



Review article

Caffeic acid phenethyl ester alleviates asthma by regulating the airway microenvironment via the ROS-responsive MAPK/Akt pathway

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ABSTRACT

In the pathophysiology of asthma, structural cell dysfunction and concomitant microenvironment changes in airways are crucial to pathological progression, which involves oxidative stress. Caffeic acid phenethyl ester (CAPE) is an active anti-oxidative component obtained from propolis, and has been shown to have beneficial effects on several respiratory disorders, such as chronic obstructive pulmonary disease and lung cancer. However, the impact of CAPE on asthma is not well understood. Therefore, this study investigated the advantages of using CAPE to treat asthma and demonstrated the roles of CAPE in the regulation of airway microenvironments. In ovalbumin (OVA)-sensitized mice, CAPE treatments notably reduced airway hyperresponsiveness, attenuated extensive inflammatory cell infiltration and inhibited goblet cell hyperplasia and collagen deposition and fibrosis. In addition, CAPE improved the airway microenvironment in a dose-dependent manner by inhibiting OVA-induced increases in immunoglobulin E, tumor necrosis factor alpha (TNF- α), transforming growth factor- β 1 (TGF- β 1), interleukin (IL)-4 and IL-13 and suppressing matrix metalloproteinase-9 and alpha-smooth muscle actin expression as well as malondialdehyde production. To determine the underlying mechanisms responsible for these effects, we used TNF- α -stimulated BECs and TGF- β 1-challenged human ASMCs to explore the impacts of CAPE on pro-inflammatory proteins and ASMC proliferation. The results indicated that CAPE significantly limited the secretion of eotaxin-1, monocyte chemoattractant protein-1, IL-8 and intercellular adhesion molecule-1 and dramatically inhibited the proliferation of ASMCs. These effects were shown to be associated with decreased reactive oxidant species (ROS) levels. The phosphorylation of Akt and Mitogen-Activated Protein Kinase (MAPK) caused by increased ROS was significantly decreased by CAPE, which implied a contribution of ROS-MAPK/Akt signaling to the attenuation of asthma. Our findings indicated for the first time that CAPE alleviates airway inflammation and remodeling in chronic asthma by balancing the airway microenvironment, which highlights a novel profile of CAPE as a potent agent for asthma management.

1. Introduction

Asthma is a complex clinical syndrome characterized by airway hyperresponsiveness (AHR), inflammation and remodeling, and it is linked to an increased generation of reactive oxidant species (ROS) and impaired antioxidant defenses [1,2]. Airway structural cells, such as human bronchial epithelial cells (BECs) and airway smooth muscle cells (ASMCs), play important roles in the development of asthma [3,4]. Insults such as infection, allergens or environmental factors can activate BECs, initiate airway inflammation by polarizing T-helper type 2 (Th2) lymphocytes and promote pro-inflammatory protein secretion,

including eotaxin-1, monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-8 and intercellular adhesion molecule-1 (ICAM-1) [5–7]. These pro-inflammatory proteins have complex cross-talk signaling and contribute to the change of airway microenvironment, which refers to the intercellular substance and secretion of various cytokines [8,9]. Insults can also alter the profile of ASMCs to produce a range of extracellular matrix (ECM) proteins, such as collagen and matrix metalloproteinases (MMPs), which leads to increases in the ASMC size (hypertrophy) and number (hyperplasia). Such changes contribute to the development of AHR and airway remodeling, which in turn affect airway inflammation and determine the outcome of asthma

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[10,11].

Furthermore, insults can induce high levels of ROS, which can be generated by activated inflammatory cells and airway structural cells [12–15]. Accumulating data have reported that ROS in airway structural cells exacerbate responses to allergen challenge and cause a number of detrimental effects on airway functions, including airway smooth muscle contraction, AHR induction, mucus hypersecretion, epithelial shedding, and vascular exudation, and these changes produce a vicious cycle [14,16–19]. However, current knowledge of the pathophysiologic roles of ROS in asthmatic airway microenvironment remain incomplete.

ROS has been confirmed to correlate with tumor necrosis factor alpha (TNF- α) and transforming growth factor- β 1 (TGF- β 1) [20–24]. TNF- α is an important pro-inflammatory molecule that induces ROS generation, elicits pro-inflammatory mediators and evokes the activation of various cells, thereby leading to an amplification of inflammatory responses [23,25]. TGF- β 1 primarily contributes to airway smooth muscle dysfunctions in asthma by eliciting ASMC proliferation and hypertrophy, which is probably attributed to the generation of intracellular ROS [26]. Herein, we established an inflammatory model with TNF- α and a proliferation model with TGF- β 1 in vitro.

Galangin and caffeic acid phenethyl ester (CAPE) (Fig. 1A) are two potent ingredients of propolis. Our previous findings established that galangin could alleviate ovalbumin (OVA)-induced airway inflammation and remodeling by inhibiting the NF- κ B and MAPK/Akt pathways, respectively [1,27]. Recently we found that the anti-oxidant effect of CAPE is more pronounced than that of galangin. As previous studies have reported, CAPE presents anti-tumor and anti-allergic properties in various disorders. CAPE attenuates cigarette smoke-induced airway inflammation in chronic obstructive pulmonary disease (COPD) through NF- κ B inactivation [28]. More recently, CAPE was reported to prevent TGF- β 1-induced epithelial-mesenchymal transition in human alveolar epithelial cells via PPAR γ activation, suggesting a regulatory potential of CAPE in pulmonary fibrosis and remodeling [29]. Evidence from animal studies suggested that CAPE alleviates OVA-induced airway inflammation while the mechanism has not been determined [30]. And the contribution of CAPE to the pathophysiology of chronic asthma remains incompletely defined. The present study was designed to investigate the effects of CAPE on airway inflammation and remodeling in chronic asthma and to identify the mechanisms in vivo and in vitro.

2. Materials and methods

2.1. Animals

Specific pathogen-free female BALB/c mice (18–22 g) aged 6–8 weeks were obtained from Vital River Laboratories (Beijing, China). The mice were maintained in a temperature-controlled room under a 12 h dark/light cycle and provided with food and water ad libitum. All experiments that involved animal and tissue samples were performed in accordance with the guidelines of the National Institutes of Health and Nanjing Medical University, and all of the procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China).

2.2. OVA sensitization and challenge

Fig. 1B schematically depicts the protocols used in this study. In total, 42 specific pathogen-free female BALB/c mice were randomly divided into 6 groups as follows: control, OVA (Grade V, Sigma-Aldrich, Milwaukee, WI, USA), OVA+CL (1 mg/kg CAPE, Sigma-Aldrich), OVA+CH (5 mg/kg CAPE), OVA+dexamethasone (1 mg/kg DEX, Sigma-Aldrich) and OVA+dimethylsulfoxide (DMSO, Sigma-Aldrich). The asthmatic models were established by sensitization to OVA. Specifically, all of the mice in the OVA, OVA+CL, OVA+CH, OVA

+DEX and OVA+DMSO groups were sensitized on days 0, 7 and 14 by intraperitoneal injection of 20 μ g OVA emulsified in 2 mg aluminum hydroxide gel (Invivo-Gen, San Diego, CA, USA) in a total volume of 200 μ l. These sensitized mice were exposed to aerosolized 5% OVA in sterile saline for 30 min three times a week for 8 weeks beginning on the 16th day of the experiment. We placed the mice in 51 $\times$ 31 $\times$ 21 cm chambers that were connected to a jet nebulizer (NE-U11B; OmronCorp, Tokyo, Japan) to create a whole-body inhalation system. CAPE (1 and 5 mg/kg), DEX (1 mg/kg, positive control) and DMSO (0.4 μ l in 200 μ l saline, solvent control) were administered by intraperitoneal injection 30 min before each OVA challenge. The control subjects were sensitized and challenged using the same protocol with saline alone. The mice were sacrificed at 24 h after the final challenge, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected for analyses.

2.3. Measurement and analysis of airway responsiveness

Lung function was evaluated by direct measurement of lung resistance and dynamic compliance in restrained, tracheostomized, mechanically ventilated mice via the FinePointe RC system (Buxco Research Systems, Wilmington, NC) under general anesthesia as described before [31].

2.4. BALF collection and differential cell counts

After the AHR measurements, the tracheae were exposed, and then the BALF were collected by lavage with ice-cold phosphate-buffered saline (PBS, 400 μ l $\times$ 3; 85–90% of the lavage volume was recovered) via a tracheal catheter. The lavage samples from each mouse were centrifuged at 1000 rpm for 10 min at 4 $^{\circ}$ C. The total number of inflammatory cells in the BALF was counted using a hemocytometer. Differential cell counts were performed using Wright's staining based on the morphological criteria. The number of cells in the BALF was determined by two independent investigators in a single-blind study, and at least 200 cells each were analyzed from three random locations using a microscope. Then, the supernatant was collected, divided into four equal portions and frozen at –80 $^{\circ}$ C for enzyme-linked immunosorbent assay (ELISA).

2.5. Lung histology

After the BALF samples were obtained, the right lungs were immersed in 4% paraformaldehyde prior to being embedded in paraffin. A series of microsections (5 μ m) were cut with a microtome and stained with hematoxylin and eosin (H & E) to assess inflammatory cell infiltration. The inflammation score was determined as follows: grade 0, no inflammation; grade 1, occasional cuffing with inflammatory cells; and grades 2, 3, and 4, most bronchi or vessels surrounded by a thin layer (1–2 cells: grade 2), a moderate layer (3–5 cells: grade 3), or a thick layer (>5 cells: grade 4) of inflammatory cells, respectively. The total inflammation score was calculated by the addition of the peribronchial (PB) and perivascular (PV) inflammation scores. Periodic acid-Schiff (PAS) staining was used to quantify the airway goblet cells, and Masson's trichrome staining was used to visualize the collagen deposition and fibrosis. Both staining methods were scored as follows: 0, none; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75% goblet cells [32–34]. Sections were also immunohistochemically stained for matrix metalloproteinase-9 (MMP-9) and α -smooth muscle actin (α -SMA). For the semi-quantitative evaluation of MMP-9 and α -SMA expression, we used the modified scoring method by Sinicrope and Lu [32,33]. The mean percentages of positive epithelial cells in the bronchi were determined in at least five areas at 400 $\times$ magnification and assigned to one of the following categories: 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%. The immunostaining intensity of MMP-9 and α -SMA was scored as 1+(weak),

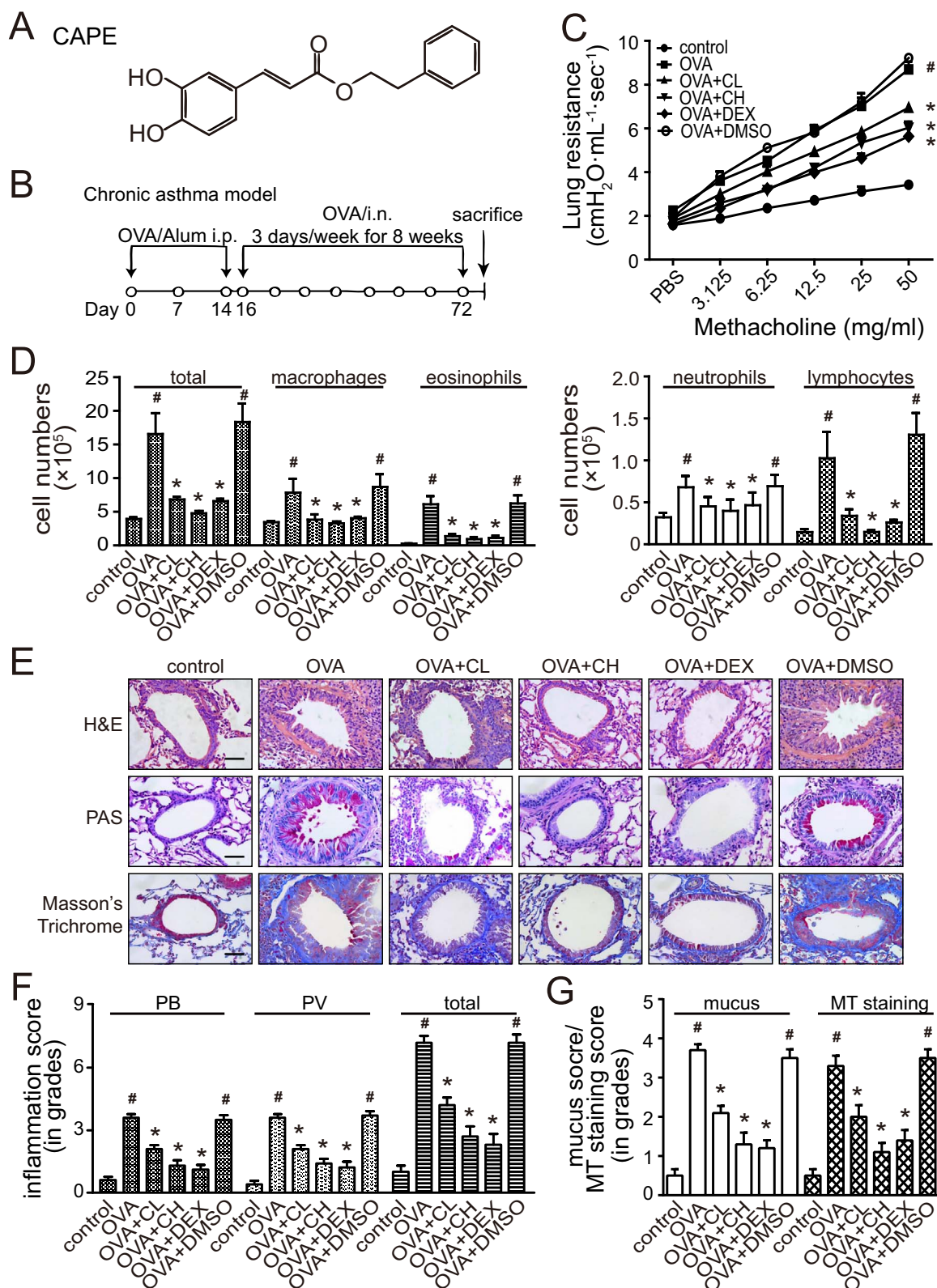


Fig. 1. Chemical structure of CAPE and experimental protocol for the chronic asthma model. Treatment with CAPE decreased AHR, inflammatory cell infiltration, goblet cell hyperplasia and collagen deposition. (A) Chemical structure of CAPE. (B) BALB/c mice were sensitized with OVA and aluminum hydroxide gel by intraperitoneal injection on days 0, 7 and 14 and then challenged with aerosolized 5% OVA for 30 min per day three days per week for eight weeks beginning on the 16th day of the experiment. The control mice were sensitized and challenged only with saline. CAPE, DEX or a vehicle (DMSO) were administered by intraperitoneal injection at 30 min before each OVA challenge. (C) Mice inhaled increasing doses of methacholine (3.125–50 mg/ml), and AHR was measured. (D) Cell numbers and differentiation in BALF were determined by hemocytometer, and at least 200 cells were counted. (E) Lung sections were stained with H & E to analyze the infiltration of inflammatory cells, PAS to assess goblet cell hyperplasia, and Masson's trichrome to evaluate the subepithelial deposition of collagen and fibrosis (magnification, 400 $\times$; scale bar, 50 μm). (F) Layers of inflammation cells were counted, and the total inflammation score was summed with the peribronchial (PB) and perivascular (PV) inflammation scores. (G) PAS-positive and PAS-negative epithelial cells were counted, and the percentage of PAS-positive cells per bronchiole was calculated. Masson's trichrome staining analysis of collagen deposition was also calculated. Values are the mean $\pm$ SEM. (n=8 per group). *P < 0.05 compared with the control group, and #P < 0.05 compared with the OVA group.

2+(moderate) or 3+(intense). The percentage of positive epithelial cells and the staining intensity were multiplied to produce a weighted score for each case. All of the scores were calculated by 2 independent observers who were blinded to the experiment, and at least three different fields were examined for each lung section.

2.6. *In situ* detection of ROS

In situ production of ROS can be evaluated microscopically in frozen sections using dihydroethidium (DHE) (Beyotime Institute of Biotechnology, Jinan, Shandong, China) [34]. Briefly, snap-frozen lung tissue samples were embedded in Tissue-Tek OCT compound, cryosectioned at 7 mm, allowed to air-dry at room temperature, and stored at -80°C until needed. Slides were placed into PBS for 30 min at room temperature and stained with DHE (10 mM) in PBS for 30 min in a moist chamber in the dark. The slides were rinsed extensively with PBS, cover slipped, and imaged with fluorescence microscope (Zeiss LSM 5 LIVE, Germany). DHE fluorescence was quantified by averaging the mean value of fluorescence intensity using Image Pro Plus 6.0. Four sections from each mouse were analyzed using this procedure, and the mean value of fluorescence was calculated.

2.7. Culture and treatment of normal human BECs and ASMCs

Normal human BECs were purchased from the Beijing Institute for Cancer Research (Beijing, China) and cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Invitrogen-Gibco, Paisley, Scotland) supplemented with 20 U/L penicillin, 20 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal bovine serum (HyClone, Logan, UT, USA). Normal human ASMCs were purchased from Scien-Cell Research Laboratories (Carlsbad, CA, USA) and cultured at 37°C and 5% CO_2 in smooth muscle cell medium (Scien-Cell) supplemented with 20 U/L penicillin, 20 $\mu\text{g}/\text{ml}$ streptomycin, 1% smooth muscle cell growth supplement and 2% fetal bovine serum (Scien-Cell). The cells between passages 4 and 8 were used for the experiments. After serum starvation for 6–8 h, the BECs were stimulated with 10 ng/ml TNF- α (Peprotech, Rocky Hill, USA) or hydrogen peroxide (H_2O_2) (20 μM) (Sigma-Aldrich) alone or in combination with CAPE (10 μM). The ASMCs were stimulated with 1 ng/ml TGF- β 1 (Peprotech) or H_2O_2 (20 μM), alone or in combination with CAPE (10 μM). Then, the cells were further cultured for the indicated durations. The cells were treated in the same manner with N-acetylcysteine (NAC) as a positive control.

2.8. Cell viability assay

The proliferation of ASMCs was determined by a cell counting kit (CCK)-8 assay and 5-ethynyl-2'-deoxyuridine (EDU) assay. The ASMCs were cultured in a 96-well plate at a density of 5×10^3 cells per well and treated with TGF- β 1 at concentrations ranging between 0.1 and 100 μM for 72 h. Then, CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to the cell culture medium at a 1:10 dilution, and the cultures were incubated for another 1–2 h at 37°C . The absorbance at 450 nm (A450) was measured with a microplate reader (CANY, Shanghai, China). Similarly, the cytotoxicity of CAPE and H_2O_2 and the effect between TGF- β 1 and CAPE on the ASMCs were examined using the CCK-8 assay. For the EDU assay, the ASMCs were cultured via the aforementioned procedure. After 48 h of treatment, an EDU assay kit (Ribobio, Guangzhou, China) was used for labeling according to the manufacturer's instructions. Images were taken using a fluorescence microscope (Olympus IX71, Japan). The cytotoxicity of CAPE and H_2O_2 on BECs was also examined using the CCK-8 assay as previously described. Each sample was measured in triplicate.

2.9. ELISA

To explore the effects of CAPE and H_2O_2 on airway inflammation in vitro, human eotaxin-1, MCP-1, IL-8, and ICAM-1 (R & D Systems, Minneapolis, MN, USA) levels were measured. The BECs were cultured using the aforementioned procedure. After starvation in serum-free medium, the cells were stimulated with TNF- α or H_2O_2 alone or with CAPE for 6–8 h, and then ELISAs were performed. The total IgE (Immuno-Biological Laboratories Co., Hamburg, Germany), TNF- α , TGF- β 1, IL-4, and IL-13 levels (R & D Systems) in the BALF of the mice were also measured by ELISA according to the manufacturer's instructions.

2.10. Determination of malondialdehyde (MDA) level

The MDA levels in the cell culture medium and lung tissues were determined using the thiobarbituric acid reacting substances (TBARS) assay (Senbeijia Corp., Nanjing, China) as previously described [35]. MDA reacts with thiobarbituric acid under acidic conditions at 95°C to form a pink-colored complex that can be measured at 532 nm. In this test, 1,3,3-tetra ethoxy propane (TEP) was used as a standard.

2.11. Determination of intracellular ROS production

Intracellular ROS levels were measured using the 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) assay. Briefly, 1.5×10^4 ASMCs were seeded into each well of a 6-well plate and cultured for 24 h. They were then divided into 5 groups: control, TGF (1 ng/ml TGF- β 1), TGF+C (1 ng/ml TGF- β 1+10 μM CAPE), TGF+N10 (1 ng/ml TGF- β 1+10 mM NAC), and C (10 μM CAPE). The cells were cultured for an additional 24 h and then incubated with 10 μM DCFH-DA for 30 min at 37°C in the dark. Next, the cells were washed twice with PBS and analyzed within 30 min using a FACScan instrument (Becton Dickinson, San Jose, CA, USA) with an excitation setting of 488 nm. The specific fluorescence signals corresponding to DCFH-DA were determined using a 525-nm band-pass filter. For consistency, 10,000 cells were analyzed for each determination. Intracellular ROS production was also measured using a laser scanning confocal microscope (Zeiss). After incubation with DCFH-DA, the cells were fixed with 4% paraformaldehyde for 10 min and washed three times with PBS before being imaged. The excitation and emission wavelengths were identical to those previously described. For each culture, a minimum of 5 random fields were captured. The intracellular ROS of the BECs were measured using the same method except for two differences. First, the BEC groups included the control, TNF (10 ng/ml TNF- α), TNF+C (10 ng/ml TNF- α +10 μM CAPE), TNF+N10 (10 ng/ml TNF- α +10 mM NAC), and C (10 μM CAPE). Second, after the administration of TNF- α , CAPE or NAC, the cells were cultured for 6 h and then incubated with DCFH-DA.

2.12. Western blot analysis

Total cellular protein was collected following lysis in lysis buffer (Cell Signaling Technology, Inc., Beverly, MA, USA) on ice and centrifugation for 15 min at 14,000 rpm at 4°C . The supernatant was transferred to a fresh tube and denatured in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer (Beyotime, Shanghai, China) with heating to 100°C for 5 min. The supernatant was then stored at -80°C . The total protein concentration was determined using a BCA protein assay kit (Thermo, Rockford, IL, USA). The proteins were separated by 10% SDS-PAGE. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using the wet transfer method. Nonspecific sites were blocked with 5% nonfat milk in TBS Tween 20 (TBST; 25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) for 2 h, and the blots were incubated with primary

antibodies (Cell Signaling Technology, Inc.), including anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-phospho-Akt, anti-Akt, anti-phospho-p38, anti-p38, anti-phospho-ERK, anti-ERK, anti-phospho-JNK, and anti-JNK antibodies overnight at 4 °C. Goat anti-rabbit horseradish peroxidase-conjugated IgG (Cell Signaling Technology, Inc.) was used to detect the antibody binding. After treating the membranes with enhanced chemiluminescence system reagents (Thermo), the binding of specific antibodies was visualized using a Bio-Rad Gel Doc/Chemi Doc Imaging System and analyzed by Quantity One software.

2.13. Statistical analysis

The data are expressed as the mean $\pm$ standard error of the mean (SEM). All of the tests were performed using Prism 6.00 (GraphPad Software, San Diego, CA, USA) and SPSS version 20 (SPSS, Inc., Chicago, IL, USA). The results were analyzed by a one-way analysis of variance for repeated measures followed by Dunnett's post hoc test to determine differences among multiple comparisons. The significance level was set to $P < 0.05$.

3. Results

3.1. Effect of CAPE on AHR

The results of the AHR analysis showed that the airway resistance increased with increasing methacholine doses in the OVA-sensitized mice (Fig. 1C). Overall, the CAPE treatment significantly suppressed airway resistance in the asthmatic mice.

3.2. Effect of CAPE on chronic airway inflammation

Lung sections were stained with H & E, and the inflammatory cells in the BALF were counted at 24 h after the final OVA challenge. Compared with the mice in the control group, those in the OVA and OVA+DMSO groups displayed severe airway inflammatory responses, including extensive infiltration of inflammatory cells into the BALF (Fig. 1D) and around the respiratory tracts and vessels (Fig. 1E). Treatment with CAPE or DEX suppressed the infiltration of inflammatory cells to varying degrees. The high-dose administration of CAPE (5 mg/kg) induced remarkable decreases in the total cell counts and the number of macrophages, eosinophils, neutrophils and lymphocytes compared with those observed in the untreated asthmatic mice ($P < 0.05$). The low-dose administration of CAPE (1 mg/kg) did not cause such drastic decreases in cell number (Fig. 1D). These results were further confirmed by the H & E analyses and inflammation scores. The mice treated with CAPE (1 and 5 mg/kg) and DEX had fewer inflammatory cells compared with the untreated asthmatic (Fig. 1F), and the total inflammation scores were 4.2 ± 0.36 , 2.7 ± 0.47 , and 2.3 ± 0.52 , respectively ($P < 0.05$) (Fig. 1F). All of these findings indicated that the administration of CAPE before the OVA aerosol challenge attenuated the inflammatory responses in the asthmatic airways in a dose-dependent manner.

3.3. Effect of CAPE on goblet cell hyperplasia and collagen deposition/fibrosis

To evaluate the extent of goblet cell and mucus production, the lung sections were stained with PAS and the percentage of PAS-positive cells in the airway epithelium was determined. We observed that the OVA-challenged mice developed marked goblet cell hyperplasia and mucus hypersecretion in the lumen of the bronchioles (Fig. 1E). Mice treated with DEX and high doses of CAPE had fewer goblet cells in the airway epithelium and reduced mucus scores of 1.2 ± 0.20 and 1.3 ± 0.30 ($P < 0.05$), respectively, which indicated equivalent effects of the treatments (Fig. 1G).

The area of collagen deposition/fibrosis was assessed by Masson's trichrome stain. The extent of collagen deposition/fibrosis was profoundly enhanced over the interstitia of the airways and vessels of the tissues in the OVA-challenged mice compared with the control mice (Fig. 1E). These serious pathophysiological changes were significantly ameliorated by the administration of 5 mg/kg CAPE, which presented a score of 1.1 ± 0.23 ($P < 0.05$), whereas modest effects were observed with the administration of DEX and low doses of CAPE (Fig. 1G).

3.4. Effect of CAPE on MMP-9 and α -SMA expression

Representative photomicrographs of the immunohistochemical staining for α -SMA and MMP-9 in the airways are shown in Fig. 2. The densities of α -SMA staining in the airway smooth muscles in the OVA-challenged mice were higher than those in the control mice, and the score was 8.2 ± 0.29 ($P < 0.05$). As shown in Fig. 2B, the administration of CAPE markedly decreased the areas of α -SMA-stained smooth muscle layer (CL 2.9 ± 0.50 , $P < 0.05$; CH 1.6 ± 0.34 , $P < 0.05$). Treatment with DEX also decreased the density of α -SMA staining and presented a score of 4.8 ± 0.61 ($P < 0.05$); however, the effect was not as obvious as that of CAPE. In addition, the immunohistochemistry assays showed strong MMP-9 staining around the bronchioles and in the infiltrated inflammatory cells in the OVA-challenged mice compared with the control mice, and the score was 8.2 ± 0.29 ($P < 0.05$). These increases were dramatically reversed by the administration of DEX or high doses of CAPE, which presented scores of 1.5 ± 0.34 and 1.5 ± 0.37 , respectively ($P < 0.05$) (Fig. 2C). These observations implied that CAPE may have potential anti-remodeling effects and the mechanisms between CAPE and DEX may not be completely the same.

3.5. Effect of CAPE on IgE and Th2-cytokines levels

High levels of IgE and Th2 cytokines have been well established as features of asthma. After performing the sensitization and challenges, the total IgE levels in the BALF were significantly elevated in the OVA and OVA+DMSO groups (391.02 ± 7.33 μ g/ml and 385.67 ± 11.51 μ g/ml, respectively) compared with the control group (184.85 ± 3.01 μ g/ml) ($P < 0.05$). This elevation was abolished by both CAPE and DEX administration (CL 259.83 ± 7.87 μ g/ml, $P < 0.05$; CH 227.16 ± 4.97 μ g/ml, $P < 0.05$; DEX 218.60 ± 5.15 μ g/ml, $P < 0.05$) (Fig. 2D). Similarly, the IL-4 and IL-13 levels in the BALF were notably increased by airway challenge with OVA. The administration of CAPE reduced IL-4 (CL 72.40 ± 0.60 pg/ml, $P < 0.05$; CH 32.02 ± 1.37 pg/ml, $P < 0.05$) and IL-13 levels (CL 25.63 ± 0.73 pg/ml, $P < 0.05$; CH 16.79 ± 0.49 pg/ml, $P < 0.05$) in a dose-dependent manner compared with the levels in the BALF of the OVA-group mice (Fig. 2E and F). These findings indicated that CAPE could inhibit allergic airway reactions by modifying Th2-predominant immune activity in the OVA-induced asthma model.

3.6. Effect of CAPE on TNF- α and TGF- β 1 levels

TNF- α and TGF- β 1 are key mediators involved in asthmatic airway inflammation and remodeling. After performing the sensitization and challenges, the BALF TNF- α levels in the OVA and OVA+DMSO groups were markedly elevated to 107.99 ± 1.33 pg/ml and 102.41 ± 3.04 pg/ml, respectively. However, the high and low doses of CAPE decreased the TNF- α levels to 28.81 ± 0.37 pg/ml and 51.40 ± 1.10 pg/ml, respectively (Fig. 2G). The TGF- β 1 levels in the BALF were also upregulated in the OVA (1037.47 ± 177.73 pg/ml) and OVA+DMSO groups (1054.44 ± 269.12 pg/ml) compared with the control (54.24 ± 18.10 pg/ml) ($P < 0.05$). This upregulation was almost reversed by CAPE and DEX (CL 351.99 ± 13.79 pg/ml, $P < 0.05$; CH 161.07 ± 16.74 pg/ml, $P < 0.05$; DEX 162.32 ± 35.47 pg/ml, $P < 0.05$) (Fig. 2H). These observations suggested that the protective effect of CAPE may be attributed to the downregulation of TNF- α and TGF- β 1

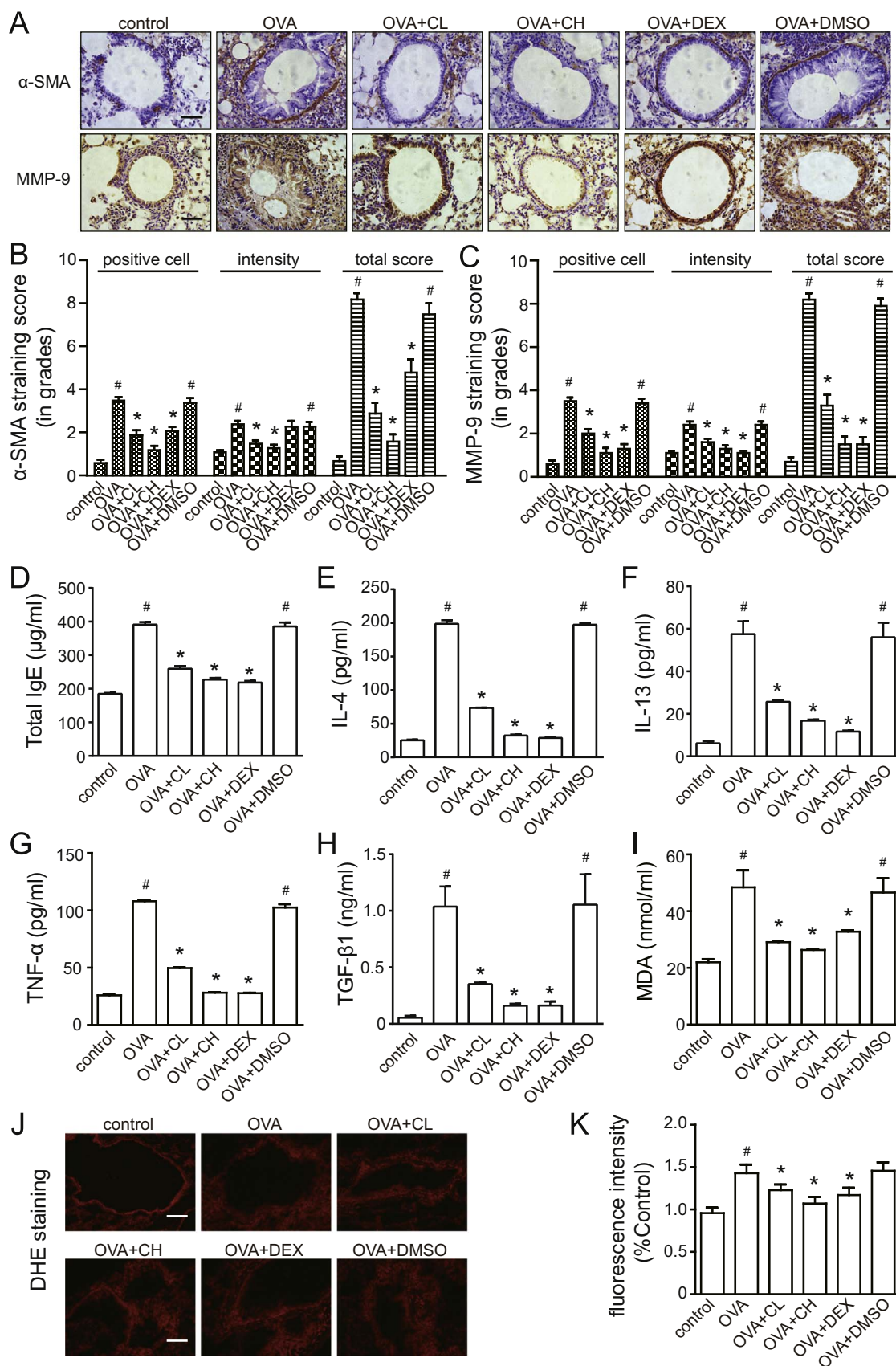


Fig. 2. Treatment with CAPE inhibited the expression of α-SMA, MMP-9, MDA and in situ ROS levels in lung tissue and inhibited IgE, Th2 cytokines, TNF-α and TGF-β1 levels in BALF. (A) Immunohistochemical staining was performed to assess the distribution of α-SMA and MMP-9 (magnification, 400×; scale bar, 50 μm). (B–C) α-SMA and MMP-9 expression were evaluated. The total α-SMA and MMP-9 staining scores were multiplied by the percentage of positive epithelial cells and staining intensity scores, respectively. (D–I) The concentrations of MDA, IgE, IL-4, IL-13, TNF-α and TGF-β1 were measured by ELISA. (J) Frozen lung sections were stained with DHE, imaged and analyzed by Image Pro Plus6.0. (K) The fluorescence values are expressed as the ratio to the levels in the control cells. Values are the mean ± SEM. (n=8 per group). *P < 0.05 compared with the control group, and #P < 0.05 compared with the OVA group.

levels.

3.7. Effect of CAPE on MDA levels and ROS generation in vivo

To determine whether CAPE inhibits OVA-induced airway inflammation and remodeling by the scavenging of free radicals, we detected the MDA and in situ ROS levels in the lung tissue to evaluate the changes in OVA-induced oxidative damage. As shown in Fig. 2I, the concentrations of MDA in the lung tissues in the OVA and OVA+DMSO groups (48.43 ± 5.99 nmol/ml and 46.59 ± 5.11 nmol/ml, respectively) were significantly higher than that in the control group (21.99 ± 1.11 nmol/ml) ($P < 0.05$). The MDA levels in the lung tissues of the CAPE pretreatment groups (CL 29.10 ± 0.42 nmol/ml; CH 26.34 ± 0.28 nmol/ml) were significantly decreased compared with that in the OVA group ($P < 0.05$). Treatment with DEX also promoted a decrease in the MDA levels (32.78 ± 0.43 nmol/ml) but not as dramatically as that observed with the CAPE treatment ($P < 0.05$). The in situ ROS level in lungs was also assessed by DHE staining (Figs. 2J and K). In control mice, DHE was predominately detected in airway epithelium. Challenge of OVA enhanced the fluorescence not only in epithelium but also in other cells around airway, including ASMCs. Administration with CAPE notably inhibited the elevation both in epithelium and ASMCs.

3.8. Effect of CAPE on the expression of pro-inflammatory protein in human BECs

The toxicity of CAPE (0.1, 1, 5, 10, 50, 100 and 200 μ M) on human BECs was first determined. Cell viability was $96\% \pm 5\%$ in the 10 μ M group at 24 h (Fig. 3A). BECs are known to release chemokines and adhesion molecules to induce inflammatory responses and stimulate eosinophil migration in asthmatic patients [36]. To ascertain the anti-inflammatory mechanism of CAPE, we studied the effects of CAPE on the TNF- α -induced upregulation of eotaxin-1, IL-8, MCP-1 and ICAM-1 in the BECs. The results showed that CAPE (10 μ M) dramatically blocked the TNF- α -induced upregulation of eotaxin-1, IL-8, MCP-1 and ICAM-1 expression in the human BECs (Fig. 3B–E).

To investigate whether oxidative stress is involved in the induction of pro-inflammatory proteins in BECs, further studies were performed. The toxicity of H_2O_2 (1, 2, 5, 10, 20, 50 and 100 μ M), an important instigator of ROS, on human BECs was determined. Cell viability was $100\% \pm 2\%$ in the 20 μ M group at 8 h. (Fig. 5A). Eotaxin-1, IL-8, MCP-1 and ICAM-1 expression were elevated in 20 μ M H_2O_2 -treated BECs ($P < 0.05$), although the levels were not increased as dramatically as TNF- α -treated BECs. These over-expression of pro-inflammatory proteins were also attenuated by pretreatment with CAPE ($P < 0.05$) (Fig. 5B–E). These observations implied that 20 μ M H_2O_2 induced pro-inflammatory chemokines and adhesion molecules from BECs, although this effect was reversed by pretreatment with CAPE. Accordingly, oxidative stress may be involved in TNF- α -induced airway inflammation.

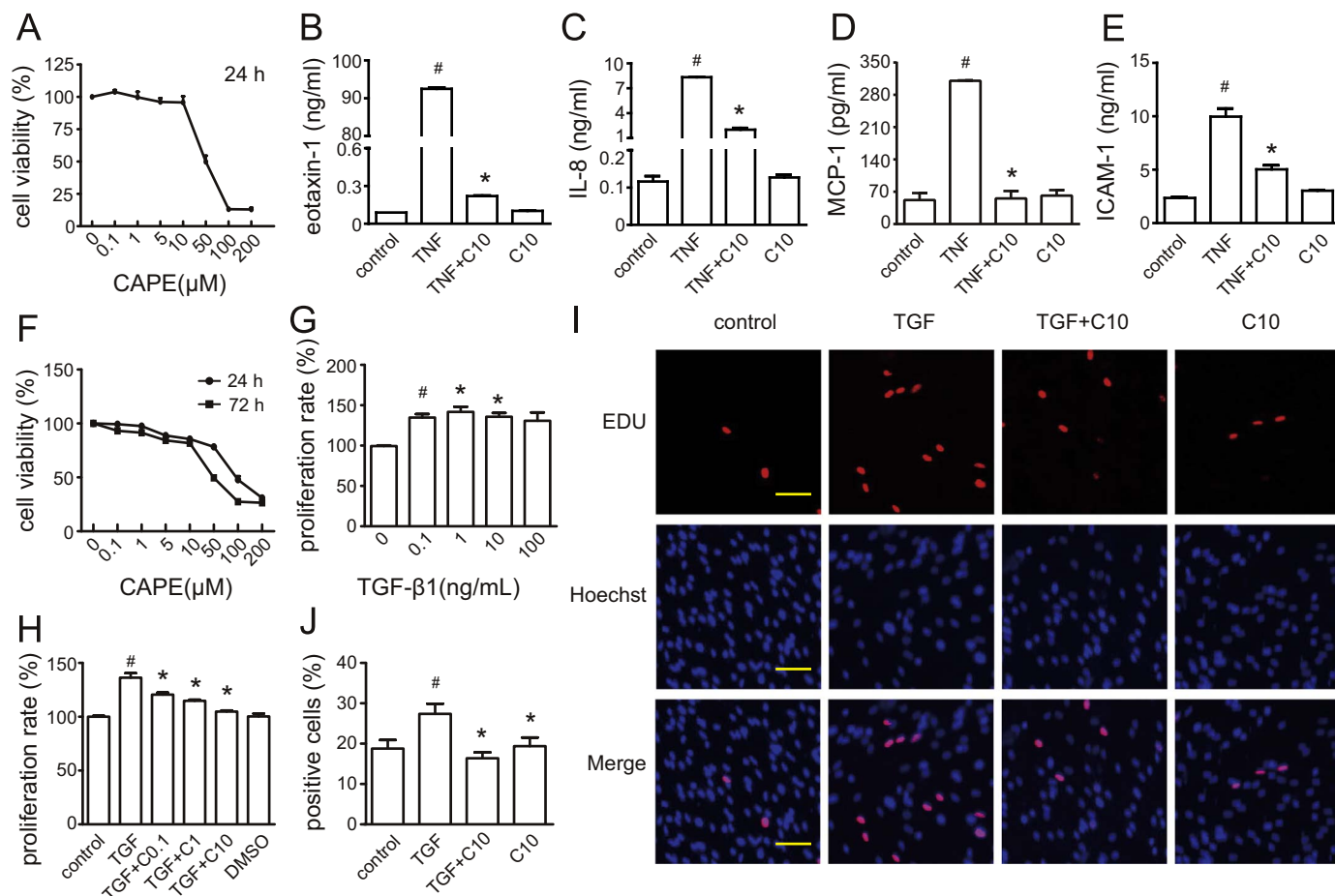


Fig. 3. Treatment with CAPE reduced the production of pro-inflammatory proteins induced by TNF- α in BECs and the proliferation induced by TGF- β 1 in ASMCs. (A) Effects of CAPE on the viability of human BECs as assessed by CCK-8 assay. (B–E) The eotaxin-1 (B), IL-8 (C), MCP-1 (D), and ICAM-1 (E) levels in supernatant were measured by ELISA. (F) Effect of CAPE on the viability of human ASMCs as assessed by CCK-8 assay. (G) Effect of stimulation with different concentrations of TGF- β 1 on the proliferation of human ASMCs as assessed by CCK-8 assay. (H–J) Effect of CAPE on TGF- β 1-stimulated human ASMCs as assessed by CCK-8 and EDU assays (magnification, 200 $\times$; scale bar, 100 μ m). Values are the mean $\pm$ SEM of at least four independent experiments performed in triplicate. ^{*} $P < 0.05$ compared with the control group, and [#] $P < 0.05$ compared with TNF- α group.

3.9. Effect of CAPE on the proliferation of human ASMCs

CAPE decreased ASMC proliferation *in vitro* in a dose-independent manner, and the cell viability was $86\% \pm 1\%$ and $82\% \pm 3\%$ in the 10 μm group at 24 h and 72 h, respectively (Fig. 3F). The proliferation of human ASMCs was enhanced following TGF- β 1 stimulation. The concentrations of TGF- β 1 (1 ng/ml) induced significant ASMC proliferation as determined by the CCK-8 assays at 72 h (Fig. 3G). CAPE (0.1–10 μm) inhibited the proliferation of ASMCs induced by 1 ng/ml TGF- β 1 in a dose-dependent manner. Treatment with 10 μm CAPE dramatically decreased the proliferation of ASMCs induced by TGF- β 1 from $136\% \pm 4\%$ – $105\% \pm 1\%$ ($P < 0.05$) (Fig. 3H). Furthermore, we used the EDU incorporation assay, which is a more sensitive and specific method [37], to evaluate the effects of CAPE on the TGF- β 1-induced cell proliferation. As shown in Fig. 3I and J, the number of cells incorporating EDU significantly increased after TGF- β 1 stimulation compared with that of the control, whereas the CAPE-pretreated cells distinctly limited the TGF- β 1-induced proliferation.

Similarly, to investigate whether oxidative stress is involved in the proliferation of human ASMCs, CCK-8 and EDU assays were performed. H_2O_2 at 10 and 20 μm induced significant ASMC proliferation at 72 h ($113\% \pm 5\%$ and $125\% \pm 2\%$, respectively) (Fig. 5F). As confirmed by the EDU assays, the number of proliferative cells increased significantly after stimulation with 20 μm H_2O_2 , whereas the CAPE-pretreated cells suppressed H_2O_2 -induced proliferation (Fig. 5G). These results suggested that 20 μm H_2O_2 greatly induced the proliferation of ASMCs, which was inhibited by CAPE pretreatment. Thus, oxidative stress may be involved in TGF- β 1-induced ASMC proliferation and airway remodeling.

3.10. Effect of CAPE on MDA levels and ROS generation *in vitro*

Mounting evidence has suggested that ROS play essential roles in the pathogenesis of airway inflammation and remodeling. To determine whether ROS are involved in TNF- α -induced inflammation and TGF- β 1-induced proliferation and demonstrate the mechanisms underlying these effects, further studies were performed. In BECs, the content of MDA from group TNF- α (194.219 ± 4.49 nmol/ml) were significantly higher than that from the control (105.625 ± 2.60 nmol/ml) ($P < 0.05$). Administrated with CAPE (176.56 ± 7.17 nmol/ml) or NAC (149.22 ± 3.50 nmol/ml), BECs produced less MDA compared to group TNF- α ($P < 0.05$) (Fig. 4E). A similar effect was also observed in ASMCs (Fig. 4J). Moreover, as shown in Fig. 4A, C, the activation of ROS was achieved by treatment with TNF- α , which is an effective activator of BECs. Flow cytometry showed that the pretreatments with CAPE (10 μm) or NAC (10 mM) resulted in decreases of intracellular ROS production to $79\% \pm 1\%$ and $80\% \pm 4\%$, respectively. In the ASMCs, the activation of ROS was achieved by treatment with TGF- β 1. However, pretreatments with CAPE (10 μm) or NAC (10 mM) resulted in decreases in intracellular ROS production to $105\% \pm 1\%$ and $98\% \pm 1\%$, respectively (Fig. 4F and H). These data were significant because the anti-oxidant effect of CAPE may be similar to that of NAC, which is a potent ROS scavenger. Additionally, similar results were obtained when the ROS levels in the BECs and ASMCs were monitored using a laser scanning confocal microscope (Fig. 4B, D, G and I). Thus, we found that CAPE apparently suppressed intracellular ROS production, thus implying a significant protective effect against oxidative stress (Fig. 5).

3.11. Effect of CAPE on the mitogen-activated protein kinase (MAPK)/Akt pathway

To determine the signaling mechanisms involved in the effects of CAPE on airway inflammation and remodeling, the phosphorylation statuses of JNK, ERK, p38 and Akt were investigated. As shown in Fig. 6A, p38 and ERK phosphorylation showed notable peaks in the

BECs at 1 h after TNF- α treatment. The phosphorylation of JNK was not as dramatic compared with that of p38 and ERK. CAPE significantly inhibited the activation of p38 and ERK but failed to affect JNK activation at the examined time points. As shown in Fig. 6B, we found that TGF- β 1 could activate ERK, JNK and Akt in ASMCs within 1 h after stimulation. This activation was partially blunted in the group pretreated with CAPE compared with the vehicle control group. Moreover, NAC markedly suppressed MAPK/Akt signaling both in BECs and ASMCs. These results suggested that CAPE might attenuate the inflammation and remodeling of the airways by depressing the activation of the ROS-dependent MAPK/Akt pathway.

4. Discussion

Asthma is a complex chronic lung disease that has considerably increased worldwide [1]; and many efforts have been directed toward the management of asthma. However, current therapeutic approaches are merely ameliorative rather than curative, and they are associated with diverse unexpected side effects [2]. Thus, developing safe and effective options for asthma treatment is extremely urgent.

As previously demonstrated, CAPE has attracted much attention for its potential anti-inflammatory, anti-fibrotic and anti-oxidant properties. To study the effect of CAPE on chronic asthma, we established an OVA exposure model and identified several characteristic features of allergic asthma, including AHR, eosinophil-rich inflammation, goblet cell metaplasia and collagen deposition [38–40]. These features were accompanied by robust increases in the airway expression of IgE, IL-4 and IL-13, and these changes are consistent with the known roles of these cytokines in the generation of allergic asthma [41]. Our results showed that treatment with CAPE suppressed AHR, inhibited inflammatory cell infiltration, mucus hypersecretion and collagen deposition and fibrosis and downregulated IgE and Th2 cytokines in the OVA-induced mice. These findings indicate that CAPE may be valuable as a new anti-inflammatory and anti-remodeling agent for asthma management.

Furthermore, CAPE markedly inhibited OVA-induced α -SMA and MMP-9 expression *in vivo*. The growth of ASMCs (including hypertrophy and hyperplasia) is a major pathogenic feature of asthma and believed to contribute to increased airway resistance and airway remodeling. As a marker for ASMCs, α -SMA is pivotal for the contraction, migration and proliferation of ASMCs and ECM [42,43]. Higher α -SMA levels have been reported in patients with severe asthma relative to control subjects [44]. Our results demonstrated that the effect of CAPE on α -SMA was more pronounced than that of DEX. Because DEX failed to attenuate the expression of α -SMA in lung fibroblasts [45], this evidence suggests that CAPE has an advantage over DEX with respect to the suppression of ASMC hyperplasia and hypertrophy.

Similarly, MMP-9 participates in the physiological and pathological processes of asthma, especially airway inflammation, ECM deposition and fibrosis [46]. Accumulating evidence has verified that patients with classic asthma have elevated levels of MMP-9 in their serum, sputum and BALF [47]. MMP-9-deficient animals have exhibited reduced airway inflammation, decreased collagen deposition, and attenuated peribronchial fibrosis [48]. Our results indicated that CAPE significantly restrained OVA-induced MMP-9 expression and thereby contributed to the attenuation of airway inflammation, ECM deposition and fibrosis.

Regardless of the observed regional differences of α -SMA and MMP-9, significant enrichment of TNF- α and TGF- β 1 was observed in the BALF samples from subjects with asthma compared with the samples from the normal controls. TNF- α plays a central role in asthmatic inflammation [22,23]. By activating the MAPK or ROS pathways, TNF- α can upregulate multiple pro-inflammatory mediators, including pro-inflammatory cytokines and chemokines (e.g., eotaxin-1, MCP-1, and IL-8) and adhesion molecules (e.g., ICAM-1) [23,49,50].

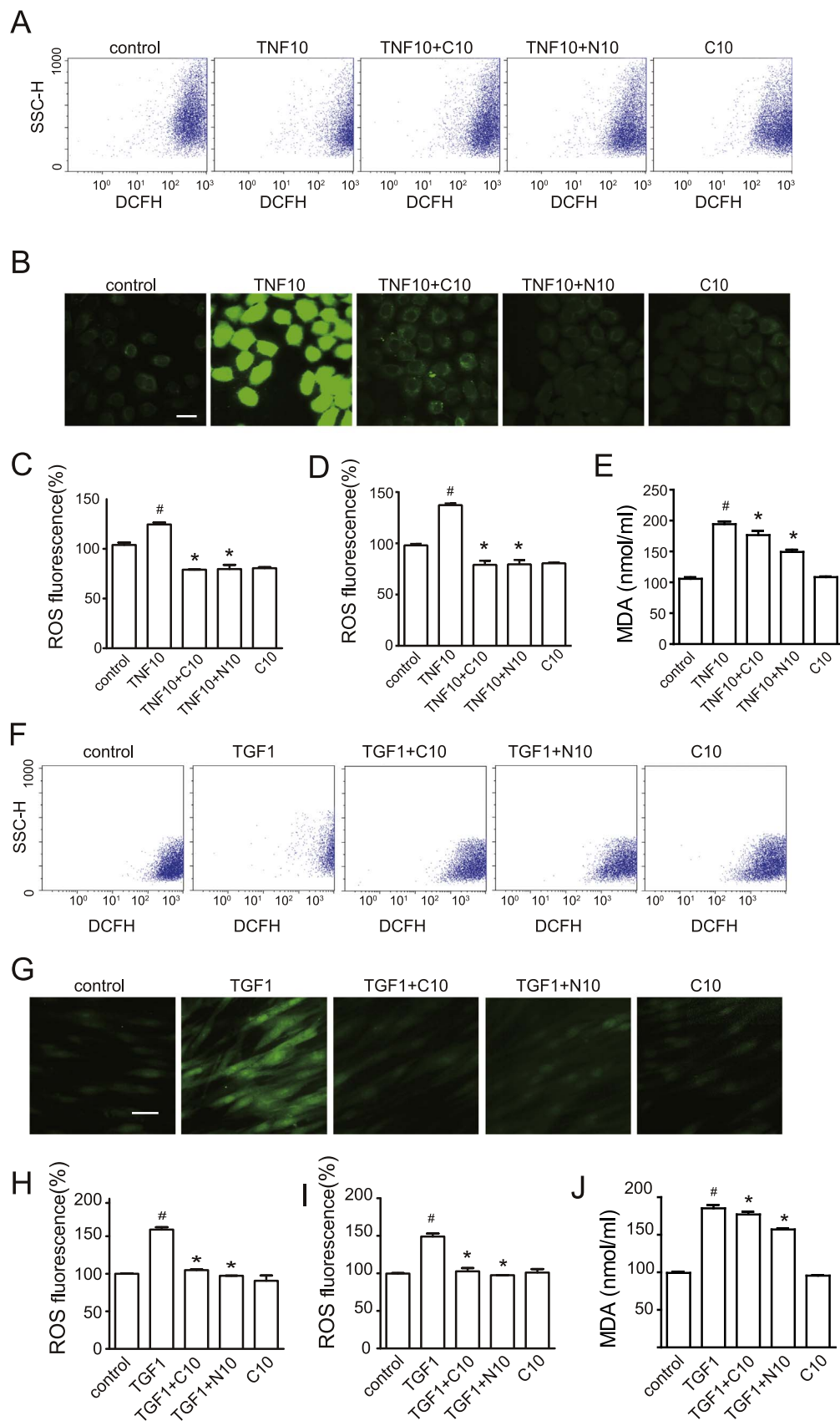


Fig. 4. Treatment with CAPE reduced the intracellular ROS production and MDA induced by TNF-α in BECs and TGF-β1 in ASMCs. (A, C) Fluorescence-activated cell sorting profile of reactive oxygen species (ROS) production by flow cytometry. Summary of the average ROS production by BECs treated with CAPE (10 μM) or NAC (10 mM) after TNF-α (10 ng/ml) stimulation in three independent experiments. (B, D) DCFH-DA fluorescence (green) imaging of ROS in BECs evaluated with a laser scanning confocal microscope (magnification, 400×; scale bar, 50 μm). (E) The concentrations of MDA level in BECs were measured by ELISA. (F, H) Fluorescence-activated cell sorting profile of reactive oxygen species (ROS) production by flow cytometry. Summary of the average ROS production by ASMCs treated with CAPE (10 μM) or NAC (10 mM) after TGF-β1 (1 ng/ml) stimulation in three independent experiments. (G, I) DCFH-DA fluorescence (green) imaging of ROS in ASMCs evaluated with a laser scanning confocal microscope (magnification, 400×; scale bar, 50 μm). (J) The concentrations of MDA level in ASMCs were measured by ELISA. Values are the mean ± SEM of at least four independent experiments performed in triplicate. *P < 0.05 compared with the control, and #P < 0.05 compared with the TGF-β1 group.

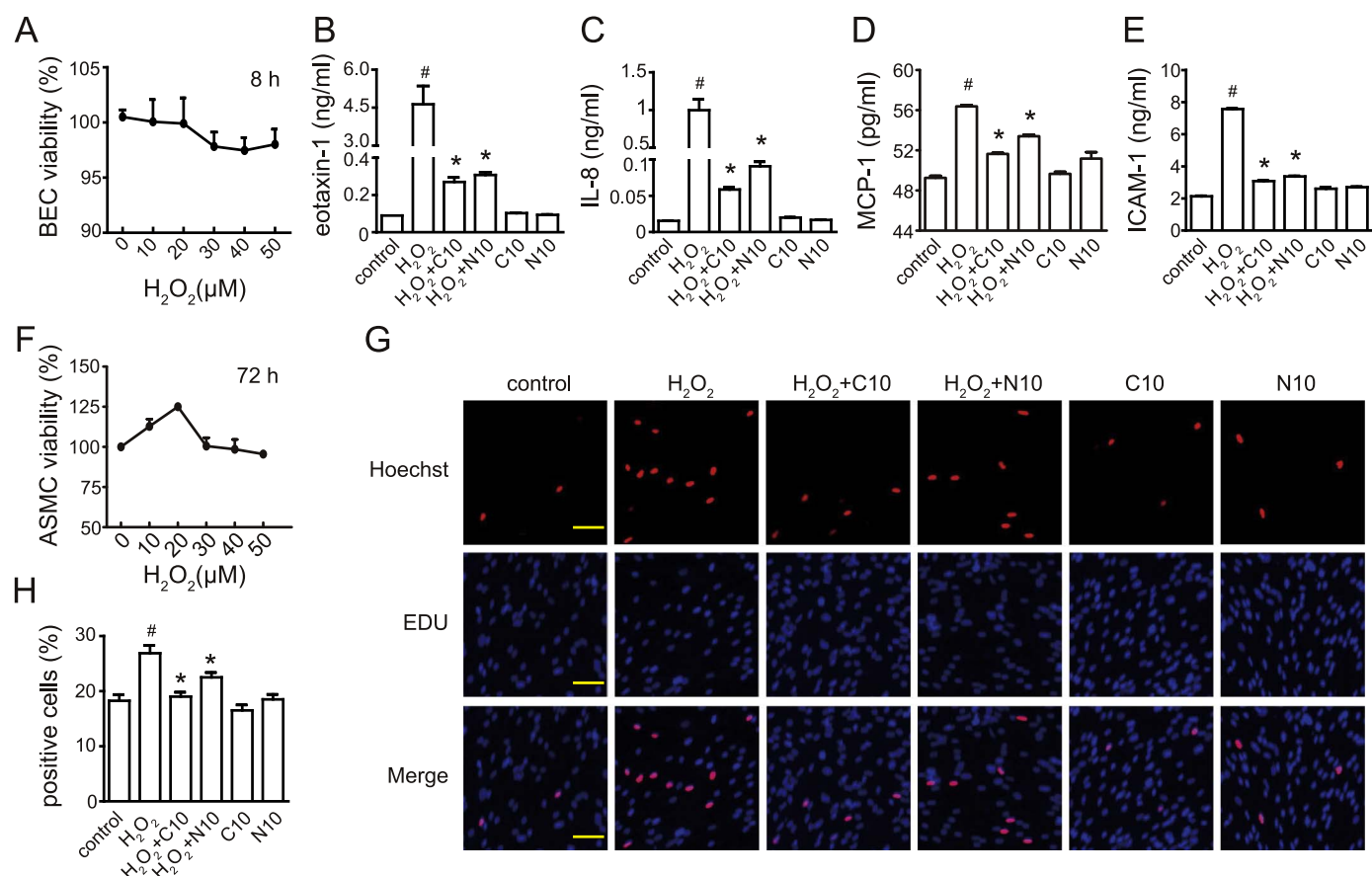


Fig. 5. Treatment with CAPE inhibited the H₂O₂-induced expression of pro-inflammatory protein in BECs and proliferation in ASMCs. (A) Effects of H₂O₂ on the viability of human BECs as assessed by CCK-8 assay. (B–E) Eotaxin-1 (B), IL-8 (C), MCP-1 (D), and ICAM-1 (E) levels in supernatant were measured by ELISA. (F) Effect of H₂O₂ on the viability of human ASMCs as assessed by CCK-8 assay. (G, H) Effect of CAPE on H₂O₂-stimulated human ASMCs as assessed by EDU assay (magnification, 200×; scale bar, 100 μm). Values are the mean ± SEM of at least four independent experiments performed in triplicate. [#]P < 0.05 compared with the control, and ^{*}P < 0.05 compared with the H₂O₂ group.

These pro-inflammatory mediators disturb the airway microenvironment and contribute to airway inflammation and remodeling. Eotaxin-1 has been well established as a crucial factor for the delivery of eosinophils to the airway; furthermore, eosinophils release highly cytotoxic granular proteins, thereby eliciting detrimental tissue damage and severe inflammation [51]. MCP-1 has been described as increasing the risk of allergic asthma by monocyte and lymphocyte chemotactic activity [52]. IL-8 is an important chemoattractant for neutrophil recruitment to the airways of patients with severe asthma [53]. ICAM-1 has been known to mediate cell-to-cell interactions during antigen presentation and outside-in cell signaling pathways [54]. The evidence presented herein and in the literature indicate that these pro-inflammatory mediators play vital roles in airway inflammation. Recently, Malavia et al. compared human ASMCs with injured human BECs after 8 days of in vitro co-culture [8], and they found that the concentrations of IL-8 and MCP-1 increased in the BEC co-culture, which correlated with enhanced ASMC growth, and concluded that the airway epithelium likely modulated ASMC proliferation via a mechanism that involved the secretion of soluble mediators, including potential smooth muscle mitogens such as IL-8 and MCP-1 [8]. Another study confirmed that eotaxin-1 and IL-8 can directly influence ASMC mass by increasing the rate of proliferation or enhancing the survival of these cells [9], which indicates that these pro-inflammatory mediators also induce the proliferation of ASMCs and contribute to airway remodeling via the airway microenvironment modification. Accordingly, blockades of these cytokines and adhesion molecules may provide novel therapeutic strategies for airway disorders.

Several reports have indicated that TGF-β1 plays crucial roles in the remodeling of asthmatic airways and the proliferation of ASMCs

[4,10,11]. These roles may be related to the overexpression of MMPs and the activation of MAPK/ROS signaling [24,55]. TGF-β1 can induce multiple effects in airway structural cells. For example, TGF-β1 can induce ASMC proliferation, hypertrophy, and inflammatory mediator and collagen release. These effects contribute to chronic airway inflammation and subepithelial fibrosis and thereby regulate the airway remodeling process in asthma [4,10,11]. Clinical results have been consistent in previous reports, which have demonstrated high levels of TGF-β1 in the BALF of patients with COPD or asthma [55,56]. Thus, we concluded that when allergens are inhaled, the protective barrier of structural cells may be destroyed, which has the potential to lead to the overexpression of TNF-α and TGF-β1. This reaction likely involves the modification of the airway microenvironment, which causes airway inflammation and remodeling.

Our results showed that CAPE markedly suppresses OVA-induced TNF-α and TGF-β1 expression in vivo, which implies that these cytokines may serve as key modulators in the anti-inflammatory or anti-remodeling activity of CAPE. Through in vitro experiments, we further observed that CAPE reduced the expression of TNF-α-induced eotaxin-1, MCP-1, IL-8 and ICAM-1 in human BECs and blocked the proliferation of human ASMCs induced by TGF-β1. Based on our findings, we can confirm that CAPE decreases the local lung inflammatory response and pathological manifestations of airway remodeling. These protective effects may be attributed to the downregulation of TNF-α and TGF-β1. Because accumulating data have shown that the expression of ROS is regulated by TNF-α and TGF-β1, we speculate that CAPE may suppress TNF-α-induced inflammation and TGF-β1-induced proliferation by inhibiting ROS.

ROS can be produced by inflammatory cells recruited to the

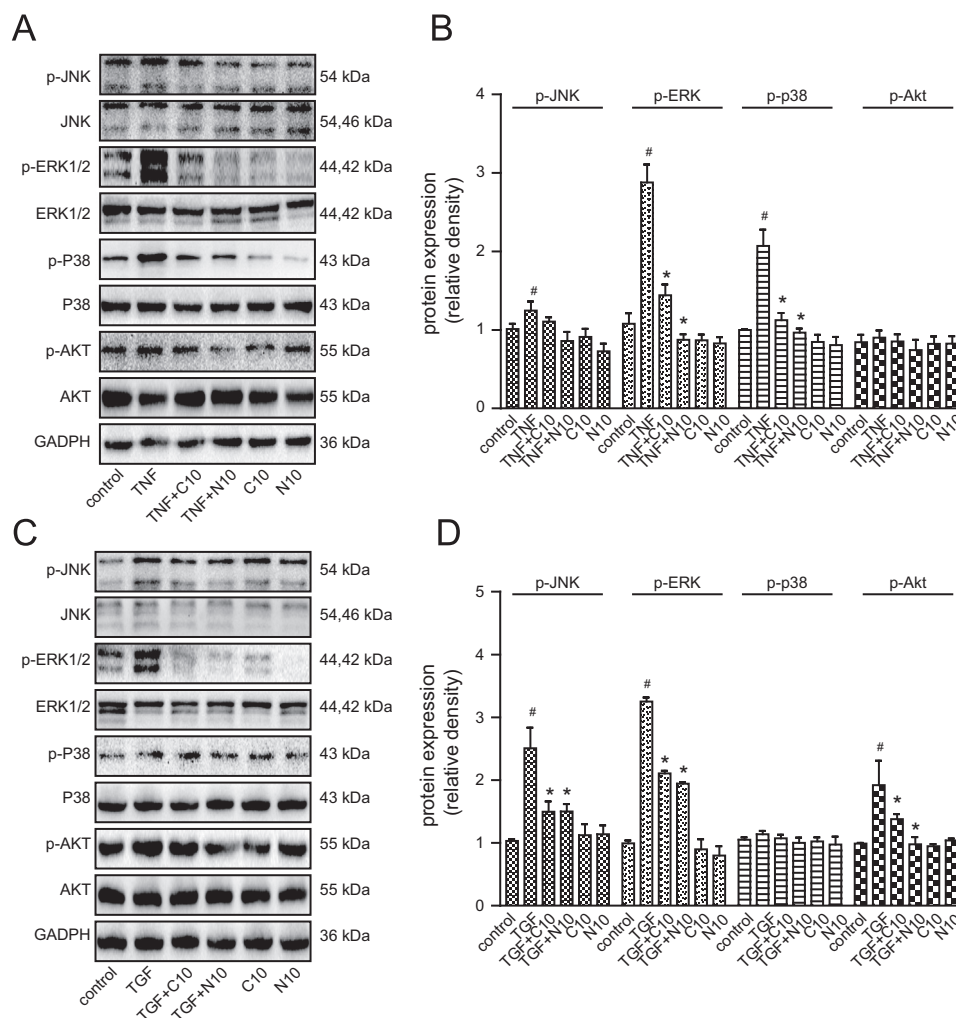


Fig. 6. Treatment with CAPE suppressed the activation of the MAPK/Akt pathway. (A and B) Treatment with CAPE suppressed the TNF- α induced phosphorylation of ERK and p38 in BECs. (C, D) Treatment with CAPE suppressed the TGF- β 1-induced phosphorylation of JNK, ERK and Akt in ASMCs. The phosphorylation and total protein content of JNK, ERK, p38 and Akt were measured by western blotting. The relative density quantification was phosphorylated proteins relative to total proteins. Values are the mean $\pm$ SEM of at least four independent experiments performed in triplicate. * $P < 0.05$ compared with the control, and * $P < 0.05$ compared with the TNF- α group.

asthmatic airways as well as by airway structural cells, such as BECs and ASMCs [12–15]. Enhanced ROS production in allergic airways is correlated with increased airway contractions, AHR, inflammatory cell infiltration, goblet cell metaplasia, mucus hypersecretion, and epithelial shedding within respiratory cells [14,16–19], and it also contributes to the emergence and persistence of airway inflammation and remodeling. ROS causes physiological damage via oxidative stress. As an indicator of lipid peroxidation, MDA is produced by free radicals during tissue damage and detected during measurements of oxidative stress [57]. Our results showed that the MDA and in situ ROS levels were increased in the lung tissue from OVA-challenged rats. CAPE pretreatment significantly attenuated this increase and produced a stronger attenuation than DEX. Our in vitro studies showed that H₂O₂ directly induced eotaxin-1, IL-8, MCP-1 and ICAM-1 overexpression in the BECs and promoted ASMC proliferation, whereas CAPE pretreatment reduced these effects. Moreover, our findings further demonstrated that CAPE intensively inhibited the MDA and intracellular ROS induced by TNF- α or TGF- β 1 and produced similar effects as those elicited by NAC. As a free radical scavenger, NAC prevents the release of oxidant-induced inflammatory mediators, contraction of airway smooth muscles and proliferation of ASMCs. Thus, we conclude that ROS directly induces damage to structural cells and regulates the airway microenvironment. The amelioration of airway inflammation and remodeling by CAPE in chronic asthma most likely accounts for

the anti-oxidant activities of CAPE.

ROS are cytotoxicants and secondary messengers in intracellular signal transduction; therefore, ROS mediate several signaling pathways. ROS, MAPK and PI3K/Akt are closely related to airway inflammation and pulmonary cell proliferation. However, the exact nature of their interactions remains unknown. Previous studies have confirmed that ERK phosphorylation is dependent on ROS in Siglec-8-mediated eosinophil cell death [58] and that JNK phosphatases are critical molecular targets of ROS in TNF- α -induced programmed cell death [59]. Furthermore, pretreatments of cells with anti-oxidant enzymes abrogated the thalidomide-induced p38 MAPK activation in adult erythropoiesis [60]. A recent study also indicated that H₂O₂ significantly increased p38 MAPK and ERK1/2 phosphorylation and NAC effectively suppressed this phosphorylation [61]. Moreover, the inhibition of ROS production by various chemicals has been shown to reduce TNF- α -induced MAPK phosphorylation in human ASMCs [62], which suggests that ROS play key roles in mediating MAPK activation in response to pro-inflammatory mediators [63]. Additionally, several reports have indicated that TGF- β 1 promotes the proliferation of ASMCs through the MAPK pathway [10,55]. The activation of the PKC family isoenzymes has been shown to drive oxysterol-generated signals through the MAPK pathway with marked upregulation of ERK and JNK kinases but without any apparent modulation of p38 kinase [63,64]. Evidence from literature suggests that ROS play key roles in

MAPK/Akt signaling associated with TNF- α or TGF- β 1 stimulation. Therefore, we hypothesize that CAPE may suppress TNF- α -induced inflammation and TGF- β 1-mediated proliferation by inhibiting MAPK/Akt signaling via ROS.

Our data indicate that CAPE suppresses TNF- α -induced phosphorylation of ERK and p38 in BECs and ameliorates the TGF- β 1-induced activation of ERK, JNK and Akt in ASMCs. Additionally, NAC effectively suppresses the phosphorylation of MAPK/Akt stimulated by TNF- α in BECs as well as activated by TGF- β 1 in ASMCs respectively. These results suggest that CAPE specifically prevents BECs and ASMCs from developing a pro-oxidant phenotype and inhibits airway inflammation and remodeling.

This study presents the novel observation that CAPE acts in a dose-dependent manner to suppress various aspects of OVA-induced Th2-mediated asthma in mice, abrogate pro-inflammatory cytokines release, prevent aberrant airway smooth muscle function and proliferation, and modify the asthmatic airway microenvironment. These effects may provide therapeutic benefits for asthma patients. Thus, the observations and known anti-inflammatory and anti-remodeling properties of CAPE should be further evaluated to determine the in vitro/in vivo correlations and clinical utility of CAPE in delayed allergic diseases.

5. Conclusions

We demonstrated the potential therapeutic action of CAPE in an experimental model of asthma and revealed its anti-inflammatory and anti-proliferative properties in vivo and in vitro. Collectively, our findings indicate that CAPE (I) abrogated AHR and attenuated inflammatory cell infiltration, goblet cell hyperplasia and fibrotic expression in the airways; (II) decreased the over-expression of α -SMA, MMP-9 and MDA in OVA-sensitized mice; (III) diminished elevated IgE, TNF- α , TGF- β 1, IL-4, and IL-13 levels in BALF; (IV) reduced TNF- α and H₂O₂-induced pro-inflammatory protein expression in human BECs; (V) limited TGF- β 1 and H₂O₂-induced human ASMC proliferation; (VI) inhibited TNF- α -induced ROS by regulating P38 and ERK in human BECs; and (VII) suppressed TGF- β 1-induced ROS by the inhibition of ERK, JNK and Akt phosphorylation in human ASMCs. Taken together, our results indicate that CAPE has the potential to provide beneficial improvements to airway inflammation and remodeling, which most likely occur because of oxidative stress-responsive MAPK/Akt pathways. Thus, the use of CAPE may provide a new therapeutic approach for treating allergic airway diseases.

Conflicts of interest statement

The authors declare no conflict of interest.

Authors' contribution

All authors have read and approved the final paper. Yuan Ma and Jia-Xiang Zhang contributed equally to this work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2016.09>.

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