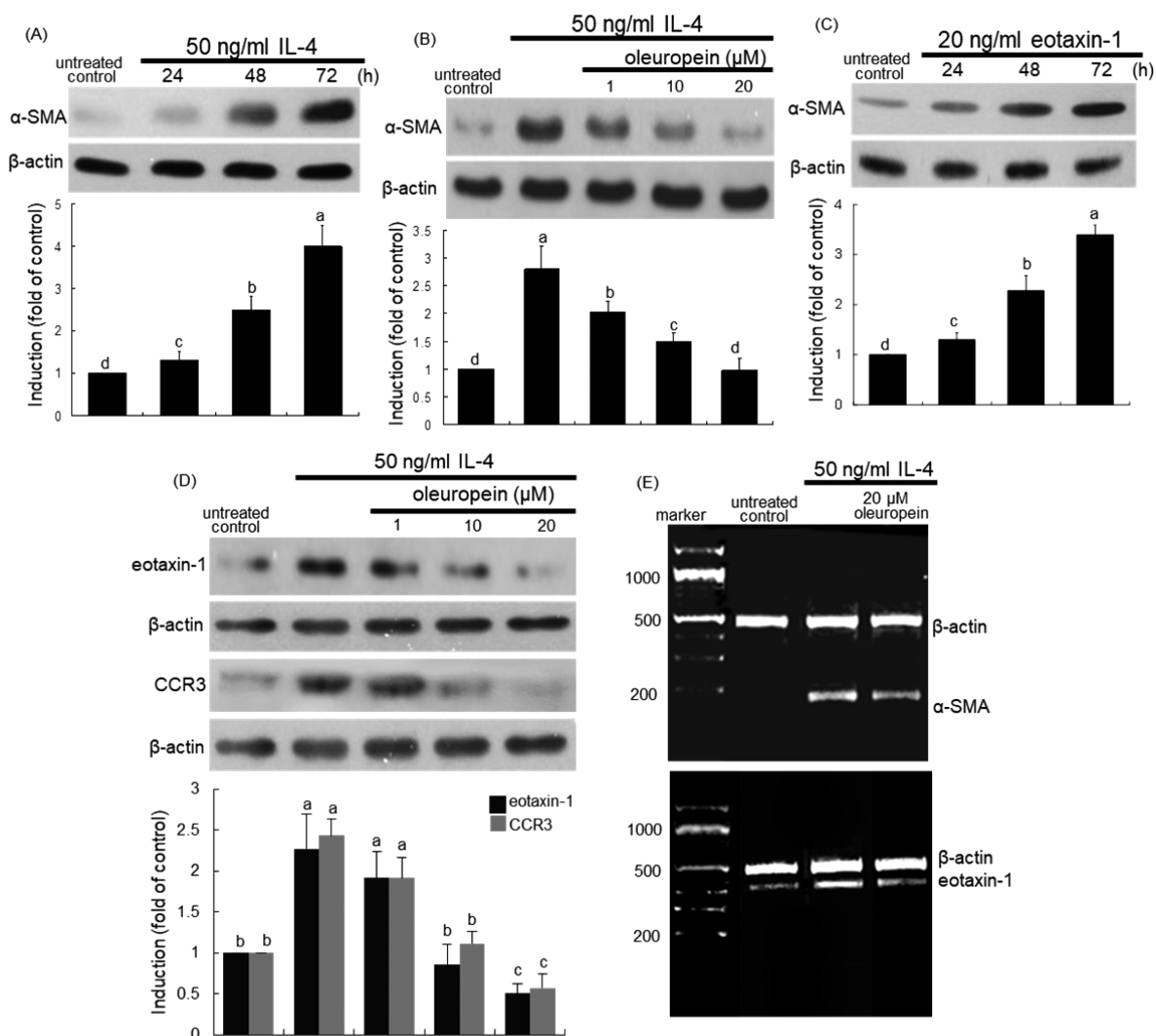


Figure 2. Western blot analysis showing inhibition of airway fibrosis and eosinophilia by oleuropein in interleukin (IL)-4- or eotaxin-1-exposed bronchial epithelial cells. Human BEAS-2B cells were treated with 1–20 μ M oleuropein and stimulated with IL-4 (A, B, D) or eotaxin-1 (C) for up to 72 h. Cell lysates were prepared for Western blot analysis with a primary antibody against α -SMA, eotaxin-1, and CCR3. β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results of the upper bands obtained from a densitometer. Values in bar graphs not sharing a same lower case indicate significant different at $P < 0.05$. RT-PCR analysis showing blockade of IL-4-stimulated transcriptional induction of eotaxin-1 and α -SMA in airway epithelial cells by oleuropein (E). β -Actin gene was used as an internal control for coamplification in RT-PCR analysis.

RESULTS

Inhibitory Effects of Oleuropein on IL-4-Induced Fibrosis and Eosinophil Infiltration. Hyperplasia and hypertrophy of airway smooth muscle result in thickening and fibrosis of the airway wall.³¹ This study examined whether 1–20 μ M oleuropein inhibited fibrosis and eosinophil recruitment in 50 ng/mL IL-4-exposed airway epithelial cells. It should be noted that oleuropein did not show any cytotoxicity within its concentrations of 1–20 μ M (Figure 1C). When IL-4 were solely applied to bronchial epithelial cells for up to 72 h, cellular expression of α -SMA was highly accelerated (Figure 2A). In contrast, 1–20 μ M oleuropein diminished IL-4-induced induction of α -SMA in a dose-dependent manner (Figure 2B). Additionally, the eosinophil chemotactic protein eotaxin-1 enhanced the α -SMA induction in bronchial epithelial cells in a similar manner to the IL-4 cytokine (Figure 2C). This study found that ≥ 10 μ M oleuropein dampened the induction of eotaxin-1 and CCR3

by IL-4 (Figure 2D). The RT-PCR analysis was employed to accomplish inhibitory effects of oleuropein on the transcription of α -SMA and eotaxin-1. IL-4 elevated the mRNA levels of α -SMA and eotaxin-1 of bronchial epithelial cells, while oleuropein attenuated the elevated transcription of these proteins (Figure 2E). Accordingly, IL-4 may induce airway fibrosis and eosinophil recruitment through augmenting airway epithelial proteins of α -SMA and eotaxin-1 at their transcriptional levels.

This study further revealed that the OVA inhalation induced the thickening of mouse airways, evidenced by HE staining with blue nuclei and pink cytoplasm and extracellular matrix. There was a marked cell infiltration and fibrosis detected in the small airways of OVA-challenged mice (Figure 3A). On the contrary, oral administration of 10–20 mg/kg oleuropein diminished the subepithelial fibrosis evoked by OVA inhalation. The OVA inhalation increased the lung tissue eotaxin-1 level, and such increase in eotaxin-1 was highly diminished in 20 mg/kg oleuropein-treated mice (Figure 3B).

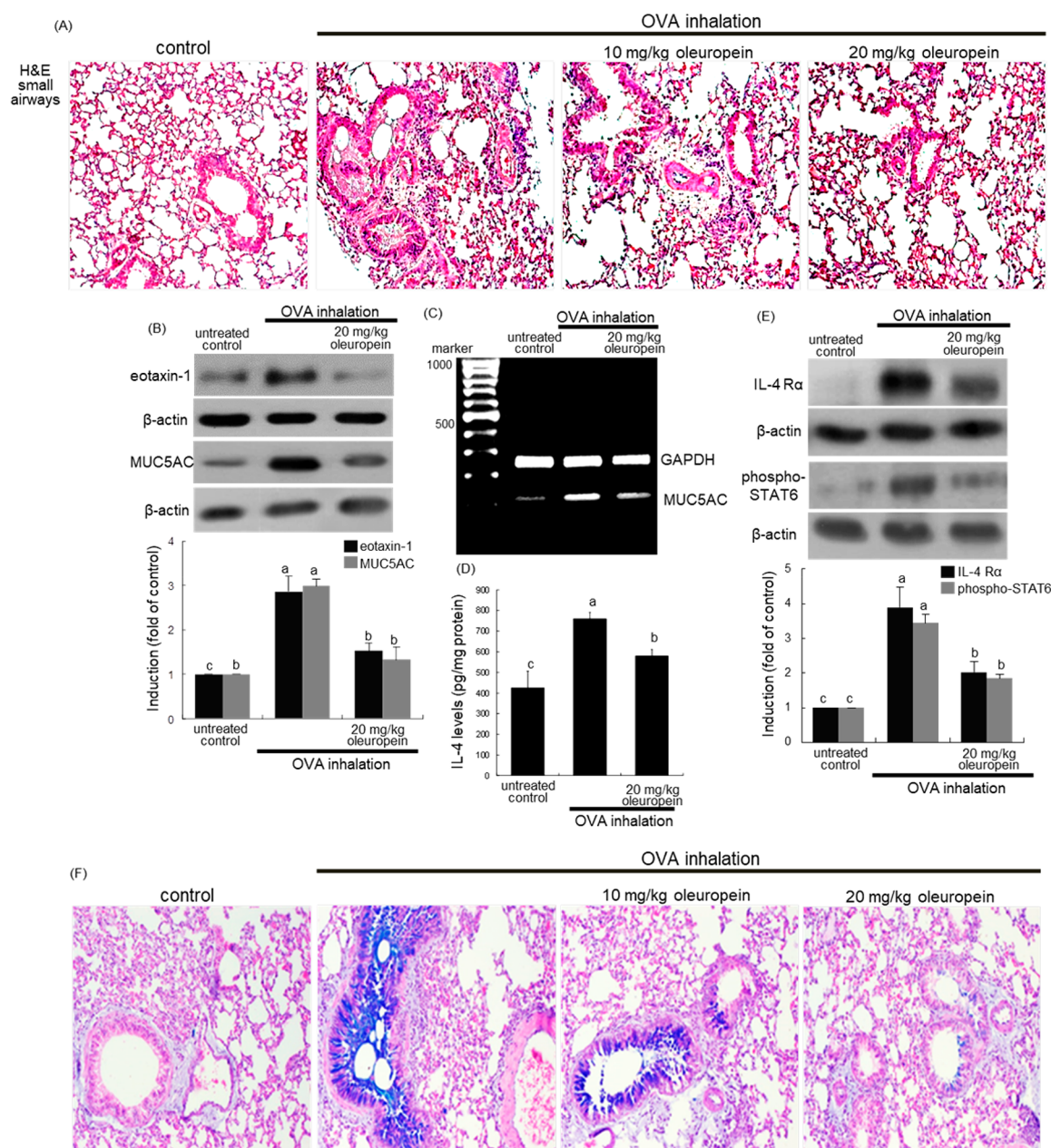


Figure 3. Suppressive effects of oleuropein on airway fibrosis, airway interleukin (IL)-4 secretion, and induction of airway target proteins in ovalbumin (OVA) inhalation-challenged mouse lungs. OVA-sensitized mice were orally administered with 10 or 20 mg/kg oleuropein. Airway tissue sections were stained by using HE reagent (A). Each photograph is representative of four mice. Magnification: 200-fold. Lung tissue extracts were subject to Western blot with a primary antibody against eotaxin-1, MUC5AC, IL-4 R α , and STAT6 (B, E), and phosphorylated STAT6 (phospho-STAT6, D). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results of upper bands obtained from a densitometer. Values in bar graphs not sharing a same lower case indicate significant different at $P < 0.05$. RT-PCR analysis showing blockade of transcriptional induction of MUC5AC in OVA-sensitized airways by oleuropein (C). β -Actin gene was used as an internal control for coamplification in RT-PCR analysis. The IL-4 secretion in lungs was measured using ELISA kit (D). Alcian blue and periodic acid schiff (AB-PAS) staining was performed in small airways and trachea of OVA-sensitized and oleuropein-treated mice (F). Nuclear counter-staining was conducted with hematoxylin. Each photograph is representative of four mice. Magnification: 400-fold.

Interestingly, oleuropein encumbered the induction of MUC5AC in lung tissues exposed to OVA, indicating its inhibition of mucus hypersecretion in the pulmonary tracts. In addition, oleuropein inhibited OVA inhalation-elevated MUC5AC mRNA level in mouse lungs (Figure 3C). Thus, oleuropein reduced the MUC5AC induction via a direct modulation at its transcriptional level. On the other hand, the OVA inhalation increased the lung tissue levels of IL-4, IL-4R α , and STAT6 in mice, which was reduced by treating 20

mg/kg oleuropein (Figure 3D,E). Accordingly, it is deemed that the subepithelial fibrosis and cell infiltration in airways contribute to the OVA-induced activation of IL-4-IL-4R α -STAT6 pathway in airways.

Alcian blue-PAS dye was employed to stain mucus produced by epithelial goblet cells. There was strong staining of mucus-producing goblet cells in small airways of OVA-exposed mice observed (Figure 3F). In contrast, 20 mg/kg oleuropein markedly reduced the staining in airway epithelium of OVA

Table 2. Cell Numbers in BALF of OVA Inhalation-Exposed Mice^a

cells groups	total cells	neutrophils	monocytes	eosinophils	basophils	lymphocytes
Cells in BALF (×2000/mL)						
untreated control	197 ± 4d	3 ± 3b	20 ± 3b	17 ± 2b	8 ± 2c	98 ± 8c
OVA inhalation	339 ± 7a	77 ± 8a	27 ± 4a	37 ± 7a	17 ± 4a	133 ± 12a,b
OVA inhalation +10 mg/kg oleuropein	308 ± 5b	82 ± 8a	20 ± 1b	20 ± 4b	10 ± 1b	138 ± 8a
OVA inhalation +20 mg/kg oleuropein	225 ± 6c	58 ± 6b	19 ± 3b	19 ± 3b	10 ± 1b	122 ± 10b

^aOvalbumin (OVA)-sensitized mice were orally administrated with 10 or 20 mg/kg oleuropein. Values are mean ± SEM (*n* = 6). Mean values in a column not sharing a same lower case indicate significant different at *P* < 0.05.

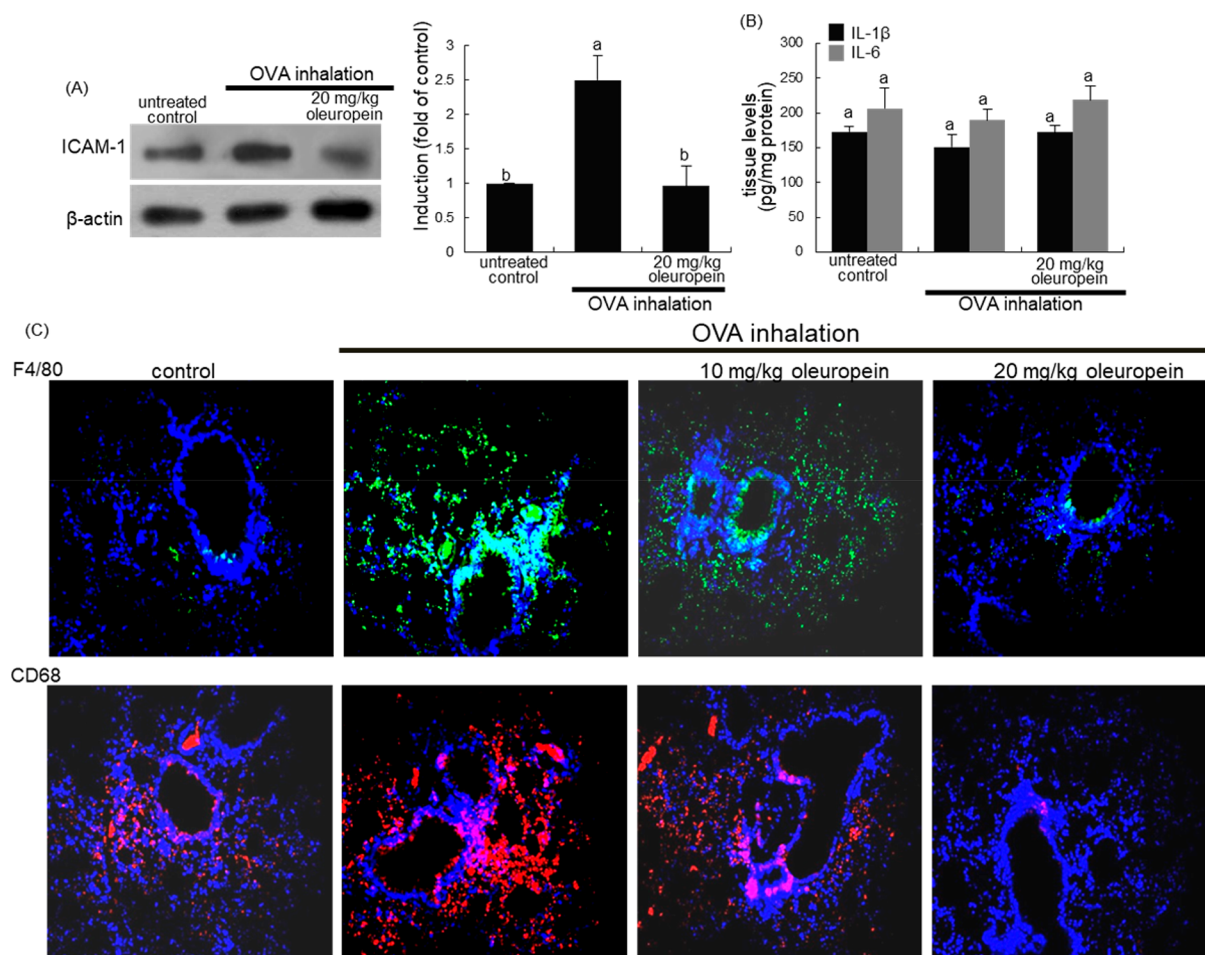


Figure 4. Inhibition of allergic inflammation by oleuropein in small airways of ovalbumin (OVA) inhalation-challenged mice. OVA-sensitized mice were orally administrated with 10 or 20 mg/kg oleuropein. Tissue extracts were subject to Western blot with a primary antibody against ICAM-1 (A). β -Actin protein was used as an internal control. The bar graphs (mean ± SEM, *n* = 3) represent quantitative results of the left bands obtained from a densitometer. Airway tissue levels of IL-1 β and IL-6 were measured by using ELISA kits (B). Values in bar graphs not sharing a same lower case indicate significant different at *P* < 0.05. Immunohistofluorescence analysis showing blockade of macrophage infiltration by oral administration of oleuropein in small airway tissues of OVA inhalation-challenged mice (C). The F4/80 localization was identified as FITC-green staining, and CD68 was confirmed by Cy3-red staining in mouse airways exposed to OVA. Nuclear staining was done with DAPI (blue). Each photograph is representative of four mice. Magnification: 200-fold.

mice (Figure 3F). Thus, oleuropein may attenuate goblet cell hyperplasia and mucus production.

Disturbance of Infiltration of Macrophages and Neutrophils by Oleuropein. Macrophages and neutrophils are penetrated into airways and alveoli of COPD, while activated eosinophils are infiltrated in asthmatic airways.¹¹ The total cell number in the BALF of mice exposed to OVA enhanced with the predominance of eosinophils (>2-fold increase) (Table 2). The administration of ≥ 10 mg/kg oleuropein hampered OVA-induced eosinophil influx, which was incomparable to that of PBS-treated mice. Interestingly,

the OVA-challenged mice had higher numbers of neutrophils and monocytes in the BALF than those of control mice, indicating that OVA induced airway inflammation responsible for infiltration (Table 2). In contrast, oleuropein diminished the numbers of these inflammatory cells in the BALF and lessened lymphocytosis.

Adhesion molecules are associated with various stimuli-responsive pulmonary inflammation.³² ICAM-1 is responsible for pulmonary neutrophil recruitment in lipopolysaccharide (LPS)-induced airway diseases.³³ Western blot data revealed that oleuropein abolished the ICAM-1 induction by OVA

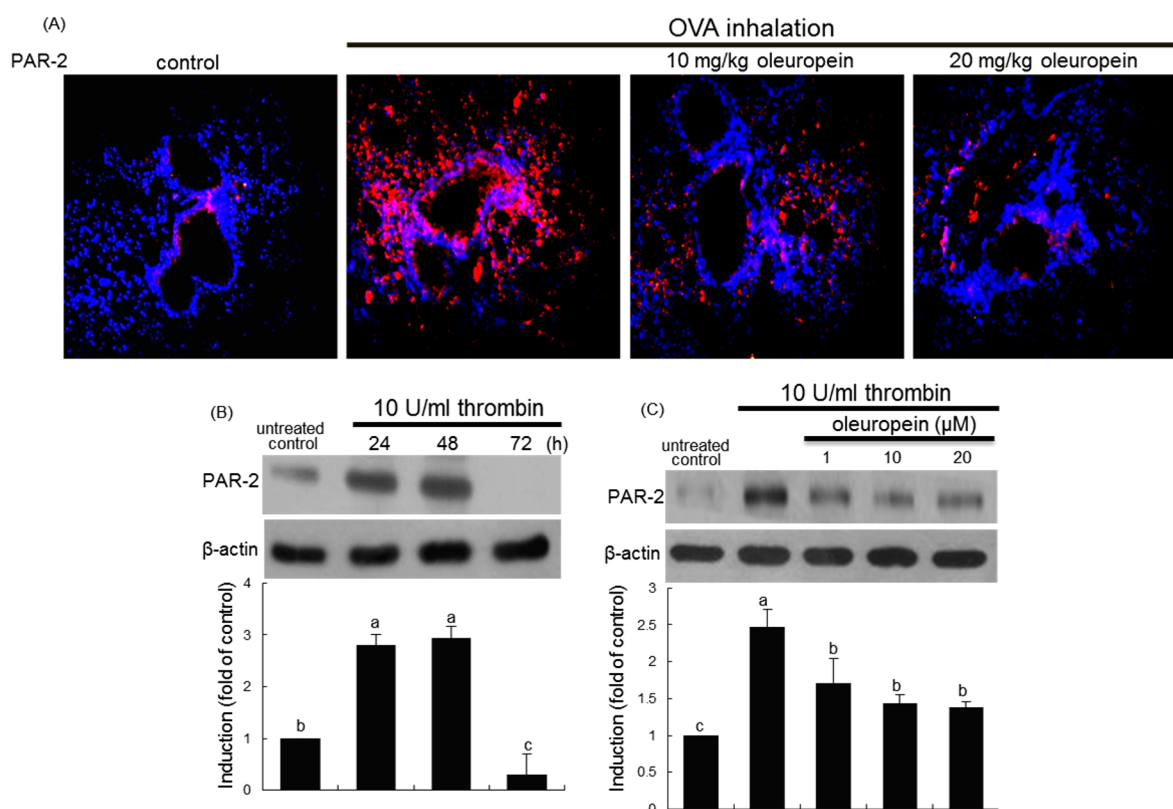


Figure 5. Inhibitory effects of oleuropein on proteinase-activated receptor (PAR)-2 induction in small airways of ovalbumin (OVA) inhalation-challenged mice and in thrombin-treated BEAS-2B cells. OVA-sensitized mice were orally administrated with 10 or 20 mg/kg oleuropein. Immunohistofluorescence analysis was done in small airway tissues of OVA inhalation-challenged mice. PAR-2 was confirmed by Cy3-red staining in mouse airways exposed to OVA. Nuclear staining was done with 4',6-diamidino-2-phenylindole (blue). Each photograph is representative of four mice. Magnification: 200-fold. Bronchial epithelial cells were incubated in media containing 10 U/ml thrombin in the absence and presence of 1–20 μ M oleuropein for up to 72 h. Tissue extracts were subject to Western blot with a primary antibody against PAR-2 (A). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results of the upper bands obtained from a densitometer. Values in bar graphs not sharing a same lower case indicate significant different at $P < 0.05$.

(Figure 4A). However, OVA did not influence the secretion of the inflammatory cytokines of IL-1 β and IL-6 (Figure 4B). The macrophage markers of F4/80 (FITC-green staining) and CD68 (Cy3-red staining) were markedly expressed in small airways of mice exposed to OVA (Figure 4C). In contrast, the immunohistochemical staining demonstrated that the oral treatment of 10–20 mg/kg oleuropein attenuated the induction of F4/80 and CD68 in small airways of OVA-challenged mice (Figure 4C). Notably, it can be assumed that the macrophage infiltration occurred in airway subepithelial wall of mice stimulated with OVA. Thus, oleuropein may prevent the infiltration of neutrophils and macrophages in OVA-challenged mouse small airways.

Blockade of Pulmonary Thrombosis by Oleuropein.

PARs, sensors for active extracellular serine proteases, may represent novel pharmacological targets in airway diseases like asthma and COPD.³⁴ The OVA inhalation evoked the PAR-2 induction in small airways of mice (Figure 5A). Its induction appeared to mainly occur in perivascular regions of airways. Oral supplementation of 10–20 mg/kg oleuropein ablated such induction in mice (Figure 5A). This study further tested whether the PAR-2 induction was enhanced in bronchial epithelial cells treated with the serine protease thrombin participating in the clotting process. The PAR-2 induction was highly promoted within 48 h after exposure of bronchial epithelial cells to thrombin (Figure 5B). However, $\geq 1 \mu$ M

oleuropein attenuated the epithelial PAR-2 induction (Figure 5C). Accordingly, oleuropein may antagonize pulmonary thrombosis triggered by asthmatic insults through suppressing the PAR-2 induction.

Suppressive Effects of Oleuropein on Allergic Inflammation in CS-Challenged Airways. The current study examined whether oleuropein allayed airway inflammation evoked by CS. Exposure of mice to CS increased total leukocyte number in the BALF by ~ 2 -fold (Figure 6A). The administration of ≥ 10 mg/kg oleuropein curtailed CS-induced leukocytosis of neutrophils, lymphocytes, and monocytes, which was comparable to that of control mice (Figure 6A). Interestingly, the CS challenge increased eosinophil influx in the BALF like the OVA inhalation, which was also attenuated by oleuropein (Figure 6A). Furthermore, the secretion of the inflammatory cytokines of IL-4 and IL-1 β was elevated in CS-challenged mouse lungs, while oleuropein reduced their secretion (Figure 6B). When mice were exposed to CS for 30 min, the lung tissue levels of IL-4 $R\alpha$, eotaxin-1, and MUC5AC increased in a similar manner to cellular IL-4 challenge (Figure 6C vs Figure 2). On the contrary, oral administration of 20 mg/kg oleuropein reduced these levels. Accordingly, oleuropein may alleviate CS-induced and IL-4-mediated allergic inflammation in airways.

The histological examination was conducted in lung tissues stained with HE. The CS challenge resulted in tissue damage

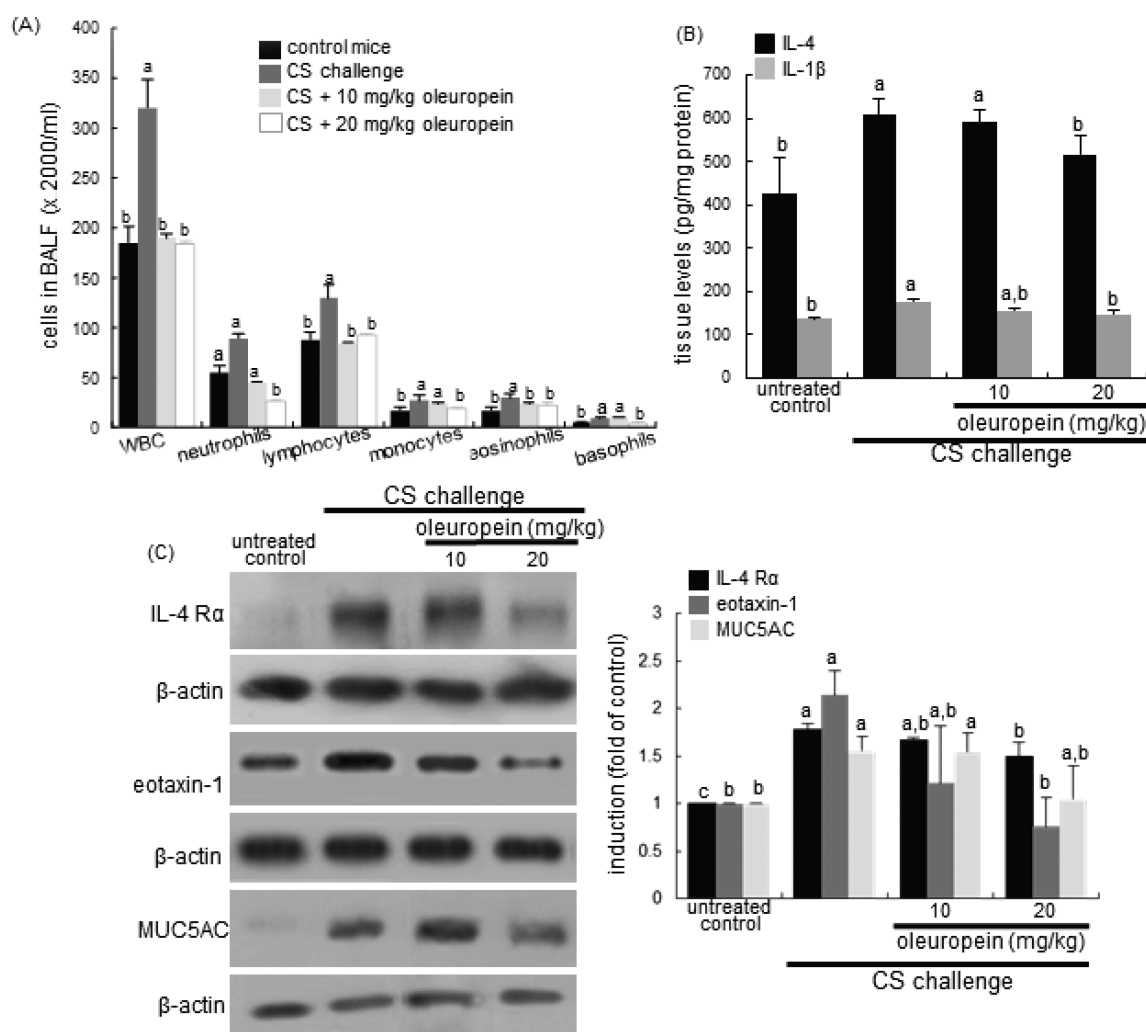


Figure 6. Cells in the BALF (A), airway secretions of IL-4 and IL-1 β (B), and target protein induction (C) in mice exposed to cigarette smoke (CS) and supplemented with oleuropein. Mice were orally administrated with 10 or 20 mg/kg oleuropein and exposed to CS for 30 min. Cells in BALF were counted using a Hemavet HV950 Multispecies Hematologic Analyzer (A). The secretion of IL-4 and IL-1 β was measured using ELISA kits (B). Tissue extracts were subject to Western blot with a primary antibody against IL-4 R α , eotaxin-1 or MUC5AC (C). β -Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results of the left bands obtained from a densitometer. Values in bar graphs not sharing a same lower case indicate significant different at $P < 0.05$.

of airway walls and emphysema in alveoli (Figure 7A). The treatment with oleuropein noticeably blocked these pathological alterations in lung tissues of CS-challenged mice. Furthermore, F4/80 and CD11b were highly expressed in mouse airways exposed to CS (Figure 7B). However, the staining showed that the oral administration of 10–20 mg/kg oleuropein reduced their induction in CS-challenged airways (Figure 7B). Therefore, oleuropein may disturb the recruitment of macrophages and neutrophils in CS-directed airways through manipulating the F4/80 and CD11b induction.

Inhibition of Apoptosis of CS-Challenged Alveolar Cells by Oleuropein. This study attempted to examine how CS resulted in emphysema in mouse alveoli and how oleuropein protected alveoli from CS-triggered damage. When 1–20% CSE was applied to alveolar epithelial A549 cells for up to 48 h, the viability dropped up to below 20% (Figure 8A). Moreover, the treatment of A549 cells with $\leq 1\%$ CSE did not lead to cytotoxicity for 48 h, while the 48 h-exposure of cells to 5–10% CSE resulted in marked cell loss by $\sim 45\%$ (Figure 8B). When A549 cells were supplemented with 5% CSE for 24 h, the viability was significantly reduced in an

apoptotic manner (Figure 8C). The cytotoxicity was much severe and cells were lost in 48 h-exposed cells with 5% CSE. On the other hand, in $\geq 10 \mu\text{M}$ oleuropein-added A549 cells, the apoptotic cell death of 5% CSE was significantly curtailed, evidenced by nuclear staining with Hoechst 33258 (Figure 8D). Accordingly, CS-induced alveolar emphysema may be ascribed to its apoptotic death of alveolar cells.

DISCUSSION

Nine major findings were extracted from this study. (1) Nontoxic oleuropein at $\leq 20 \mu\text{M}$ diminished IL-4-induced cellular expression of α -SMA, eotaxin-1 and CCR3. (2) Eotaxin-1 enhanced the α -SMA induction in bronchial epithelial cells, indicating that IL-4 may induce airway fibrosis through its induction of eotaxin-1. (3) Oral administration of 10–20 mg/kg oleuropein inhibited bronchial subepithelial fibrosis and IL-4 secretion in mouse airways augmented by OVA inhalation. (4) The OVA inhalation increased the lung tissue levels of IL-4 R α , eotaxin-1, and MUC5AC, which was highly reduced in 20 mg/kg oleuropein-treated mice. (5) Oleuropein curtailed OVA-induced cell influx in the BALF

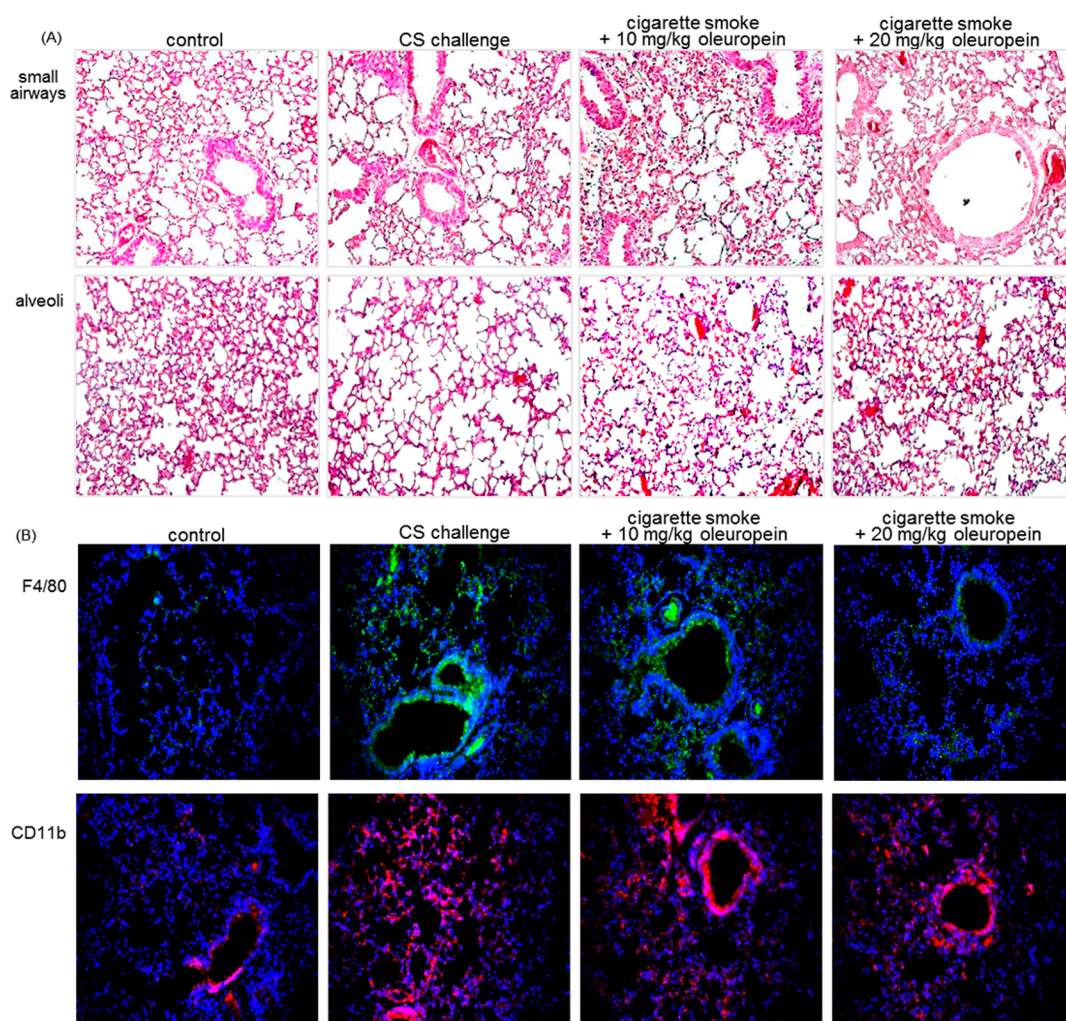


Figure 7. Blockade of airway destruction, inflammation, and alveolar emphysema and by oleuropein in cigarette smoke (CS)-challenged mouse small airways. Mice were orally administrated with 10 or 20 mg/kg oleuropein and exposed to CS for 30 min. Airway tissue sections were stained by using HE reagent (A). Each photograph is representative of four mice. Magnification: 200-fold. The arrows indicate small airway wall damage and alveolar destruction. Immunohistochemistry analysis was done in small airway tissues of CS-challenged mice (B). The F4/80 localization was identified as FITC-green staining, and CD11b was confirmed by Cy3-red staining in mouse airways exposed to CS. Nuclear staining was done with DAPI (blue). Each photograph is representative of four mice. Magnification: 200-fold.

with blocking eosinophilia. (6) This compound reduced the induction of F4/80 and CD68 in OVA-exposed lung tissues of mice, preventing the infiltration of macrophages in small airways. (7) Oleuropein dampened the OVA inhalation-evoked thrombosis via PAR-2 induction in small airways of mice. (8) Oral supplementation of oleuropein to CS-exposed mice allayed cell influx in the BALF, airway tissue damage and inflammation, and alveolar emphysema. (9) In oleuropein-treated A549 cells, the apoptotic cell death by CSE declined significantly, eventually reducing CS-induced alveolar emphysema. Accordingly, OVA- or CS-induced lung inflammation may contribute to increased fibrosis and airway tissue destruction, ultimately causing small airway thickening and emphysema. Anti-inflammatory effects of oleuropein have potential benefit in treating inflammatory lung diseases of COPD and asthma. It should be noted that pertinent clinical trials with oleuropein need to be performed.

Numerous studies have proposed various molecular targets as novel therapeutic options for asthma and COPD.^{15,16,18,35} Asthma and COPD are progressively recognized to have common syndromes that may share comparable pathophysio-

logic mechanisms and curable features.³⁶ Macrolide refers to a potential therapeutic medication for asthma with airway hyper-responsiveness by dint of their antimicrobial and immunomodulatory activities.¹⁵ Macrolides may cause antibiotic-induced cardiac arrhythmia.³⁷ Inhaled corticosteroids and long-acting β_2 agonists, long-acting muscarinic antagonists, phosphodiesterase-4 inhibitors, and statins effectively relieve symptoms and to reduce the frequency of exacerbations.^{16–18,38} However, the application of inhaled corticosteroid results in an increase in the adverse rates of respiratory events including risk of pneumonia and COPD exacerbations.^{19,39} Accordingly, one of new therapeutic strategies for chronic airway diseases is to use natural products that have mostly proven to be safe. The primary therapeutic option for pulmonary diseases is to combat airway inflammation.¹⁴ Several studies have introduced various natural agents for treating inflammatory lung diseases.^{20–22} Additionally, the immunomodulatory curative therapy may be an alternative for the relief of airway diseases.⁴⁰

Eosinophilic airway inflammation may be the most influential treatable trait of chronic airway disease.³⁶ The present study showed that oleuropein suppressed bronchial

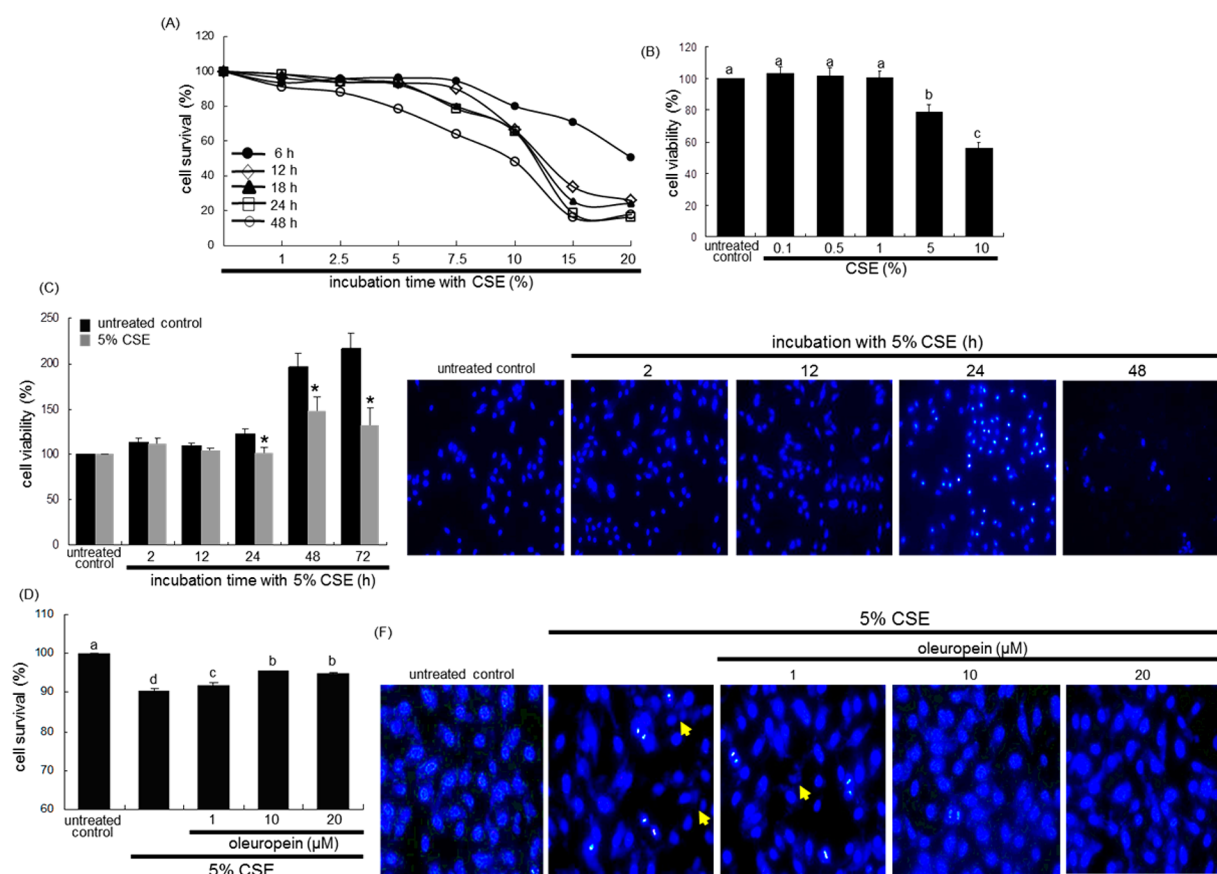


Figure 8. Viability of alveolar epithelial A549 cells exposed to different doses of cigarette smoke extract (CSE) for various times (A, B, C), and effects of oleuropein on A549 cell viability (D). Alveolar epithelial cells were incubated in media containing CSE in the absence and presence of 1–20 μ M oleuropein for up to 72 h. A549 cell viability (mean $\pm$ SEM, $n = 5$) was measured by using MTT assay and expressed as percent cell survival relative to untreated controls. Respective values not sharing a letter are different at $P < 0.05$. * $P < 0.05$, compared to respective untreated control. Nuclear staining was done with Hoechst 33258 dye for the detection of apoptotic cells (arrows, right panels of C and D). Original magnification of microscopic images ($n = 3$), 200–400-fold.

epithelial eotaxin-1 induction and eosinophil influx in BALF by OVA inhalation, indicating its blockade of eosinophilic inflammation in airways. Similarly, the antioxidant kaempferol blocks eosinophil infiltration and airway inflammation in a model of experimental asthma.⁴¹ Astragalin antagonizes oxidative stress-responsive eosinophilic inflammation and epithelial fibrosis.²⁴ Accordingly, it is deemed that the inhibition of eosinophilic inflammation of oleuropein may be ascribed to its antioxidant property. Incidentally, oleuropein reduced the lung tissue level of IL-4 but not IL-6 and IL-1 β with OVA challenge. In addition, the induction of IL-4 α , eotaxin-1, and MUC5AC was observed in OVA-challenged airways. Collectively, these results indicate that airway epithelial IL-4 may instigate eosinophilic inflammation and mucin hypersecretion in airways. Interestingly, oleuropein retarded the OVA inhalation-induced lymphocyte influx in the BALF. There is growing evidence that allergic airway inflammation depends on eosinophil-induced recruitment of effector T-cells to the lung, supporting a linkage between the innate immune effectors of eosinophils and adaptive T-cell responses.⁴² Therefore, the inhibition of allergic inflammation by oleuropein may entail a reduced communication between eosinophils and lymphocytes.

One study shows that ICAM-1 and CD11b are involved in the neutrophil recruitment in endotoxin-induced pulmonary diseases.³³ Consistent with the Western blot data and

immunohistochemical data of ICAM-1 and CD11b, oleuropein might inhibit neutrophilic inflammation by OVA inhalation and alleviated airway damage and alveolar emphysema by CS. Furthermore, oleuropein alleviated monocyte/macrophage-driven inflammation by curtailing the induction of the macrophage F4/80 and CD68 in airway subepithelial compartments by OVA challenge and CS exposure. Macrophages are responsible for airway blockade and alveolar wall damage in pulmonary emphysema as key players in the innate defense of the airways.⁴³ Collectively, one can speculate that the OVA inhalation and CS induce the recruitment of various inflammatory cells in the airways through promoting innate and adaptive immune mechanisms.^{44,45} On the other hand, the OVA inhalation and thrombin enhanced airway PAR-2 induction, suggesting a molecular link between allergic inflammation and coagulation in asthma.^{34,46} It has shown that asthma places the bronchoalveolar space in a procoagulant state.⁴⁶ Accordingly, the PAR-2 blockade by natural compounds such as oleuropein may be a novel therapeutic option targeted to prevent thrombosis as well as inflammatory lung destruction.

To identify how the CS insult can influence the pathogenesis of emphysema may prove useful in directing therapeutic approaches. In this study, CSE induced apoptosis of alveolar A549 cells with nuclear condensation, indicating that alveolar emphysema may be attributed to apoptotic cell loss in alveoli.

It has been demonstrated that the turnover of alveolar wall cells is highly up-regulated in emphysematous lungs.⁴⁷ Accordingly, the inhibition of alveolar emphysema by oleuropein may be due to its reduced apoptosis-associated cell loss. Cigarette-smoking endogenously released ROS during the inflammatory process and mitochondrial dysfunction leading to alveolar epithelial cell injury and death.⁴⁸ This study did not examine ROS release and then oxidative stress in alveolar cells exposed to CS. However, one can assume that oleuropein may ablate CS-induced alveolar emphysema through inactivation of oxidative stress-responsive mechanism(s).²⁶ Similarly, diallyl disulfide rich in garlic with antioxidant and anti-inflammatory activity is effective in reducing inflammation and cell influx in the BALF in a rat model of emphysema induced by CSE.⁴⁹ Also, ursolic acid diminishes CS-induced lung apoptosis and emphysema by retarding PERK pathway and by up-regulating Nrf2 pathway to lessen oxidant stress in rat lungs.⁵⁰

In summary, this study investigated that oleuropein combated IL-4-mediated eosinophilia and macrophage accumulation in airways in OVA inhalation-challenged mice. Oral administration of oleuropein diminished thickening and mucin hyperproduction of OVA-exposed bronchial subepithelium through blocking IL-4-associated eosinophilic inflammation. In addition, oleuropein ablated pulmonary inflammation triggered by macrophages and neutrophils infiltrated in CS-exposed mouse small airways and alveoli. Oleuropein antagonized CS-evoked lung tissue damage and alveolar emphysema through inhibiting apoptotic death of alveolar cells. Although oleuropein may serve as a modulator against airway fibrosis and alveolar emphysema due to neutrophilic inflammation, its dietary role in asthma and COPD remains unclear. In addition, the underlying mechanisms involved in regulation of pulmonary inflammation will identify the dietary manipulation of anti-inflammatory oleuropein in lung diseases.

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Y.-H. Kim and Y.-J.C. contributed equally. Y.-H. Kim, Y.-J.C., and Y.-H. Kang designed research; Y.-H. Kim, M.-K.K., E.-J.L., D.Y.K., and H.O. conducted research; Y.-H. Kim analyzed the data; Y.-H. Kim, Y.-J.C., and Y.-H. Kang wrote the paper. Y.-H. Kang had primary responsibility for final content. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; ICAM-1, intracellular adhesion molecule-1; IL-4, interleukin-4; LPS,

lipopolysaccharide; OVA, ovalbumin; PAR-2, proteinase-activated receptor-2; ROS, reactive oxygen species

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