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Platycodi Radix and its active compounds ameliorate against house dust mite-induced allergic airway inflammation and ER stress and ROS by enhancing anti-oxidation

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ABSTRACT

Allergic airway inflammation is an increasing global health problem, and novel strategies to prevent or ameliorate the condition are needed. The endoplasmic reticulum (ER) is involved in protein synthesis and maturation, and is a susceptible to sub-organelle stress including inflammation and ROS-amplifying signaling. Here, the effects of Platycodi Radix extracts (PRE) on house dust mite (HDM) extract (*Dematophagoides pteronyssius*)-induced asthma were investigated. Following 50, 100, or 200 mg/kg-PRE-treatment, the infiltration of inflammatory cells, ER stress, and NF-κB signaling were controlled. The expression of inflammatory cytokines and mucin5AC was also inhibited in the presence of PRE. Consistently, in the HDM-exposed human bronchial epithelial cells, ER stress and its associated ROS were significantly increased along with NF-κB signaling, which was also attenuated by PRE and its components. This study suggests that PRE might be useful as a therapeutic/preventive agent in HDM-associated allergic airway inflammation. ER stress and its associated ROS signaling involved in inflammation provide additional mechanistic insight into the underlying molecular mechanism.

1. Introduction

Asthma is a major health problem affecting people worldwide. The prevalence of asthma is continually increasing, with substantial associated healthcare expenditures. Asthma is a chronic airway inflammatory disorder. Along with autoimmune diseases, asthma is characterized by the presence of immune cells in the airway. It can be elicited by a variety of environmental allergens including air pollutants and smoke (Oeser et al., 2015). Common symptoms of asthma include wheezing, coughing, shortness of breath, and chest pain. These symptoms are caused by chronically hyperactive and inflamed airways, leading to contraction of the muscles surrounding the airway and airflow obstruction (Lommatzsch and Virchow, 2014).

Currently, it is an urgent priority to develop preventative and ameliorating strategies to treat asthma. Several drugs are currently available. Corticosteroids are widely used for improvement of lung function in patients with asthma. However, it is well known that inhaled corticosteroids are limited in their capacity for airway remodeling (Durrani et al., 2011). Therefore, there has been marked interest in the development of drugs with fewer side effects (Hocaoglu et al., 2012; Jang et al., 2016). Recent studies have indicated that herbal medicines can improve the symptoms of asthma through anti-inflammatory mechanisms (Jang et al., 2016; Kostic et al., 2017; Tao et al., 2015; Won Jung et al., 2012).

Exposure to house dust mite (HDM) allergens is associated with the development of allergic response, including asthma and rhinitis (Platt-Mills et al., 1995; Thomas et al., 2002). Mites from the genus *Dermatophagoides* are thought to be the primary source of indoor allergens associated with human asthma (Thomas et al., 2002). However, few studies exist which demonstrate that herbal medicines can improve the symptoms of HDM-associated asthma.

The endoplasmic reticulum (ER) is an intracellular organelle that is specialized for protein folding and secretion in cells. A number of physiological and pathological conditions, including redox alterations, calcium equilibrium, and protein degradation can alter ER function leading to ER stress (Bhandary et al., 2012; Mahdi et al., 2016). In addition, ER stress has been implicated in various conditions including pulmonary fibrosis, lung injury, and chronic airway disorders (Lawson et al., 2011; Lee et al., 2016; Park et al., 2015). Under pathological conditions, excessive reactive oxygen species (ROS) accumulation has

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been identified as a key deleterious signal. Moreover, ROS are associated with ER stress in other pathological conditions (Bhandary et al., 2012; Zeeshan et al., 2016). Recent studies have suggested that ROS and ER stress have a role in allergic lung inflammation (Jeong et al., 2017; Kim and Lee, 2015). Therefore, the identification of interventions that can control ROS-associated ER stress is important for preventing HDM-associated allergic airway inflammation.

Platycodi Radix, *Platycodon grandiflorum* root, has been used as a source of food and traditional medicine to treat bronchitis and asthma in Korea, and has been recently demonstrated to be a potential novel approach for treating inflammatory and allergic conditions, including asthma. In animal models, platycodin saponins (derived from PRE) exhibits anti-obesity and anti-hypolipidemic effects based on the ability to inhibit intestinal absorption of dietary fat (Han et al., 2000; Kim et al., 2000). The most potent pharmacological constituents of Platycodi radix, Platycoside E (PLE) and Platycodin D_3 (PLD) exhibit many pharmacological activities including anti-inflammatory (Kim et al., 2001), anti-diabetic (Han et al., 2002), and anti-cancer effects (Luan et al., 2014). Although Platycodi Radix extracts (PRE) and its major compounds PLE and PLD traditionally have been used to treat colds, coughs, and asthma, the anti-inflammatory and anti-asthma effects of these agents have yet to be established. This study sought to investigate the anti-inflammatory effects of PRE, PLE, and PLD in HDM-induced airway inflammation. Based on our results, we hypothesized that PRE and its major compounds, PLE and PLD, would have anti-inflammatory effects against asthma in humans.

2. Materials and methods

2.1. Materials

Platycoside E (PLE) and platycodin D_3 (PLD) were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Primary antibodies targeting the following proteins were used in this study: rat anti-GRP78 (sc-13539), mouse anti-eIF2α (sc-133132), rabbit-anti−NF−kB p65 (sc-8008), rabbit anti-tubulin (sc-365791), goat-anti-histone (sc-393358), rabbit anti-IκB-α (sc-1643), rabbit anti-NOX4, mouse anti-β-actin (sc-130300, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), mouse anti-GADD153/C/EBP homologous protein (CHOP, 2895S), rabbit anti-IRE1α (3294S), and rabbit anti-p-eIF2 (9721, Cell Signaling Technologies, Inc., Danvers, MA, USA), Mucin5AC (ab77576, Abcam, Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies were obtained from Novus Biologicals, Inc. (Littleton, CO). 4-HNE (4-hydroxynonenal, ab48506) and DHE (dihydroergotamine, D7008) were purchased from Sigma-Aldrich (St. Louis, MO). The enzyme-linked immunosorbent assay (ELISA) kits for cytokine measurement were purchased from Pierce Biotechnology (Rockford, IL) and R& D Systems (Minneapolis, MN).

2.2. Preparation of Platycodon Radix

Platycodon grandiflorum was grown for 3–4 years on Jeju Island, Korea, and used in the current study. Dried and ground powder of Platycodon Radix was supplied by the Hangirim JK Milk Thistle Research Institute (Jeju, Korea). Powdered Platycodon Radix (100 g) was extracted with 900 mL of 70% ethanol twice by shaking for 24 h at 25 °C. The precipitates were removed by centrifuging at 8000 *g* for 30 min (Beckman, Brea, CA) and lyophilized (Il Shin, Korea).

2.3. HPLC analyses

HPLC analyses for the identification of Platycoside E and Platycodin D_3 were performed on an HPLC system equipped with an LC-10AD pump, a CBM-10A control bus, and an SPD-M10AVP UV/Vis detector (Shimadzu, Tokyo, Japan). Stock standard solutions of platycoside E and platycodin D_3 were prepared in ethanol at a concentration of 1.0 mg/mL. The appropriate amount of each standard solution was mixed and diluted with ethanol as indicated. The sample solutions were filtered through a 0.45 μm pore size (170 mm diameter) nylon membrane filter (Millipore, Billerica, MA) and analyzed by a HPLC-UV detector. Chromatographic separation was carried out using a Phenomenex Luna C18 column (4.6 × 250 mm, 5 μm; Torrance, CA) heated to room temperature. The injection volume was 20 μL, and the following solvent ratios were used for the mobile phase with a flow rate of 1 mL/min: solvent A is distilled water and solvent B was acetonitrile in water). Gradient conditions were as follows: 0 min, 20% B; 40 min, 60% B; 45 min, 78% B; 45.1 min, 100% B; 60 min, 100% B; 60.1 min, 20% B; and 71 min, 20% B. The detection wavelength (203 nm) was scanned at a flow rate of 1 mL/min at 35 °C. Peak analyses and assignments were performed using platycoside E and platycodin D_3, which were identified in accordance with UV spectra and retention times in the HPLC chromatograms.

2.4. Cell culture

Human bronchial epithelial cells (BEAS-2B) were obtained from the Department of Allergy and Clinical Immunology (Ajou Research Institute for Innovation Medicine, Ajou University Medical Center, Suwon, South Korea). BEAS-2B cells were cultured and maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) serum at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. The cells were maintained at 37 °C and 80% confluence in an air-ventilated and humidified incubator maintained with 5% carbon dioxide.

2.5. Quantitative real-time PCR

BEAS-2B cells were exposed to either HDM, platycoside E (PLE), or Platycodin D_3 (PLD) for 24 h. Total RNA was extracted and reverse transcribed into cDNA using 1 μg RNA. cDNA corresponding to human interleukin IL-4, -13, −25, and −33 was amplified by PCR from 1 μL of the cDNA synthesis reaction using iQSYBR Green Supermix (Bio-Rad, Hercules, CA) and the following primers: MUC5AC sense 5′- CGACAA CTACTTCTGCGGTGC-3′, antisense 5′-GCACTCATCCTTCCTGTCGTT-3'; IL-25: sense, 5′—CCAGGTGGTTGCATTCTTGG-3′; antisense, 5′-TGGCT GTAGGTGTGGGTTCC-3'. IL-33: sense, 5′-CAAAGAAGTTTGCCCCA TGT-3′; antisense, 5′-AAGGCAAAGCACTCCACAGT-3′. IL-4: sense, 5′—CCAACTGCTTCCCCCTCTG-3′; antisense, 5′-TCTGTTACGGTCAACTCG GTG-3′. IL-13: sense, 5′—CCTCATGGCGCTTTTGTTGAC-3′; antisense, 5′-TCTGGTTCTGGGTGATGTTGA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously amplified as an internal standard.

2.6. Animal experiments

To generate HDM-induced asthma models, female C57BL/10 mice 7 weeks of age and free of murine-specific pathogens (Orient Bio Inc. Seoungnam, Korea) were sensitized intraperitoneally with HDM extract (100 μg, *Demarophagoides pteronyssius*, GREER Laboratories, Lenoir, NC) on day 1. Fourteen days following sensitization, mice were challenged intranasally with 100 μg of HDM extract. On day 21, mice were challenged intratracheally with 100 μg of HDM extract. After the initial sensitization, PRE extract was orally administered at doses of 50, 100, or 200 mg/kg from the day 2–20 (Supplementary Fig. 1). Forty-eight hours after the last challenge with HDM, bronchoalveolar lavage fluid (BALF) was collected and analyzed. At the time of lavage, the mice were killed by i.p. injection of ketamine. The chest cavity was opened and the trachea was intubated. The catheter was secured with ligatures. Warmed 0.9% NaCl solution was slowly instilled into the lung and removed. The collected solutions were pooled and stored at 4 °C. The supernatants from each pool were stored at −70 °C until use. Cell numbers were counted with a hemocytometer. Smears of BAL cells were

prepared using the cytospin method (Thermo Electron, Waltham, MA) and stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, Puerto Rico) in order to examine cell properties. All experimental animals used in this study were cared for under a protocol approved by the Institutional Animal Care and Use Committee of Chonbuk National University Hospital (cuh-IACUC-2017-12-2). All surgery was performed under ketamine anesthesia. All efforts were made to minimize suffering during animal experiments.

2.7. Group size, randomization, and blinding

Mice were randomly divided into six groups of 10 mice for the following treatments: sham induction using saline challenge (control group), HDM plus vehicle (HDM group), HDM plus 50, 100, or 200 mg/kg PRE (HDM with PRE group), or 200 mg/kg PRE only (PRE group). The authors who participated in the animal studies were blind to animal groupings.

2.8. Analysis of bronchoalveolar lavage fluid (BALF) samples

Bronchoalveolar lavage fluid (BALF) is commonly used to obtain inflammatory cells (alveolar macrophages, neutrophils and monocytes) and other soluble components present in alveoli (Almatroodi et al., 2015). BALF samples (1 mL) were obtained for each mouse. Samples were centrifuged at 600 *g* for 3 min, and the supernatants were stored at −20 °C for cytokine analysis. The cell pellets were pooled for total cell counts using a Model Z1 instrument (Beckman-Coulter, Miami, FL) after lysis of erythrocytes (Zap-Oglobin II, Beckman-Coulter, Fullerton, CA). Slides were loaded with cells, centrifuged at 700 *g* for 3 min, and stained with Diff-Quick (Baxter, Detroit, MI). Cells were counted under light microscopy.

2.9. Immunohistochemistry and periodic acid-Schiff staining

Lung tissues were fixed in 4% formalin solution for 24 h, embedded in paraffin, and stained with antibodies or periodic acid-Schiff (PAS) reagent. Serial 4-μm-thick sections were prepared, blocked with 5% serum after antigen retrieval, and incubated at 4 °C overnight with one of the following primary antibodies: mucin5AC or 4-HNE. Immunostaining was visualized with 3, 3′-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin and eosin (H&E). Quantification of mucous cell metaplasia was performed by analyzing present large (conducting), medium (central) and small (distal) airways within each representative lung section. The number of airways containing PAS-positive cells were counted. Mucous cell metaplasia is presented as the percentage of airways with PAS-positive cells. Image analysis was performed using Metamorph Image Analysis Software and is expressed as positive staining (4-hydroxynonenal, malondialdehyde) percentage of the microscope field.

2.10. Dihydroethidine (DHE) staining

DHE was used to evaluate the formation of ROS. Lung tissues were cut into 5-μm-thick sections, treated with DHE (5 μM), and incubated in a light-protected humidified chamber at 37 °C for 30 min. ROS levels were determined using microscopy and whole tissue fluorescence was quantified with the microscope assistant CKX41 (Olympus, Melville, NY).

2.11. Measurement of SOD activity

SOD activity was measured using a superoxide dismutase assay kit from Biovison (Mountain View, CA, USA), which uses a colorimetric assay to measure the concentration of formazan crystals. Lung tissues were suspended in 20 mM HEPES buffer (pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose), sonicated, and placed on ice. To measure SOD activity, the diluted radical detector and lysed tissue supernatant or standard solutions were added to the wells of a 96-well plate. Xanthine oxidase was then added. Following a 20-min incubation, absorbance was measured at a wavelength of 460 nm after 20 min on a microplate reader. The results are expressed as IU/mL.

2.12. Measurement of cytokines in BALFs

The cytokines IL-4, IL-5, IL-13, and IL-17 were in BALF supernatants by enzyme immunoassay according to the manufacturer's protocol (BD Biosciences).

2.13. Western blotting

Lung tissues were lysed in RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phosphatase inhibitor cocktail, and 1 mM protease inhibitor cocktail) for 30 min on ice. Equal amounts of protein were separated on 10% SDS-PAGE gels and subsequently transferred to PVDF membranes with a Bio-Rad mini-transfer tank. Membranes were probed with the indicated primary antibodies. After incubation with second antibody, blots were developed using a chemiluminescence detection system. Images were acquired and analyzed with ImageJ software. Protein expression levels (band intensities) were normalized to that of β-actin.

2.14. Lipid peroxidation assay

Lipid peroxidation assay was performed with a lipid hydroperoxide assay kit from Cayman Chemicals (Ann Arbor, MI). Lung tissue samples (1 mg) were homogenized in 1 mL of ice-cold 2% SDS buffer. The sample homogenates were incubated with SDS and 0.8% thiobarbituric acid (in 20% acetic acid, pH 3.5) in the presence of 0.8% butylated hydroxytoluene at 95 °C for 1 h. The malondialdehyde (MDA) standards were applied to the same assay. After incubation, samples were cooled on ice and centrifuged at 3000 rpm for 15 min. Peroxidation levels in supernatants were assessed on a spectrophotometer by absorbance at 532 nm.

2.15. GSH/GSSG ratio assay

Pulmonary oxidative stress was examined using a glutathione assay kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's instructions (Wheeler et al., 1990).

2.16. OxyBlot assay

Oxidative protein carbonylation assays were performed on lung tissue following western blot using an OxyBlot protein detection kit (Millipore, Billerica, MA). Carbonyl groups in protein side chains were derivatized to DNP-hydrazone with 2,4-dinitrophenylhydrazine (DNPH) according to the manufacturer's instructions. After derivatization, 1-dimensional electrophoresis was carried out by 10% SDS-PAGE. Proteins were transferred to PVDF membranes. After incubation with anti-DNP antibody, each blot was developed using a chemiluminescence detection system.

2.17. Measurement of intracellular ROS

Intracellular ROS content was measured with 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes). Cells were stained with 2 μM H_2DCF-DA for 30 min at 37 °C, washed and analyzed.

2.18. MitoSOX analysis of mitochondrial ROS production

BEAS-2B cells were labelled with mitoSOX fluorescent

mitochondrial superoxide production indicator with phenol-red free OptiMEM. Images were collected and cellular fluorescence was quantified.

2.19. Statistical analysis

All data and statistical analyses were performed using appropriate experimental design and analysis criteria. Data are expressed as the mean ± SEM. GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. Comparisons between two groups were made using Student's unpaired *t*-test for normally distributed data or the Mann-Whitney *U* test as the non-parametric equivalent. Comparisons among three or more groups were performed using a one-way ANOVA followed by Tukey's *post hoc* test for normally distributed data or with a Kruskal-Wallis *H* test for non-normally distributed data. The threshold of $P < 0.05$ was designated as statistically significant for all tests.

3. Results

3.1. Analysis of compounds in Platycodon Radix

PRE (Platycodon Radix Extract) was extracted with 100% ethanol (v/v) by reflux (Supplementary Figs. 2 and 3). In HPLC analysis, platycoside E and platycodin D_3 were identified as major components. The retention times of platycoside E (9.09 min) and platycodin D_3 (11.33 min) are presented in the chromatogram data. The abundance of platycoside E and platycodin D_3 was 4.02% and 0.04%, respectively (Supplementary Fig. 3).

3.2. PRE inhibits HDM-induced allergic airway inflammation response in mice

The mouse model of HDM-induced asthma is characterized by airway wall remodeling with structural alterations including thickened epithelium (Dumez et al., 2014). To better understand the effect of PRE on HDM-associated airway mucosal thickening and bronchial hyperresponsiveness, PRE was administered to previously HDM-challenged mice in advance of a final HDM challenge. In hematoxylin and eosin (H &E) and periodic acid-Schiff (PAS) staining analysis, the infiltrated cells increased in lungs of the HDM-challenged groups relative to control, which is a characteristics of pulmonary airway inflammation.

The inflammation was alleviated by PRE, as indicated by a reduced surface area with inflammatory infiltration observed in lungs from mice in the PRE group (Fig. 1a and b). To further evaluate the mucus secretion, MUC5AC and mucin5AC expression was examined in the HDM-challenged mice using immunostaining. In a dose-dependent manner, PRE significantly inhibited the induction and secretion of mucin5AC (relative to the HDM-treated group), indicating that submucosal edema and mucus hyper-secretion are effectively controlled by PRE (Fig. 2). The MUC5AC transcript was significantly increased in HDM-challenged mice (Fig. 2b). We also observed a decrease in muc5ac transcript level in HDM-challenged mice exposed to PRE. The protein level of mucin5AC was measured with mucin5AC antibody immunostaining. The level of mucin5AC in the HDM-challenged mice was elevated relative to saline treatment. However, mucin5AC expression was alleviated by PRE (Fig. 2c). Taken together, these findings indicate that PRE decreases mucus secretion, mucin5AC transcript and protein expression in mice exposed to HDM.

3.3. PRE reduces proinflammatory lung cell numbers in the HDM-induced asthma model

Numbers of total cells, lymphocytes, eosinophils, and neutrophils in the bronchoalveolar lavage (BAL) fluid of HDM-challenged mice were significantly higher than cell numbers from saline-induced mice administered drug vehicle alone. HDM in particular increased eosinophils in BALF. In a dose-dependent manner, PRE significantly decreased the total number of cells (including eosinophils) in BALF relative to the HDM-challenged group (Fig. 3a and b).

3.4. PRE protects HDM-induced nuclear translocation of NF-κB p65 and cytokine expression in the HDM-induced asthma model

Subcellular fractionation indicated that the nuclear translocation of NF-κB, and the degradation of cytosolic IκB-α, were significantly increased in the lungs of HDM-challenged mice receiving vehicle alone. However, elevated nuclear translocation of NF-κB and degradation of cytosolic IκB-α were not observed in PRE-treated, HDM-induced mice (Fig. 4a). Levels of the inflammatory cytokines, IL-4, IL-5, IL-13, and IL-17 were examined in the BALF of HDM-challenged mice. Cytokine levels in the HDM-challenged mice were significantly higher relative to control. However, the PRE-treated group showed significantly reduced levels of IL-4, IL-5, IL-13 and IL-17 (Fig. 4b).

3.5. PRE regulates HDM-induced ER stress, lipid peroxidation and ROS accumulation in mice

To evaluate whether PRE could protect against ER stress following HDM-challenge, mice were sensitized intratracheally with HDM extract (100 μg) on days 1 and 7. On day 14 after sensitization, mice were challenged intratracheally with 100 μg of HDM extract for 24 h to induce ER stress. To examine the ER stress response induced by HDM, we analyzed the expression of UPR proteins in HDM-induced asthma mice. As expected, the levels of GRP78, CHOP, p-IRE1-α, and p-eIF2α were significantly increased in lung tissue. However, treatment with PRE inhibited the levels of GRP78, CHOP, p-IRE1-α, and peIF2α (Fig. 5a and b), indicating that PRE affects the regulation of ER stress in HDM-induced allergic airway inflammation.

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and removal (Birben et al., 2012). ROS, which are produced as by-products of oxidative metabolism, frequently damage cellular components such as lipids, proteins, and DNA. Levels of the lung lipid peroxides malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and DHE were measured by staining lung sections. Peroxidation was induced by HDM treatment and reduced by PRE in a dose-dependent manner (Fig. 6a). In a protein carbonylation assay, protein hyper oxidation was also detected in HDM-challenged mice and was reduced by PRE (Fig. 6b). Protein carbonylation was controlled by PRE in a dose-dependent manner. Similarly, both 4-HNE and MDA were markedly increased in the lung tissue of the HDM-induced group relative to control. PRE in turn alleviated levels of these lipid peroxides (Fig. 6c and d). Next, we measured glutathione redox status (GSH/GSSG balance) in lung tissue. The GSH/GSSG ratio is an indicator of redox state [17, 18]. The ratio of GSH to GSSG was significantly decreased in HDM-induced mice. However, oral administration of PRE significantly inhibited HDM-induced GSH/GSSG perturbation in a dose-dependent manner (Fig. 6e). These results suggest that PRE protects the lungs against HDM-induced alterations by attenuating oxidative stress.

3.6. Platycoside E and platycodin D_3 regulate HDM-induced cytokine gene expression and nuclear translocation of NF-κB p65 in BEAS-2B lung epithelial cells

We evaluated the effects of PLE and PLD against epithelial-driven cytokines (IL-25 and IL-33) and Th2 cytokines (IL-4 and IL-13) in response to HDM, an asthma-relevant allergen, in BEAS-2B lung epithelial cells. Exposure to HMD resulted in significantly higher IL-4, IL-13, IL-25 and IL-33 transcripts relative to control (Fig. 7a and b). However, HDM-induced increases in cytokine gene expression were attenuated following co-treatment with PLE and PLD.

Subcellular fraction data demonstrated that nuclear translocation of

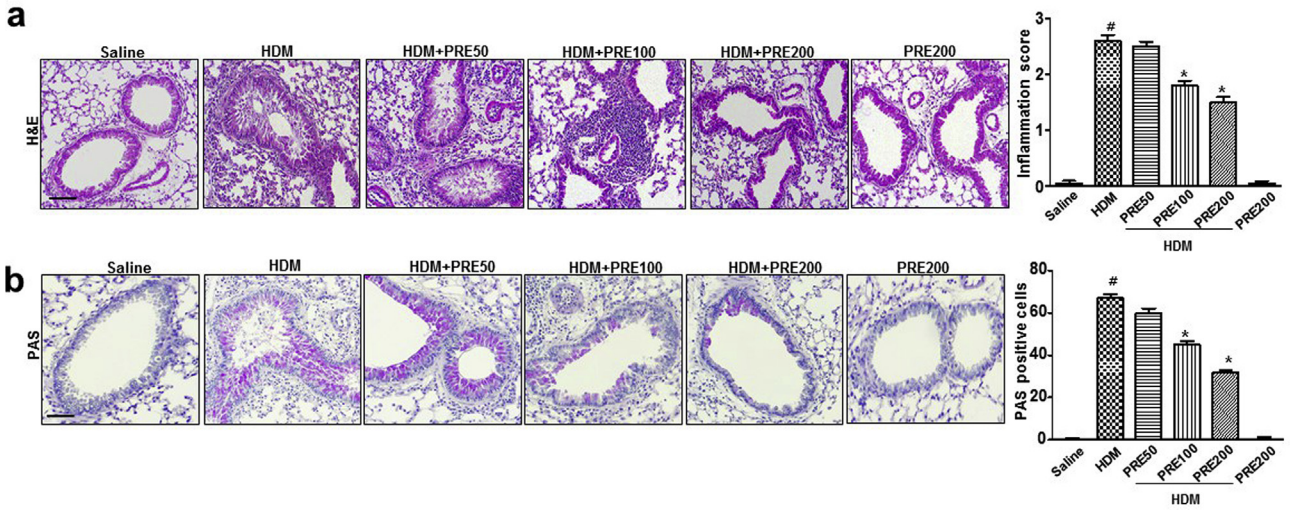

Fig. 1. Platycodon Radix extracts protect against histological alterations in the lungs of HDM-challenged mice. Lung tissues were obtained 48 h after the final challenge in HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. Another group of mice received 200 mg/kg PRE only. H&E (a) and PAS (b) staining assays were performed using the lungs from these mice (100× magnification). Experiments were repeated three times with at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin.

NF-κB and the degradation of cytosolic IκB-α were significantly increased in HDM-induced BEAS-2B lung epithelial cells. However, elevated nuclear translocation of NF-κB and degradation of cytosolic IκB-α were not observed after co-treatment with PLE and PLD (Fig. 7c). Increases in cytokine gene expression, nuclear translocation of NF-κB and the degradation of cytosolic IκB-α recovered following treatment with NAC and 4-PBA (Fig. 7a-c).

3.7. Platycoside E and platycodin D_3 regulate HDM-induced ER stress and ROS production in BEAS-2B lung epithelial cells

Considering that ROS and NF-κB signaling are associated with ER stress in inflammatory asthma (Hotamisligil, 2010; Kim et al., 2018), it is necessary to examine the effects of platycoside E and platycodin D_3 on ER stress. The intensity of ER stress was quantified as the expression of the following ER stress representative proteins: GRP78, CHOP, P-IRE1α, and P-eIF2α. HDM-induced ER stress increases levels of GRP78, CHOP, P-IRE1α, and P-eIF2α, all of which were significantly reduced following administration of platycoside E, platycodin D_3 in BEAS-2B cells (Fig. 8a and b). To evaluate the antioxidant effects of platycoside E and platycodin D_3, we investigated their effects on HDM-mediated ROS production. We found that treatment with HDM markedly increased ROS production. However, treatment with platycoside E and platycodin D_3 markedly attenuated HDM-mediated increases in intracellular ROS (Fig. 9a). As shown in Fig. 9b, HDM treatment markedly decreased the GSH/GSSG ratio. However, PRE prevented these HDM-induced effects, resulting in higher GSH/GSSG ratios. Next, we investigated the effects of platycoside E and platycodin D_3 on SOD and GPx activity. HDM

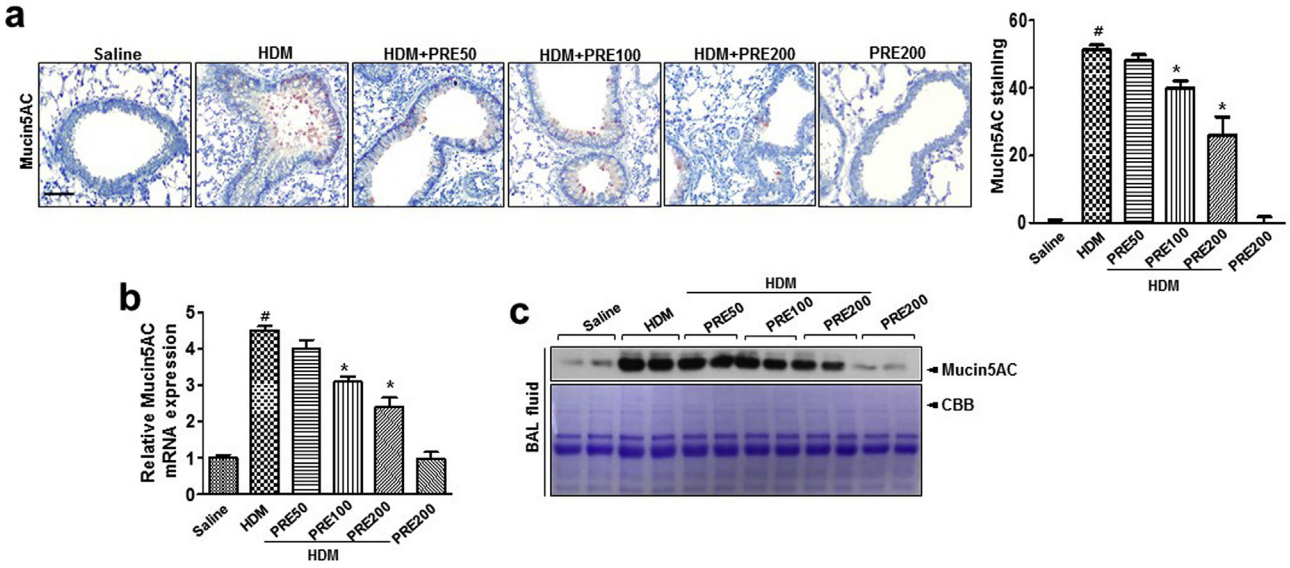

Fig. 2. Platycodon Radix extracts protect against mucous hyperproduction in the lungs of HDM-challenged mice. Lung tissues were obtained 48 h after the final challenge in HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. Another group of mice received 200 mg/kg PRE only. (a) Mucin5AC staining assays were performed using the lungs from these mice (100× magnification). RNA levels (b) and protein expression (c) were analysis using the lungs and BALFs from these mice. The experiments were repeated three times with at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; BALFs, Bronchoalveolar lavage fluid.

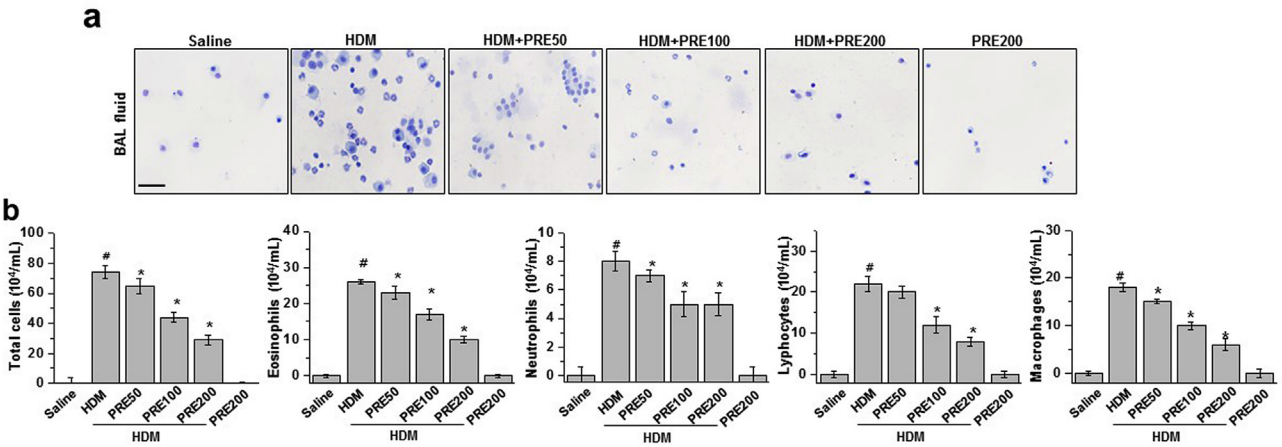

Fig. 3. Platycodon Radix extracts reduce cell counts in BALFs from HDM-challenged mice. BALFs were obtained 48 h after the final challenge from HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. BALFs were also obtained from mice that received 200 mg/kg PRE only. (a) BALFs were stained with Diff-Quick solution (100× magnification). (b) Total cells and specific cell types in BALFs (refer to Materials and Methods). Experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; BALFs, Bronchoalveolar lavage fluid.

treatment markedly inhibited SOD activity, this effect was alleviated by PRE and its active compounds, platycoside E and platycodin D_3 (Fig. 9c and d). These results suggest that the two active components protect the lung against HDM-induced damage by attenuating oxidative stress. Mitochondrial ROS are an abundant source of intracellular ROS (Gorlach et al., 2015; Sena and Chandel, 2012). MitoTracker Red (CM-H_2Xros), which stains the mitochondria in live cells upon oxidation (Lambeth, 2004), was applied to cells. As expected, HDM increased mitochondrial oxidative stress in BEAS-2B cells, as shown by the enhanced red fluorescence signal observed. This effect was attenuated by PRE and its active compounds, platycoside E and platycodin D_3 (Fig. 9e). To explain the relationship between ER stress and ROS, we applied N-acetyl cysteine (NAC, ER stress inhibitor), 4-PBA, and the NOX4 inhibitor GKT137831, to BEAS-2B cells. Intracellular and mitochondrial ROS were also inhibited by NAC, 4-PBA, and GKT137831, suggesting that ER stress and ROS are intimately involved in pulmonary inflammation controlled by PRE. ER localized NADPH oxidase-4 (NOX4) physically interacts with PDI under conditions of ER ROS accumulation (Lee et al., 2017). We thus speculated that NOX4 may be involved in ER stress. Indeed, both NOX4 protein expression and activity levels were increased in HDM-induced asthma and reversed by PRE and its active compounds, platycoside E, platycodin D_3 and NOX4 inhibitor (Fig. 9f and g). Collectively, our findings indicate that HDM sensitization induces ER stress involving NOX4 overexpression, which is controlled by PRE and its active components, in addition to GKT137831.

4. Discussion

The current study demonstrates that HDM-induced allergic airway inflammation, as indicated by increased cell infiltration (eosinophils) and abnormal lung histology, is inhibited in the presence of PRE. HDM-induced allergic airway inflammation and ER stress, including NF-κB-mediated pro-inflammatory cytokine expression and oxidative stress, is controlled by PRE and its major compounds, PLE and PLD. These results indicate that PRE, PLE, and PLD could represent novel therapeutics for dust mite-associated allergic inflammation.

One of the causative mechanisms of allergic airway inflammation and airway obstruction is oxidative stress, which is caused by disruption of the cellular redox state (Kim et al., 2011). Allergen-activated and recruited inflammatory cells including eosinophils, neutrophils, and macrophages from patients with asthma produce more ROS than those from healthy people (Teramoto et al., 1996). Constitutive airway cells are also a potential source of ROS (Rochelle et al., 1998). Intracellular sources of ROS and several asthma mediators including eosinophil granule proteins, chemokines, lipid mediators, and adhesion molecules, are potential stimuli of ROS production (Chihara et al., 1994; Nagata et al., 1999; Tenscher et al., 1996). Based upon the clinical relevance of ROS in severe inflammation, efforts to develop antioxidants and related anti-inflammatory agents, including those based on natural products, have been ongoing to control allergic airway inflammation (Agra et al., 2016; Rahal et al., 2014).

Throughout this study, we observed that PRE and its major compounds, Platycoside E and Platycodin D_3, inhibit allergic airway inflammation. In traditional Chinese medicines, *Platycodon grandiflorum* has been used as an antitussive and expectorant to treat sore throats, coughs, colds, tonsillitis, lung congestion cough and chest congestion (Nyakudya et al., 2014). *Platycodon grandiflorum* possesses antioxidant activity, suggesting that root extracts might also be utilized as an effective and safe antioxidant source (Li et al., 2015). In fact, extracts and active saponins from *Platycodon grandiflorum* root have been reported to possess a wide range of functions, including anti-inflammatory properties (Choi et al., 2009, 2017; Nyakudya et al., 2014). *Platycodon grandiflorum* is used to treat asthma, bronchitis, pulmonary tuberculosis, and inflammatory disease (Choi et al., 2009; Lee, 1973; Takagi and Lee, 1972). Recently, butanol and petroleum ether fractions of dried *Platycodon grandiflorum* extracts were shown to inhibit both cellular oxidation and NO production (Lee et al., 2004; Nyakudya et al., 2014). In the current study, inflammation was consistently attenuated by PRE and its major compounds, Platycoside E and Platycodin D_3. A reduced surface area harboring inflammatory infiltrates was observed in lungs from mice treated with PRE (Fig. 1). Histopathological alterations in lung tissue, such as goblet cell hyperplasia, mucin5AC hypersecretion, and inflammatory cell infiltration were also controlled by PRE. PRE and its major compounds, Platycoside E and Platycodin D_3, inhibits inflammation via regulating Th2 response by IL-4 and IL-13 in HDM-challenged allergic lung inflammation *in vivo* or *in vitro*. These cytokines are produced by a variety of cell types in the innate immune system, and they play a crucial role during asthma (Oeser et al., 2015). Cytokines generated by Th2 cells, including IL-4, IL-5, IL-9, and IL-13 play central roles in the initiation and maintenance of allergic inflammation (Finkelman and Urban, 2001). The development of airway eosinophilia and allergen-induced airway inflammation is associated with increased levels of IL-5 or IL-13 in BALF, consistent with development of a Th2-mediated allergic response. IL-5 is implicated in the persistence of inflammation and the mechanism linking inflammation

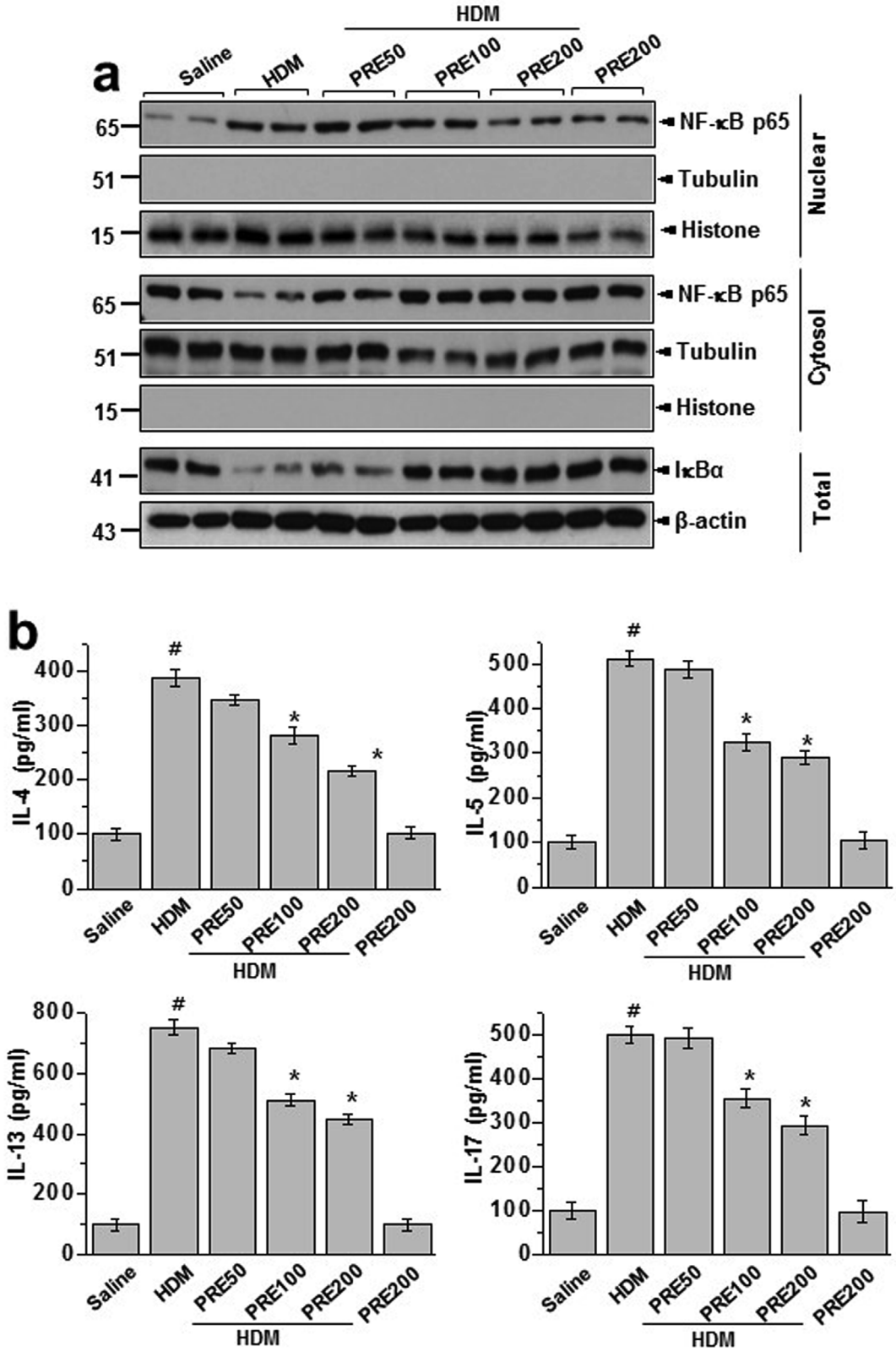

Fig. 4. Platycodon Radix extracts reduce inflammatory signaling in lungs and BALFs of HDM-challenged mice. Lung tissues and BALFs were obtained 48 h after the final challenge in HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. Lung tissues and BALFs were also obtained from mice that received 200 mg/kg PRE only. (a) Immunoblotting was performed to detect nuclear NF-κB p65/nuclear histone, cytosolic p65/cytosolic tubulin, and IκB-α/β-actin. (b) BALFs were collected and analyzed by ELISA for the cytokines IL-4, IL-5, IL-13, and IL-17. The experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts.

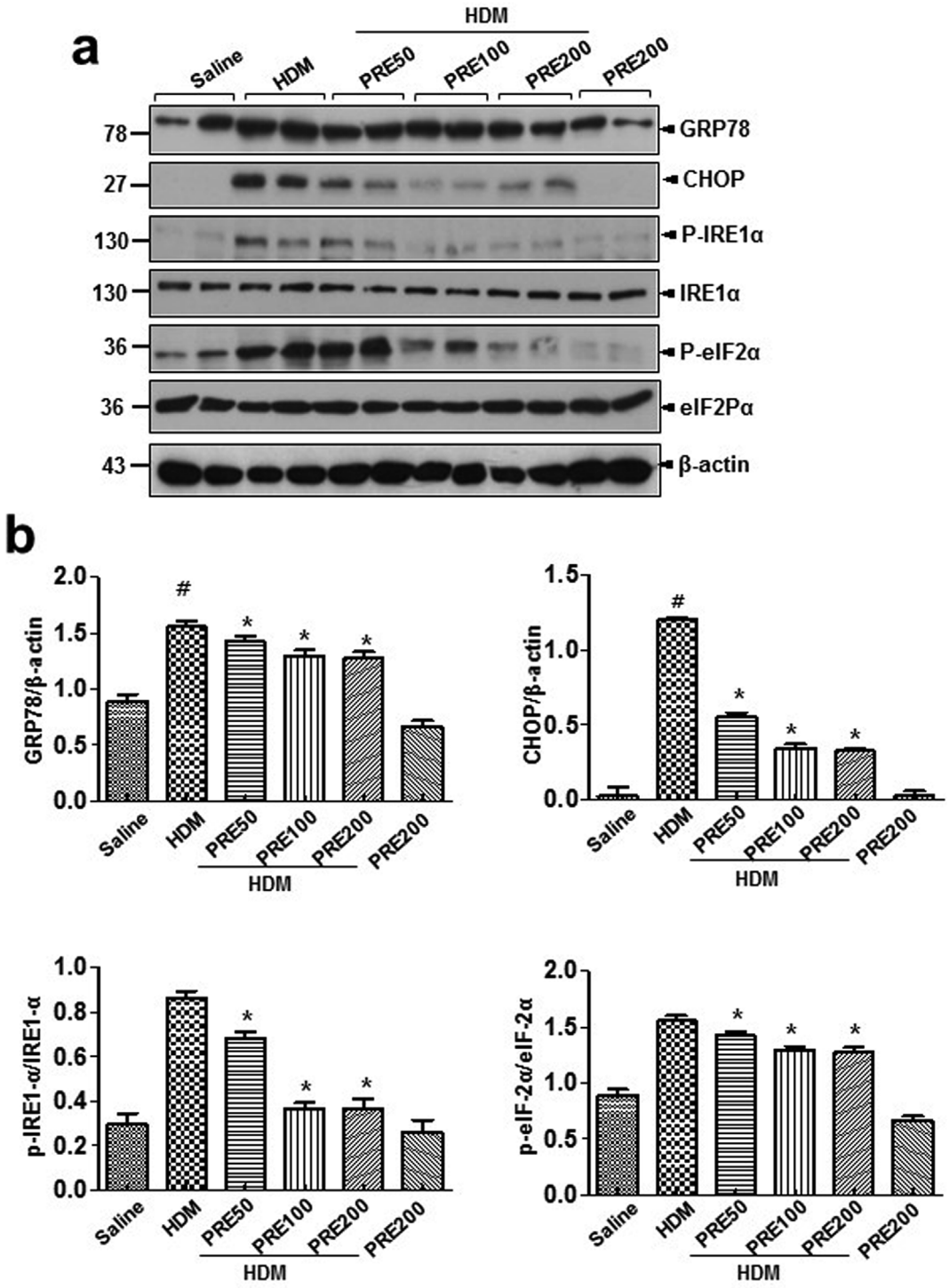

Fig. 5. Platycodon Radix extracts regulate ER stress in lungs of HDM-challenged mice. Lung tissues were obtained 48 h after the final challenge in HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. Lung tissues were also obtained from mice that received 200 mg/kg PRE only. (a) Immunoblotting was performed using antibodies against GRP78, CHOP, p-IRE1α, IRE1α, p-eIF2α, eIF2α, and β-actin. (b) Quantitative analysis of protein expression was also performed. The experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts.

to altered airway hyperresponsiveness. Our data show that PRE inhibits the pulmonary accumulation of eosinophils and decreases IL-5 levels in BALF. These results suggest that PRE plays a key role in blocking mucus secretion, and in partially blocking the recruitment of eosinophils to the lungs via inhibition of IL-4, IL-5, and IL-13. This study further revealed that PRE and its major compounds, Platycoside E and Platycodin D_3, substantially suppressed airway inflammation and ROS production in HDM-challenged mice or cells (Figs. 3 and 6). These observations suggest that airway inflammation, including bronchial asthma, might be controlled therapeutically by PRE and its major compounds, Platycoside E and Platycodin D_3, through a mechanism that includes regulation of ROS. At the cellular level, inflammatory cytokines, ROS and ER stress have been consistently observed (Figs. 7–9). More specifically, mitochondrial ROS were controlled by the chemical chaperone 4-PBA

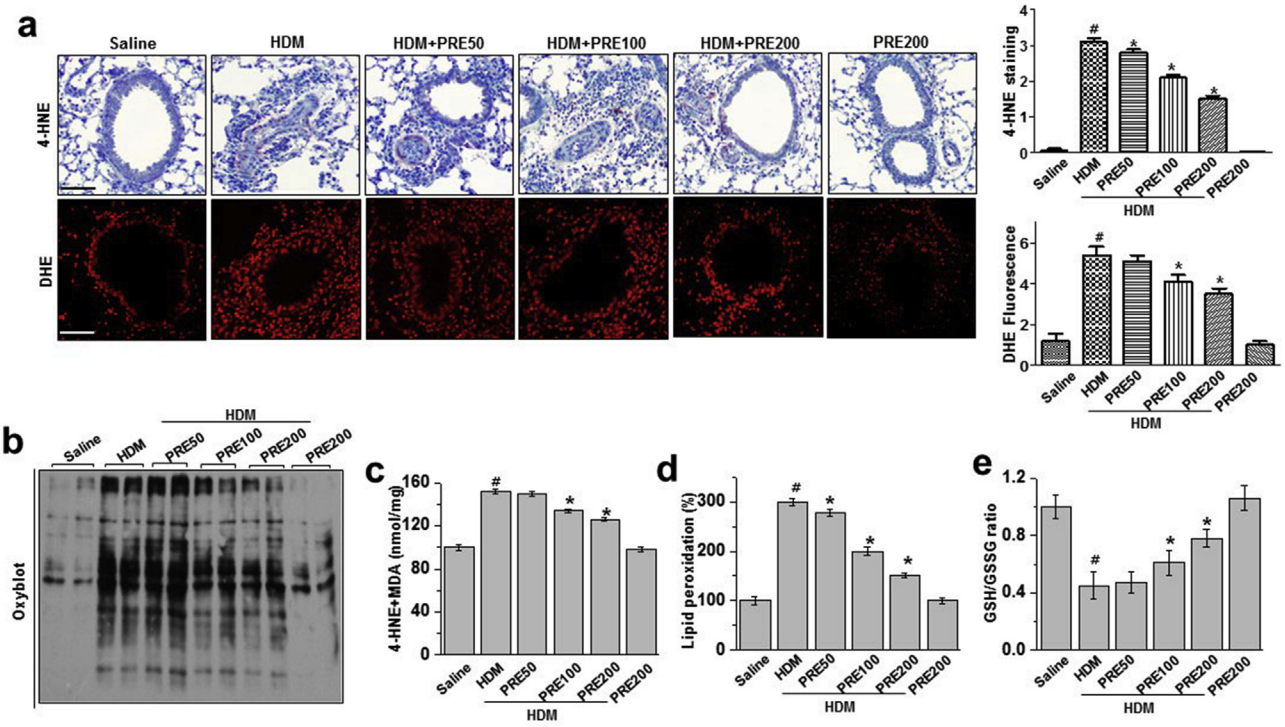

Fig. 6. Platycodon Radix extracts reduce lipid peroxidation and ROS production in the lungs of HDM-challenged mice. Lung tissues were obtained 48 h after final challenge in HDM-challenged mice administered saline or 50, 100, or 200 mg/kg PRE. Lung tissues were also obtained from mice that received 200 mg/kg PRE only. Staining assays to detect 4-HNE and dihydroethidine hydrochloride (DHE) were performed (a). Lysates from lung tissues were analyzed for oxidized proteins via OxyBlot analyses (b). Analyses of 4-HNE (c) and malondialdehyde (MDA) (d) were performed as described in the Materials and Methods. GSH/GSSG ratios in lung tissues were also analyzed (e). These experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; 4-HNE, 4-hydroxynonenal.

(Fig. 9e), strongly indicating that ER stress is amplified to the juxtaposed organelle, mitochondria, during inflammatory reactions. The ER plays an essential role in cellular homeostasis by facilitating protein folding and transport. ER stress is a condition triggered subsequent to the disruption of ER homeostasis (Cao et al., 2016), and is present in numerous inflammatory diseases including obesity, diabetes, autoimmune, infectious, and cardiovascular disease (Cao et al., 2016). In addition, HDM-induced allergic airway disease pathologies have been

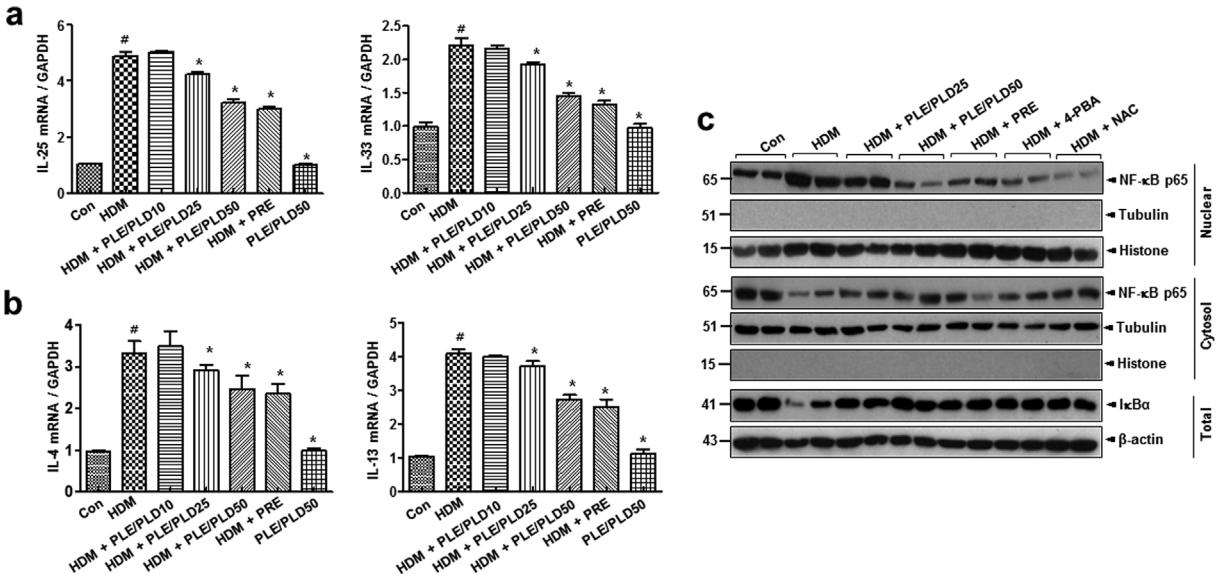

Fig. 7. Platycoside E and Platycodin D_3 modulate the effects of HDM on inflammatory gene expression in BEAS-2B lung epithelial cells. IL-25, IL-33 (a), IL-4, or IL-13 (b) expression levels in cells treated for 24 h with 200 μg/mL HDM in the presence or absence of a 10, 25, or 50 μg/mL mixture of PLE and PLD, 200 μg/mL PRE, 5 mM 4-PBA, or 2 mM NAC, were analyzed by RT-PCR assay. (c) Immunoblotting was performed to detect nuclear NF-κB p65/nuclear histone, cytosolic p65/cytosolic tubulin, and IκB-α/β-actin. Representative bands are shown from three independent experiments. Quantitative analysis of RNA expression was also performed. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; PLE, Platycoside E; PLD, Platycodin D_3; 4-PBA, 4-phenylbutyrate; NAC, N-acetylcysteine.

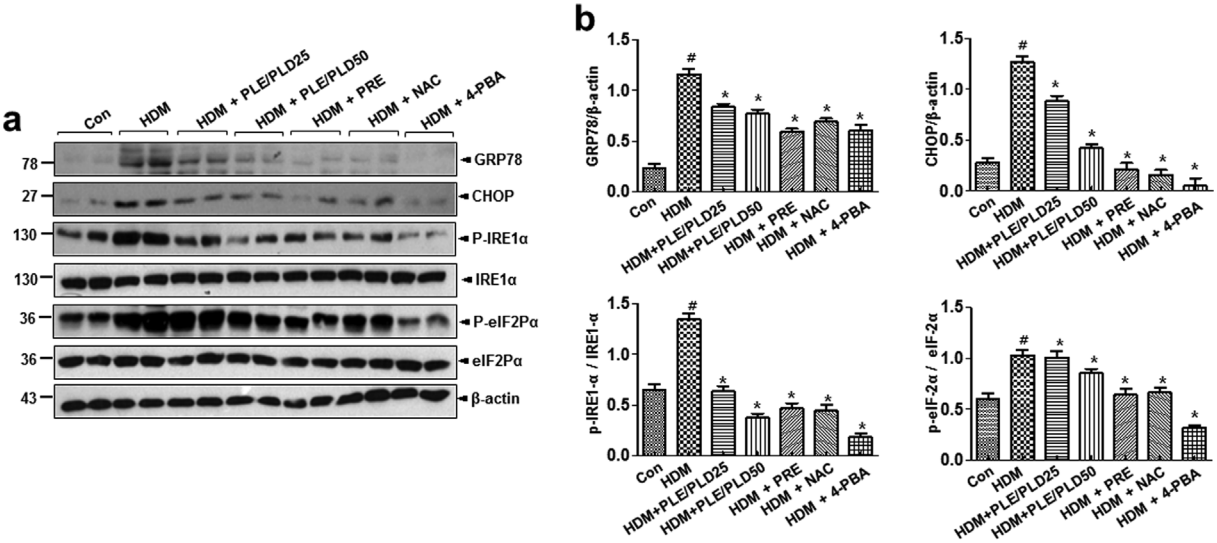

Fig. 8. Platycoside E and Platycodin D_3 regulate ER stress in BEAS-2B lung epithelial cells. Cells were treated with 200 μg/mL HDM in the presence or absence of a 25 or 50 μg/mL mixture of PLE and PLD, 200 μg/mL PRE, 5 mM 4-PBA, or 2 mM NAC for 24 h. (a) Immunoblotting was performed using antibodies against GRP78, CHOP, p-IRE1α, IRE1α, p-eIF2α, eIF2α, and β-actin. (b) Quantitative analysis of protein expression was also performed. The experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; PLE, Platycoside E; PLD, Platycodin D_3; 4-PBA, 4-phenylbutyrate; NAC, N-acetylcysteine.

linked to ER stress (Hoffman et al., 2013). Recent studies identified an airway epithelial-specific deletion of endoplasmic reticulum resident protein 57 (ERp57) is significantly decreased in HDM-induced airway inflammation, ER stress, and airway hyperresponsiveness (Hoffman et al., 2016). In our study, HDM-induced ER stress was consistently alleviated by PRE and its major compounds PLE and PLD. Considering that ER stress contributes to the pathogenesis of inflammatory diseases, the inhibition of ER stress might be a useful therapeutic strategy in the management of allergy. In this study, anti-inflammatory signaling allowed PRE to suppress HDM-associated asthma. Specifically, the HDM-induced nuclear translocation of NF-κB and the degradation of cytosolic IκB-α were shown to be inhibited by PRE and its major compounds, Platycoside E and Platycodin D_3 (Figs. 3a and 7c). PRE and its major compounds, Platycoside E and Platycodin D_3, likely suppress inflammatory responses by modulating the expression of NF-κB-associated genes. These results strongly suggest that PRE and its major compounds, Platycoside E and Platycodin D_3, protect against allergic airway inflammation, including pulmonary dysmetabolism, ER stress, and ROS accumulation in bronchial asthma.

Throughout this study, ER stress was first initiated, thereby activating ROS and inflammatory signaling. A positive feedback loop forms, wherein the accumulated ROS can induce ER dysfunction

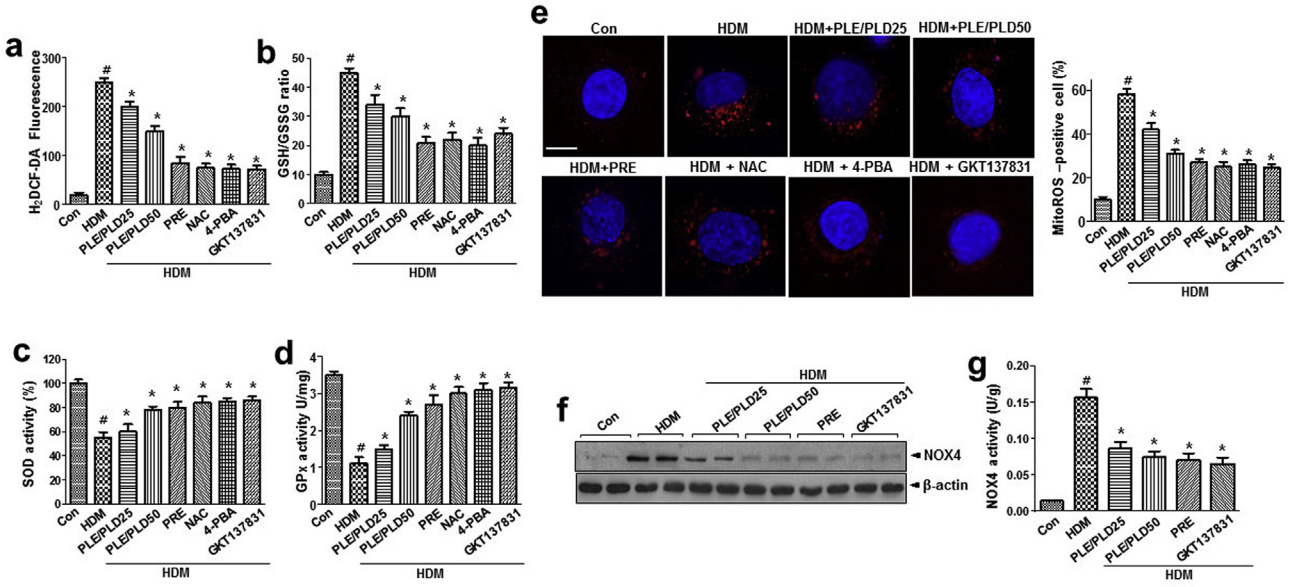

Fig. 9. Platycoside E and Platycodin D_3 regulate cellular or mitochondrial ROS production in BEAS-2B lung epithelial cells. Cells were treated with 200 μg/mL HDM in the presence or absence of a 25 or 50 μg/mL mixture of PLE and PLD, 200 μg/mL PRE, 2 mM NAC, 5 mM 4-PBA, or 10 μM GKT137831 for 24 h H_2DCF-DA (a), GSH/GSSG ratio (b), and the levels of SOD (c), GPx (d), and mtoSOX (e) were measured. (f) Immunoblotting was performed using antibodies against NOX4 and β-actin. (g) NOX4 activity was measured. The experiments were repeated three times using at least three different samples. Data are expressed as mean ± SEM ($^{\#}p < 0.05$ vs. saline; $^{*}p < 0.05$ vs. HDM). HDM, house dust mite; PRE, Platycodon Radix extracts; PLE, Platycoside E; PLD, Platycodin D_3; 4-PBA, 4-phenylbutyrate; NAC, N-acetylcysteine.

leading to further ER stress. The extract of Platycodon Radix and its major compounds, Platycoside E and Platycodin D_3, protect HDM-induced allergic airway inflammation, where ER stress and its associated ROS are involved. PRE treatment improved histopathological damage in the lungs of asthmatic mice, especially damage involving eosinophils. This study suggests that PRE and its major compounds, Platycoside E and Platycodin D_3, might be effective in preventing chronic, HDM-induced allergic airway inflammation and ER stress. Our results provide new insights into the potential pharmacologic benefits of PRE and its major compounds, Platycoside E and Platycodin D_3, including the ability to prevent allergic inflammation and bronchial asthma.

Conflicts of interest

The author(s) declare no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.001.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.001.

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