



Original Contribution

Ankaflavin, a novel Nrf-2 activator for attenuating allergic airway inflammation

Wei-Hsuan Hsu, Bao-Hong Lee, Yu-Chun Huang, Ya-Wen Hsu, Tzu-Ming Pan*

Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei, 10617, Taiwan

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ABSTRACT

The role of inflammation-induced oxidative stress in the pathogenesis and progression of chronic inflammatory airways diseases has received increasing attention in recent years. Nuclear factor-erythroid 2 related factor 2 (Nrf-2) is the primary transcription factor that regulates the expression of antioxidant and detoxifying enzymes. In this study, yellow pigment ankaflavin (AK), derived from *Monascus*-fermented products, elevated nuclear Nrf-2 protein translocation in both the A549 lung cell line and the lungs of ovalbumin (OVA)-challenged mice. Furthermore, AK increased the mRNA expression of antioxidant enzymes regulated by Nrf-2, leading to a reduction in allergen-driven airway inflammation, mucus cell hyperplasia, and eosinophilia in OVA-challenged mice. Additionally, AK prevented T-cell infiltration and Th2 cytokines including interleukin (IL)-4, IL-5, and IL-13 generation in bronchial alveolar lavage fluid. The adhesion molecules ICAM-1, VCAM-1, and eotaxin were substantially reduced by AK treatment. Importantly, the inhibitory effect of AK on adhesion molecule production and immune cell infiltration was abolished by Nrf-2 small interfering RNA. This is the first study to illustrate that AK acts as a novel Nrf-2 activator for modulating the oxidative stress pathway to improve the lung injury and ameliorate the development of airway inflammation.

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Introduction

The incidence of allergic airway diseases such as asthma is rising prodigiously in developed and developing countries. Allergic airway inflammation is a central feature of these diseases and involves a variety of inflammatory, structural, and epithelial cells of the airways. Airway epithelial cells play a key role in asthma and act as the first line of defense against allergens entering the respiratory system by triggering the release of inflammatory

mediators such as cytokines [1]. Reactive oxygen species (ROS) are generated in the airway epithelial cells in response to a variety of stimuli. In addition to releasing inflammatory cytokines and chemokines, airway epithelial cells express adhesion molecules on their cell surface. The production of ROS causes airway inflammation, which involves narrowing of airways and thickening of the epithelium; secretion of large amounts of mucus; and infiltration of eosinophils, T cells, and other inflammatory cells [2–5].

The transcription factor nuclear factor-erythroid 2 related factor 2 (Nrf-2) is involved in lung defense against oxidative injury. After translocation to the nucleus, Nrf-2 binds to antioxidant response elements (AREs) located in promoter regions of relevant genes and induces the transcription of numerous antioxidant and cellular defense genes, including NAD(P)H: quinine oxidoreductase (NQO1), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [6]. The roles of these enzymes and proteins in oxidative tissue stress have been widely discussed in the literature [7,8].

Under normal conditions, Nrf-2 is inactive and bound in the cytosol by Kelch-like ECH-associated protein 1 (Keap1). Nrf-2 can be activated by diverse stimuli, including oxidants, prooxidants, antioxidants, and chemopreventive agents. Nrf-2 can induce cellular rescue pathways against oxidative damage, abnormal inflammation, immune responses, apoptosis, and carcinogenesis [9,10], highlighting the essential protective role of Nrf-2 in the lung during disease. Disruption of the Nrf-2 gene leads to severe

Abbreviations: AK, ankaflavin; AREs, antioxidant response elements; BALF, bronchoalveolar lavage fluid; CAT, catalase; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescent; ESI-MS, electrospray ionization–mass spectrometry; FBS, fetal bovine serum; GABA, γ -aminobutyric acid; GPx, glutathione peroxidase; GST, glutathione S-transferase; H&E, hematoxylin and eosin; HO-1, heme oxygenase-1; HPLC, high-performance liquid chromatography; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; Keap1, Kelch-like ECH-associated protein 1; NAC, N-acetylcysteine; NF- κ B, nuclear factor- κ B; NMR, nuclear magnetic resonance; NQO-1, NAD(P)H: quinine oxidoreductase; Nrf-2, nuclear factor-erythroid 2 related factor 2; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid–Schiff; PPAR- γ , peroxisome proliferator activated receptor- γ ; PVDF, polyvinylidene difluoride; RMR, red mold rice; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; si-Nrf-2, Nrf-2 specific small interfering RNA; SOD, superoxide dismutase; Th2, T helper type 2; TLC, thin-layer chromatography; TNF- α , tumor necrosis factor- α ; UDG, uracil DNA glycosylase; VCAM-1, vascular cell adhesion molecule-1

* Corresponding author. Fax: +886 2 33663838.

E-mail address: tmpan@ntu.edu.tw (T.-M. Pan).

allergen-driven airway inflammation and hyperresponsiveness in mice and increased expression of the T helper type 2 (Th2) cytokines interleukin (IL)-4 and IL-13 in bronchoalveolar lavage fluid (BALF) and splenocytes after allergen challenge [11]. Nrf-2 also protects the lungs against the development of pulmonary fibrosis by regulating the cellular redox level and lung Th1/Th2 balance [12].

Fermentation products of the fungus *Monascus*, especially those produced by solid-state rice fermentation, have been historically used as food colorants and dietary material. *Monascus*-fermented rice, also known as red mold rice (RMR), is a common food and traditional health remedy in Asian countries. Several chemical components of RMR have been purified and identified, including monacolins, γ -aminobutyric acid (GABA), pigments, and dimerumic acid. Ankaflavin (AK) is a naturally occurring secondary metabolite with an azaphilone structure, isolated as a yellow pigment from *Monascus*-fermented products, and has cytotoxic [13], immunosuppressive [14], and anti-inflammatory [15] activities. AK also reduces tumor necrosis factor (TNF)- α -stimulated endothelial adhesiveness as well as downregulates intracellular ROS formation, nuclear factor (NF)- κ B activation, and vascular cell adhesion molecule-1 (VCAM-1) expression in human aortic endothelial cells [16].

We have demonstrated that AK has antiobesity potential through regulation of adipogenesis and lipolysis activity. In preadipocyte 3T3-L1 cells, AK inhibited cell proliferation and differentiation and decreased triglyceride accumulation by regulating transcription factors such as C/EBPs and PPAR- γ [17]. AK also acts as a hypolipidemic and high-density lipoprotein cholesterol-raising agent [18]. Given the reported antioxidant and anti-inflammatory properties, we speculated that AK could modulate the oxidative stress pathway in order to protect against lung injury and ameliorate the development of airway inflammation.

Materials and methods

Chemicals and reagents

Aluminum hydroxide, *N*-acetylcysteine (NAC), ovalbumin (OVA), and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries (Saitama, Japan). Ham's F-12K medium was purchased from HyClone Laboratories (Logan, UT, USA). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). The Bio-Rad protein assay dye was from Bio-Rad Laboratories (Hercules, CA, USA).

Ankaflavin isolation and purification

The *Monascus purpureus* NTU 568 culture strain was maintained on potato dextrose agar (PDA, Difco Co., Detroit, MI, USA) slanted at 10 °C and transferred monthly. The long-grain rice (*Oryza sativa*) was used to produce RMR using the method of solid-state culture [19]. Briefly, a 500-g substrate was soaked in deionized water for 8 h, and excess water was removed with a sieve. The substrate was autoclaved (Model HL-341, Gemmy Corp, Taipei, Taiwan) for 20 min at 121 °C in a "koji dish," which was made of wood with the dimensions of 30 × 20 × 5 cm. After cooling, the substrate was inoculated with a 5% (v/w) spore suspension and cultivated at 30 °C for 7–10 day. During the culturing stage, 100 ml of water was added daily to the substrate from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was extracted by 95% ethanol at 50 °C for 3 day. The crude extracts of RMR were

obtained after filtering and concentrating under reduced pressure, and then coated on silica gel and subjected to dry flash chromatography. Sufficient *n*-hexane was passed through the column to remove the oily hydrophobic materials. Extensive gradient elution was then employed using different ethyl acetate in *n*-hexane ratios to yield numerous fractions. Similar fractions were combined according to thin-layer chromatography (TLC), and the solvent was removed under reduced pressure. These fractions were further analyzed by high-performance liquid chromatography (HPLC), and then fractions with a similar single peak profile were combined, respectively. Finally, the fraction with the desired compound was concentrated to dryness. AK was obtained as described with some modification [15]. The effective compound was identified by nuclear magnetic resonance (NMR, Varian Gemini, 200 MHz, FT-NMR, Varian Inc., Palo Alto, CA, USA) and electrospray ionization–mass spectrometry (ESI-MS, FinniganMAT LCQ, Thermo Electron Co., Waltham, MA, USA) analysis [20] (Supplemental Fig. 1A and B).

Cell culture

A549, a human lung adenocarcinoma epithelial cell line, was obtained from the Bioresource Collection and Research Center (BCRC 60430; Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were maintained in Ham's F-12K medium supplemented with 10% FBS, streptomycin (100 mg/ml), and penicillin (100 U/ml) in a 5% CO₂ incubator at 37 °C. All experiments were performed when cells were 80–90% confluent.

Nuclear and cytosol protein extraction

Cells were collected for nuclear protein extraction according to the fractionation kit protocol supplied from BioVision (Mountain View, CA, USA). In brief, harvested cells were added 200 μ l of cytosol extraction buffer containing DTT and protease inhibitors. After vortex and 10 min incubation on ice, the extracts were centrifuged at 16,000g for 5 min at 4 °C and the supernatant was removed to separate the cytoplasmic fraction from nuclei. The nuclei pellets were then added with 100 μ l nuclei extraction buffer, vortexed briefly, and set on ice every 10 min for a total 40 min. After centrifuging at 16,000g for 10 min, the supernatant nuclear extracts were stored at –80 °C for future use. The protein concentration in the cell extract was determined using a Bio-Rad protein assay kit.

Immunoblot analysis

The samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE). The protein spots were electrotransferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with block buffer (PBS containing 0.05% of Tween 20 and 5% w/v nonfat dry milk) for 1 h, washed with PBS containing 0.05% Tween 20 (PBST) three times, and then probed with 1:1000 diluted solution of anti-Nrf-2 human antibodies (Epitomics Inc., Burlingame, CA, USA), and 1:500 diluted solution of anti-Nrf-2 mouse antibodies (Bioss, Woburn, MA, USA) overnight at 4 °C. In addition, the intensity of the blots probed with 1:1000 diluted solution of mouse monoclonal antibody to bind Lamin B (Santa Cruz Biotechnology Inc., SC, USA) was used as the control to ensure that a constant amount of protein was loaded into each lane of the gel. The membrane was washed three times each for 5 min in PBST, shaken in a solution of HRP-linked anti-rabbit IgG secondary antibody, and washed three more times each for 5 min in PBST. The expressions of proteins were detected by enhanced chemiluminescent (ECL) reagent (Millipore, Billerica, MA, USA).

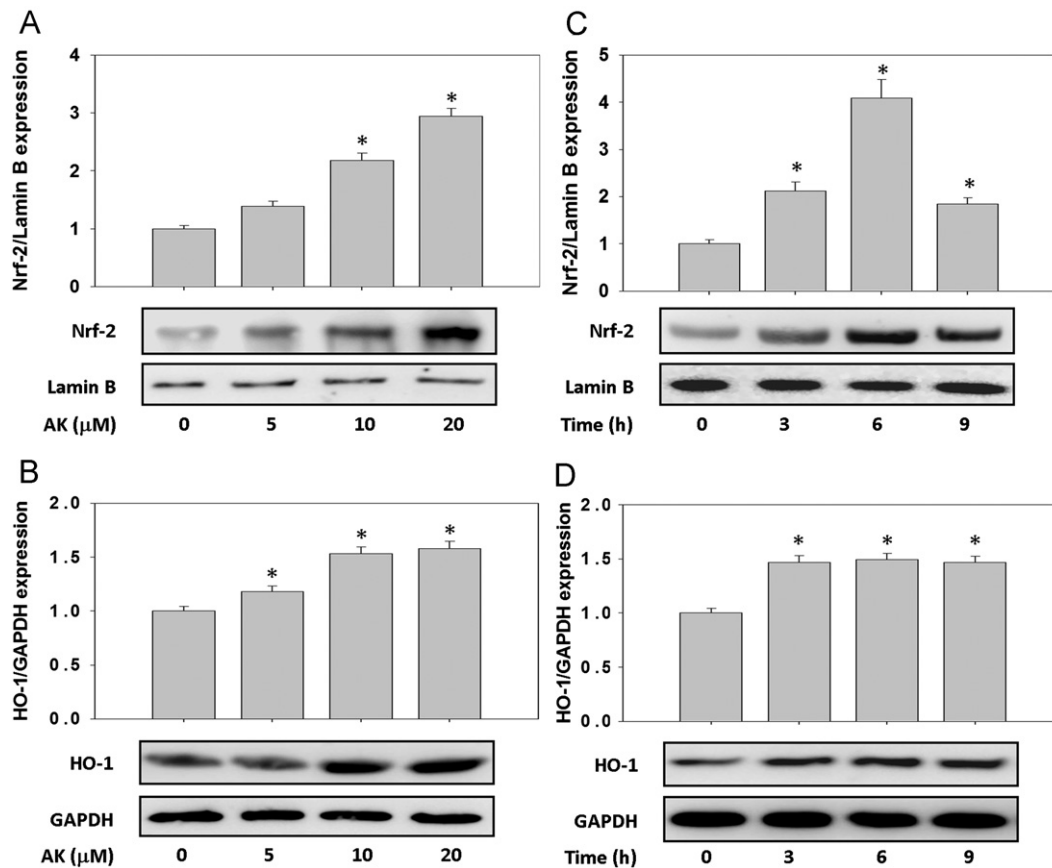


Fig. 1. AK upregulated Nrf-2 and HO-1 expression. (A, B) A549 cells were treated with different concentrations of AK for 6 h. (C, D) A549 cells were treated with 10 μM AK for 3, 6, and 9 h. Nuclear and cytosolic fractions were separated and prepared for Western blot assays. GAPDH or Lamin B was used as loading controls and the protein level in each group was normalized with GAPDH or Lamin B. Results are expressed as mean $\pm$ SD ($n=3$). *Significantly different from control group at $P < 0.05$.

Murine model of OVA-induced allergic airway inflammation

The experiment was carried out with female 6- to 8-week-old BALB/c mice obtained from National Laboratory Animal Center (Taipei, Taiwan). Mice were subjected to 12-h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C (protocol complied with guidelines described in the "Animal Protection Law," amended on Jan. 17, 2001 Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). Groups of 6 animals were used in all tests. Mice were sensitized on Days 0, 14, and 21 by intraperitoneal injection with 50 μg of OVA in 200 μl PBS mixed with 1 mg aluminum hydroxide as an adjuvant. On Days 22 to 26, mice were challenged by intranasal inhalations of OVA (100 μg) once a day and AK (10 mg/kg body weight) or NAC (200 mg/kg body weight) was administered by intraperitoneal injection per day from 12 h before the first challenge to the last challenge. In addition, siRNA knockdown of Nrf-2 in mice was performed. Nrf-2 specific small interfering RNA (si-Nrf-2) (5 nmol) (Santa Cruz Biotechnology) were given by intranasal inhalations on the day before the first challenge. The disease group of mice was immunized with OVA alone. Mice in the naive group were administered PBS. Mice were sacrificed at 24 h after last immunization (Supplemental Fig. 2A).

Collection of bronchoalveolar lavage

The tracheas of mice were cannulated and lavaged with two 0.8-ml aliquots of cold PBS. The collected fluid was called BALF, and absolute cell count for each cell type was calculated after determining the total cell count and differential cell count.

Lung histopathology

At 24 h after the last OVA challenge, mice were sacrificed and the lungs were fixed in 10% neutral buffered formalin. After fixation, 5-μm paraffin sections were stained with hematoxylin and eosin (H&E) for the evaluation of inflammatory cell infiltration, or with periodic acid–Schiff (PAS) for the identification of goblet cells. The slides were examined by a treatment-blind pathologist. The inflammatory score was graded on the following scale: 0, none; 1, minimal; 2, mild; 3, moderate; 4, severe. The number of PAS⁺ cells per millimeter of base membrane was measured for quantitative analysis.

RNA preparation and real-time PCR

Total RNA was isolated using Trizol (Life Technologies) according to the manufacturer's instructions. cDNA from 3 μg of RNA was generated using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer's instructions. The reverse-transcription product was diluted in water and a volume corresponding to 30 ng of original RNA was used for real-time PCR. Real-time PCR amplification and detection were performed using the SYBR Green qPCR SuperMix-uracil DNA glycosylase (UDG) with ROX (Life Technologies) in a fluorescence thermal cycler (StepOne Real-Time PCR System, Life Technologies) according to the manufacturer's protocol. Gene expression was normalized using β-actin as a reference gene. Relative mRNA expression levels were calculated following the $\Delta\Delta C_t$ method with the following primers: β-actin sense, 5'-CTAAGGCCAACCGTGAAAAG-3', and antisense, 5'-AGCCTG-GATGGCTACGTACAT-3', intercellular adhesion molecule-1 (ICAM-1)

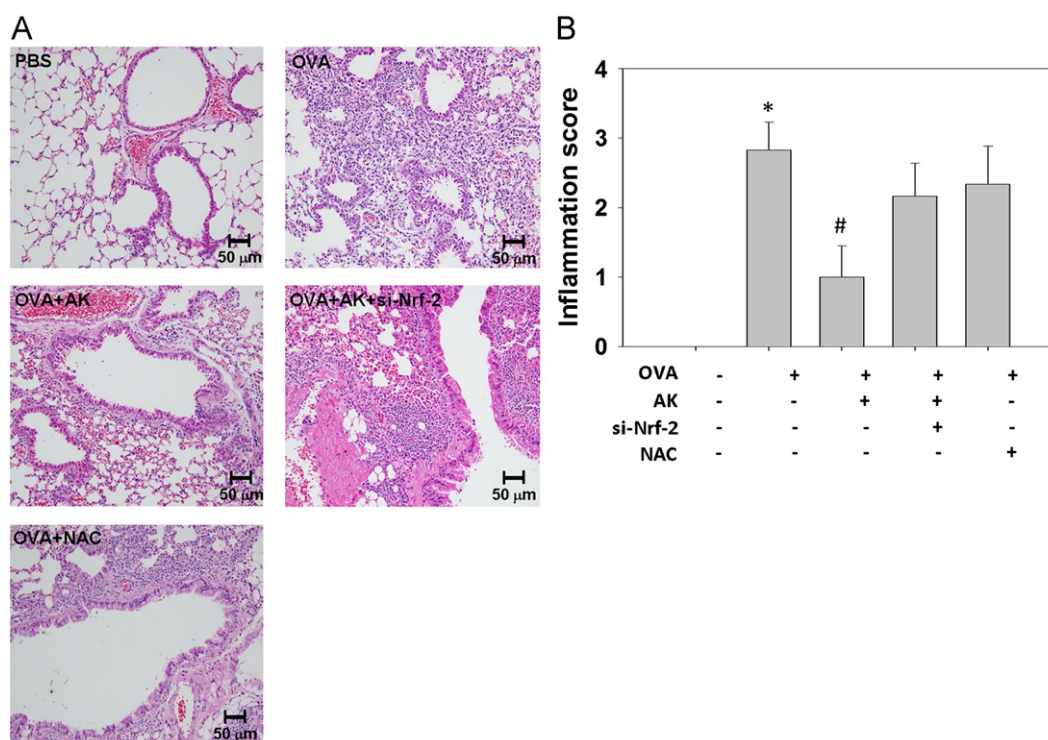


Fig. 2. Reduction of AK on infiltration of inflammatory cells into the lung tissues of OVA-challenged mice. (A) Lung tissues from mice were stained with H&E and examined by light microscopy (200 $\times$). (B) The inflammatory score was graded on the following scale: 0, none; 1, minimal; 2, mild; 3, moderate; 4, severe. Results are expressed as mean $\pm$ SEM ($n=6$). *Significantly different from control group at $P < 0.05$. #Significantly different from OVA-challenged group at $P < 0.05$. si-Nrf-2, siRNA for Nrf-2.

sense, 5'-CTGGCTGTACAGAACAGGA-3', and antisense, 5'-AAAGTAGGTGGGGAGGTGCT-3', VCAM-1 sense, 5'-CCCAAGGATCCAGAGATTCA-3', and antisense, 5'-TAAGGTGAGGGTGGCATTTC-3', eotaxin sense, 5'-GAGCCTAAGAACTGCTTGATTCCT-3', and antisense, 5'-GGGCGACTGGTGCTGATATT-3', Nrf-2 sense, 5'-ACACGGTCCACAGCTCATC-3', and antisense, 5'-TGCAATCAAATCCATGTCCTG-3', HO-1 sense, 5'-CAGCCCCACCAAGTTCAAAC-3', and antisense, 5'-AGGCGGTCTTAGCCTCTTCTG-3', NQO-1 sense, 5'-AGGAAGAGCTAATAAATCTCTTCTTGCTG-3', and antisense, 5'-TCATAITGCAGATGTACGGTGTG-GATTTAT-3', glutathione S-transferase (GST) sense, 5'-AAGCCAGGACTCTCACTA-3', and antisense, 5'-AAGGCAGTCTTGGCTTCT-3' (PREMIER Biosoft Int., Palo Alto, CA, USA). All amplifications were conducted within the linear range of the assay, normalized to respective GAPDH levels using SPSS Version 17.0 (SPSS Institute, Inc., Chicago, IL, USA).

Flow cytometry analysis

BALF cell pellet was resuspended and stained with anti-CD4 antibody (Invitrogen, Carlsbad, CA, USA) and anti-CD8 antibody (BioLegend, San Diego, CA, USA) and analyzed by flow cytometry using a FACScan-LSR flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA, USA).

Assay for cytokines

Supernatant derived from the BALF was stored at -80°C until measurement. ELISA experiments were performed according to the manufacturer's instructions. The levels of IL-4, IL-5, and IL-13 in BALF supernatants were determined with ELISA kits from eBioscience (San Diego, CA, USA).

Thiobarbituric acid-reactive substances (TBARS) assay

To determine lipid peroxidation, the TBARS assay was used. Lung tissue samples were crushed in RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 5 mM iodoacetamide, 1% bovine hemoglobin, and 0.025% NaN_3). The suspension was sonicated three times for 30 s and centrifuged at 3000g for 10 min at 4°C . One hundred microliters of supernatant was added to 200 μl of ice-cold 10% trichloroacetic acid to precipitate proteins and incubated for 15 min on ice. The samples were centrifuged at 2200g for 15 min. Two hundred microliters of supernatant was added to an equal volume of 0.67% thiobarbituric acid and incubated in a boiling water bath for 10 min. Samples were measured at 532 nm.

Statistical analysis

The statistical significance was determined by one-way analysis of variance (ANOVA) using the general linear model procedure of SPSS software (SPSS Institute, Inc.), followed by ANOVA with Duncan's test. The results were considered to be statistically significant if the P value was < 0.05 . Data are expressed as the mean $\pm$ SD or SEM.

Results

AK facilitates Nrf-2 translocation in A549 cells

Transcription factors that regulate antioxidant genes have been recently studied in diseases. Nrf-2 is regarded as a master regulator of antioxidant transcription [6]. After translocation to the nucleus, Nrf-2 binds to the ARE in the promoter of target

antioxidant genes and strongly regulates its transcription to abate lung oxidative injury in mouse models [11,21,22].

We first evaluated the regulatory potential of the antioxidation candidate AK (Supplemental Fig. 2B) on protein expression of Nrf-2 and antioxidant enzyme HO-1 in human lung epithelial cells. After treatment with AK for 6 h, the translocation of Nrf-2 from cytoplasm into nucleus increased in a dose-dependent manner (Fig. 1A). Furthermore, expression of HO-1, a downstream molecule of Nrf-2, also increased following AK treatment (Fig. 1B). We then investigated Nrf-2 and HO-1 expression after AK treatment for 3, 6, and 9 h. The translocation of Nrf-2 was markedly increased following AK treatment and was most prominent 6 h after treatment (Fig. 1C). Similarly, HO-1 expression was also enhanced by treatment with AK (Fig. 1D). These findings provide a preliminary indication that AK may protect airway epithelial cells against oxidative injury.

AK ameliorates allergen-induced airway inflammation *in vivo*

We next determined whether AK could modify ongoing OVA-induced airway inflammation *in vivo*. Real-time PCR analysis data showed that the expression of Nrf-2 was distinctly decreased in the Nrf-2 knockdown group (Supplemental Fig. 3). Histopathology revealed inflammatory cell infiltration into the airways of OVA-challenged mice. However, less inflammatory cell infiltrates were found in OVA-treated mice receiving AK compared with OVA-challenged mice. Interestingly, cellular infiltration was still evident in the OVA-treated mice that received a combination of AK and Nrf-2 siRNA. Infiltration of inflammatory cells was only slightly reduced in OVA-challenged mice following NAC treatment (Fig. 2).

Mucus hypersecretion is characteristic of airway inflammation. To assess if AK suppressed mucus overproduction as a result of goblet cell hyperplasia in OVA-challenged mice, we stained lung sections with PAS. Bright violet staining indicative of mucus

overproduction was clearly observed in the bronchial airways of OVA-challenged mice compared with PBS-treated control mice (Fig. 3). In contrast, mucus staining was markedly diminished in AK- or NAC-treated mice compared with OVA-challenged mice, but mucus overproduction was still observed in OVA-challenged mice treated with AK plus Nrf-2 siRNA and was higher than that in OVA-challenged mice treated with AK alone (Fig. 3).

AK decreases adhesion molecules expression in the lung

Leukocytes are recruited into tissues by adhesion molecules and chemokines. Adhesion molecule expression is induced in the endothelium or epithelium by several mediators, including cytokines or ROS, in a variety of chronic inflammatory conditions [23].

We sought to further elucidate the relationship between AK-regulated inhibition of adhesion molecule production and the resulting effect on the expression of Nrf-2. Fig. 4A revealed that OVA-challenged mice treated with or without Nrf-2 siRNA had substantial levels of adhesion molecules in the lung compared with PBS-challenged mice. AK reversed the elevation of VCAM-1, ICAM-1, and eotaxin levels induced by OVA, whereas treatment with NAC did not increase the mRNA abundance of adhesion molecules. The ameliorative effect of AK on adhesion molecule expression was abated by siRNA for Nrf-2. Taken together with the findings outlined above, this would suggest that the beneficial effect of AK against oxidative damage in the lung is mediated by Nrf-2 activity (Fig. 4A).

AK-mediated reduction of inflammatory cell infiltration and Th2 cytokine production

We also examined eosinophilia in BALF. BALF was collected 24 h after the last OVA challenge through intranasal instillation. Total and differential cell counts were performed. As illustrated in Fig. 4B, OVA-challenged mice showed significantly increased

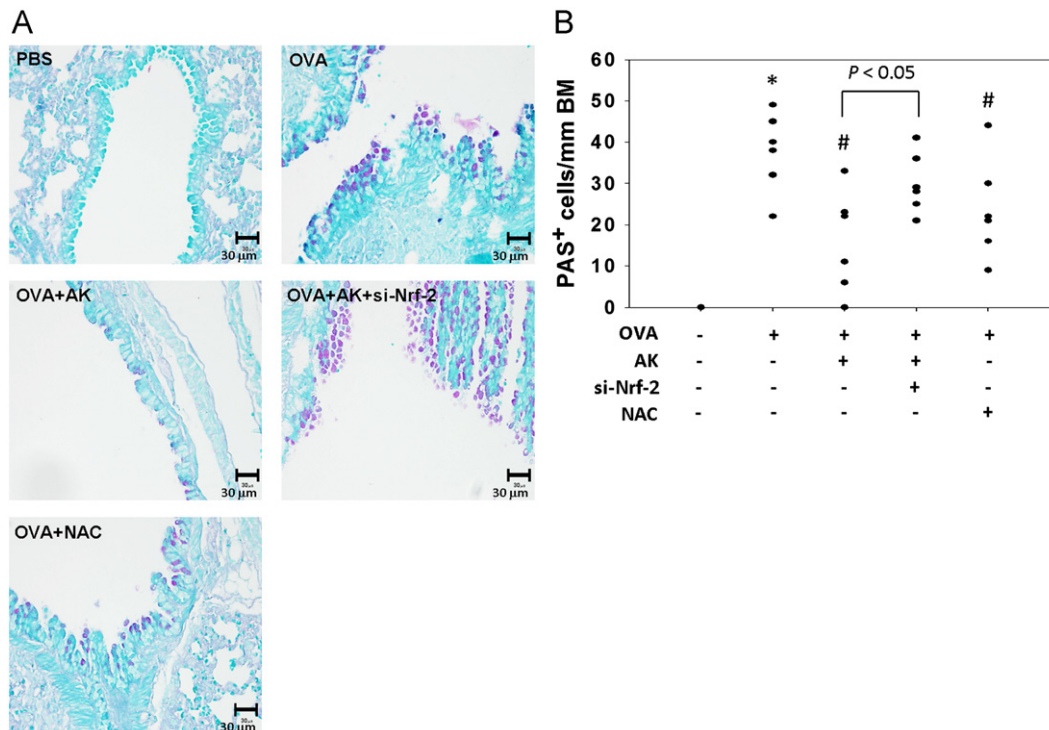


Fig. 3. Suppression of AK on hyperplasia of goblet cells into the lung tissues of OVA-challenged mice. (A) Lung tissues from mice were stained with PAS and examined by light microscopy (400 $\times$). (B) Quantitative analysis for the number of PAS-positive cells per mm of base membrane. Results are expressed as mean $\pm$ SEM ($n=6$). *Significantly different from control group at $P < 0.05$. #Significantly different from OVA-challenged group at $P < 0.05$. PAS, periodic acid–Schiff; si-Nrf-2, siRNA for Nrf-2.

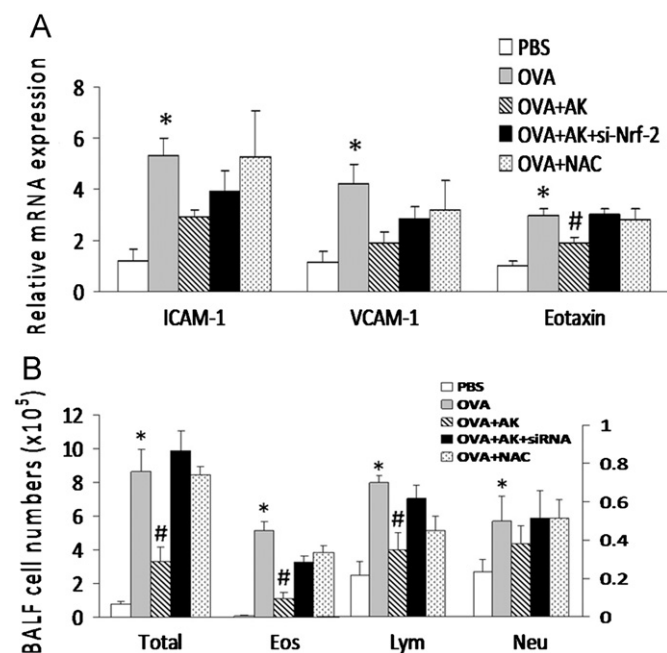


Fig. 4. Attenuation of AK in the expression of inflammatory mediators and immune cell infiltration. (A) Real-time PCR analysis of adhesion molecules expression in mouse lung tissue. GAPDH was used as a control. (B) Effects of AK on total cells and differential cellular components in BALF. The left axis represents total cell and eosinophil populations, whereas the right axis represents lymphocyte and neutrophil populations. Results are expressed as mean $\pm$ SEM ($n=6$). *Significantly different from control group at $P < 0.05$. #Significantly different from OVA-challenged group at $P < 0.05$. Eos, eosinophil; Lym, lymphocyte; Neu, neutrophil; si-Nrf-2, siRNA for Nrf-2.

numbers of total cells in BALF ($P < 0.05$) as compared to the PBS group or mice challenged with OVA in combination with AK treatment. Moreover, the numbers of lymphocytes and neutrophils were lower in AK-treated OVA-challenged mice than in mice challenged with OVA alone. In particular, the number of eosinophils was significantly reduced in OVA-challenged mice treated with AK compared with OVA-challenged mice alone ($P < 0.05$). However, total cell and eosinophil numbers were higher in OVA-challenged mice treated with a combination of AK and Nrf-2 siRNA than in OVA-challenged mice treated with AK alone, and antioxidant NAC treatment could not avoid cell and differential cell numbers in BALF (Fig. 4B).

Numerous studies performed with pulmonary inflammatory animal models have shown that the levels of Th2 cytokines increased within the affected airways [3,12,24]. Moreover, Th2 cells together with other inflammatory cells such as eosinophils and mast cells play a major role in the initiation and pathological development of airway inflammation [24]. We therefore examined Th2 cell levels in the BALF by flow cytometry. CD4⁺ and CD8⁺ T cells were clearly visible and abundant in OVA-challenged mice compared to the control mice. Conversely, T-cell infiltration was diminished in OVA-challenged mice treated with AK when compared with OVA-challenged mice, which was similar to the control mice, and this effect depended on the presence of Nrf-2. T-cell infiltration was slightly reduced in the NAC-treated group compared to the OVA-challenged group (Fig. 5). Moreover, Th2 cytokine production (including IL-4, IL-5, and IL-13) was significantly ($P < 0.05$) increased in OVA-challenged mice, while OVA-challenged mice further treated with AK had lower concentrations of Th2 cytokines in their BALF. The beneficial effect of AK treatment was again lost when OVA-challenged mice were treated with a combination of AK and Nrf-2 siRNA. Th2 cytokines secretion was only mildly reduced in

the NAC-treated group compared to the OVA-challenged group (Fig. 6). Collectively, these data indicate that AK treatment blocks allergic airway inflammation and further supports the hypothesis that the action of AK is dependent on Nrf-2.

AK acts as a novel antioxidant via Nrf-2 activation

Because increased oxidation can cause airway tissue damage by lipid degradation, we evaluated lipid peroxidation in lung homogenates using a TBARS assay. We found an increase in TBARS levels in OVA-challenged mice compared to control mice. The data suggested that AK or NAC treatment showed a decrease in TBARS levels compared to OVA-treated mice, whereas the effect of AK treatment was blocked when OVA-challenged mice were treated with a combination of AK and Nrf-2 siRNA.

To prove the involvement of Nrf-2 and downstream antioxidant enzyme activation in AK stimulation, we next investigated Nrf-2 expression following AK treatment in the lung by Western blotting. AK administration dramatically promoted Nrf-2 translocation, whereas this effect was abolished in Nrf-2 siRNA-treated mice (Fig. 7A). In addition, NAC treatment also elevated Nrf-2 expression; however, Nrf-2 activation occurred to a lesser extent in the NAC-treated group than in the AK-treated group (Fig. 7A). The effect of AK on the mRNA expression of Nrf-2 downstream genes in lung tissues of control and experimental groups of mice is depicted in Fig. 7B. The mRNA expression of HO-1, NQO-1, and GST increased in the lungs of OVA-challenged mice treated with AK and comparatively decreased in OVA-challenged mice treated with a combination of AK and Nrf-2 siRNA.

Discussion

Airway inflammation is central to the pathogenesis of asthma and other airway diseases such as chronic obstructive pulmonary disease. Epithelial cells are now known to play a central role in regulating airway inflammatory status, structure, and function in normal and diseased airways. Leucocytes, especially eosinophils, preferentially accumulate at sites of allergen-induced inflammation in the lung and are believed to play important roles in the pathophysiology of airway inflammation through the release of a variety of inflammatory mediators, including ROS and cytokines [5]. In the pathogenesis of asthma, eosinophil migration into the lung is dependent on adhesion molecules [25]. Accumulating evidence has suggested that eosinophil interaction with epithelial cells via the adhesion molecule pathway is likely to be a key step for selective eosinophil recruitment [25]. In this study, we have shown that AK modulates the expression of adhesion molecules, and we have observed and identified protein targets that are regulated by AK in allergen-induced oxidative stress. Further, we also determined that AK could regulate Nrf-2 activation to attenuate airway inflammation in OVA-challenged asthmatic mice.

The role of inflammation-induced oxidative stress in the pathogenesis and progression of chronic inflammatory airways diseases such as asthma has received increasing attention in recent years. Inflammatory airway disease is linked to increased generation of ROS and impaired antioxidant defenses, and as such, oxidative stress can be an afferent as well as efferent component of airway inflammation [2]. Nrf-2 is the primary transcription factor that regulates the expression of phase II antioxidant and detoxifying enzymes. Studies suggest that Nrf-2 has indirect modulatory effects on various immune and inflammatory pathways [26]. A lack of Nrf-2 protein expression exacerbates lung toxicity in response to multiple oxidative insults including allergens, hyperoxia, mechanical ventilation, viruses,

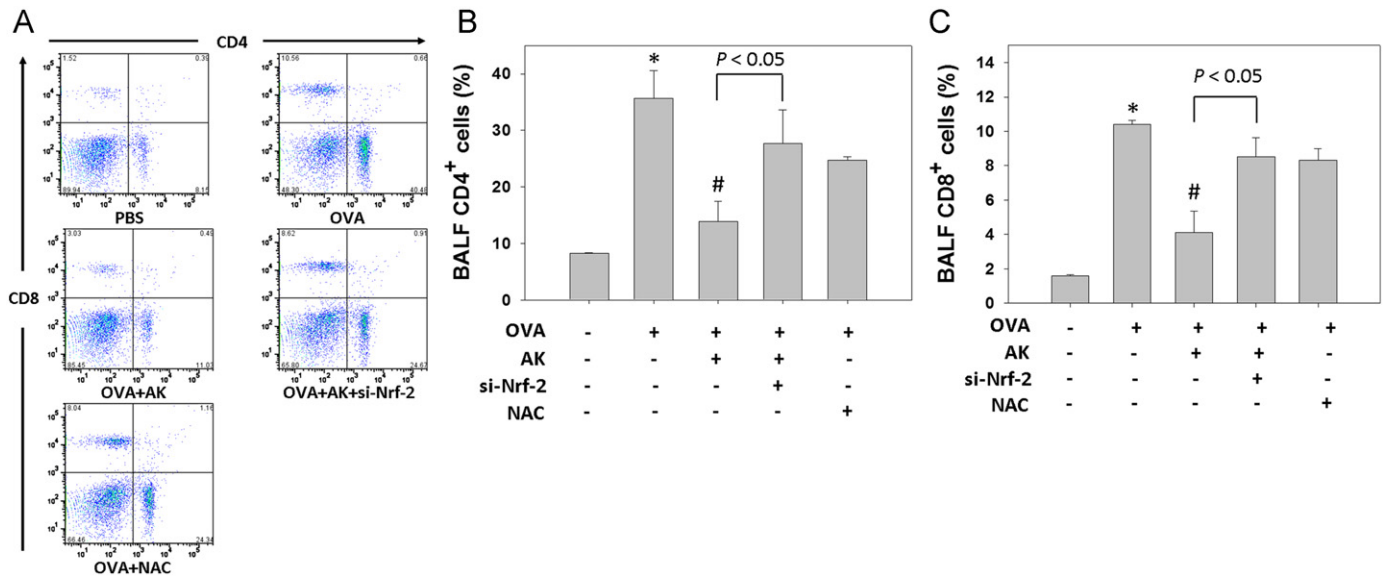


Fig. 5. AK-mediated inhibition of Th cell infiltration into BALF. (A) Analysis of Th cell infiltration was performed by flow cytometry. Cells in the lower right quadrant represented CD4-positive cells and in the upper left quadrant represented CD8-positive cells. Densitometric analysis of the percentage of (B) CD4- and (C) CD8-positive cells. Results are expressed as mean $\pm$ SEM ($n=6$). *Significantly different from control group at $P < 0.05$. #Significantly different from OVA-challenged group at $P < 0.05$. si-Nrf-2, siRNA for Nrf-2.

bacterial endotoxins, other inflammatory agents, as well as environmental pollution [6]. Pulmonary Nrf-2 expression is found mainly in the epithelium and alveolar macrophages [10]. A recent study confirmed that Nrf-2 plays a central role in the expression of survival and inflammatory genes in the lungs and might act as a critical mediator of cellular adaptation in response to proinflammatory and other noxious stimuli [27]. Disruption of the *Nrf-2* gene leads to severe allergen-driven airway inflammation and hyperresponsiveness in mice. Notably, *Nrf-2* disruption resulted in an increased expression of the Th2 cytokines IL-4 and IL-13 in BALF and splenocytes after allergen challenge [11], indicating an important protective role of the Nrf-2 pathway in allergic and asthmatic responses *in vivo*. Indeed, recent studies employing a model of mouse dendritic cells exposed to environmental particulate matter [28] and ragweed allergen [29] suggested that Nrf-2 protects against allergic airway inflammation in humans *in vivo*.

Our findings show that AK elevated Nrf-2 protein translocation from the cytoplasm to the nucleus, in both A549 cells and lung tissue of OVA-challenged mice. The result indicated a reduction in allergen-driven airway inflammation. OVA-challenged mice displayed pronounced mucus cell hyperplasia and infiltration of eosinophils into the lungs compared with the PBS-challenged control mice. There is now strong evidence that eosinophils play an essential role in the pathogenesis of allergic airway inflammation [25], and excess production of mucus and increased goblet cell differentiation are characteristic of allergic airway inflammation. Our present data showed that AK significantly reduced the numbers of eosinophils in BALF. Furthermore, AK prevented T-cell infiltration and generation of Th2 cytokines IL-4, IL-5, and IL-13 in BALF via a Nrf-2-dependent mechanism. In addition, the level of the adhesion molecules ICAM-1, VCAM-1, and eotaxin were substantially reduced. The effect of AK on adhesion molecule production and immune cell infiltration was also found to be Nrf-2 dependent.

Dietary or pharmacological antioxidants that enhance nonezymatic defenses have been extensively investigated in lung disease. Growing evidence suggests that the health benefits of dietary compounds such as curcumin [30], quercetin [31], and catechin [32] are dependent on the activation of Nrf-2 signaling.

It has been demonstrated that resveratrol, a polyphenolic phytoalexin, induces GSH synthesis by activation of Nrf-2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells [33]. The antioxidant vitamin E has been reported to prevent allergen-induced suppression of Nrf-2 and the resulting inflammation in alveolar macrophages in patients with atopic asthma [34]. Hence, the restoration of antioxidant and detoxifying enzymes by Nrf-2 provides an attractive therapeutic approach to target airway inflammation. In our study, OVA-challenged mice exhibited increased TBARS levels, whereas AK-treated mice displayed a significant decrease in TBARS levels. The inhibitory effect of AK on oxidative stress was dependent on Nrf-2, suggesting that the regulation of AK on antioxidant may be via Nrf-2 activation.

Monascus-fermented products have been used as traditional foods in Asia for several centuries. The yellow pigment AK derived from *Monascus* is a secondary metabolite with an azaphilone structure and has been reported to have cytotoxic [13], immunosuppressive [14], and anti-inflammatory activities [15]. Our group previously found that the yellow pigment from *Monascus*-fermented products can exhibit antidiabetic activity; it increases PPAR- γ mRNA expression and prevents PPAR- γ phosphorylation, thereby increasing uptake of the deoxyglucose analog 2-NBDG in C2C12 myotubes [35]. We also demonstrated that AK has antiobesity potential, through regulation of adipogenesis and lipolysis activity. In preadipocyte 3T3-L1 cells, AK inhibited cell proliferation and differentiation and decreased triglyceride accumulation by suppressing transcription factors such as C/EBPs and PPAR- γ [17]. AK also acts as a hypolipidemic and high-density lipoprotein cholesterol-raising agent [18]. In the present study, we found that AK exerts anti-inflammation potential via Nrf-2 activation. Real-time PCR data showed that antioxidant enzymes downstream of Nrf-2 were upregulated by AK, suggesting that AK increased Nrf-2 functionality. In addition, NAC treatment elicited a small increase in Nrf-2 expression, suggesting that NAC may also have the ability to activate Nrf-2 in a high dose. This would explain why airway inflammation was slightly reduced in OVA-challenged mice following NAC treatment.

In conclusion, these results provide a clear link between airway inflammation and defective Nrf-2 signaling. Nrf-2 is

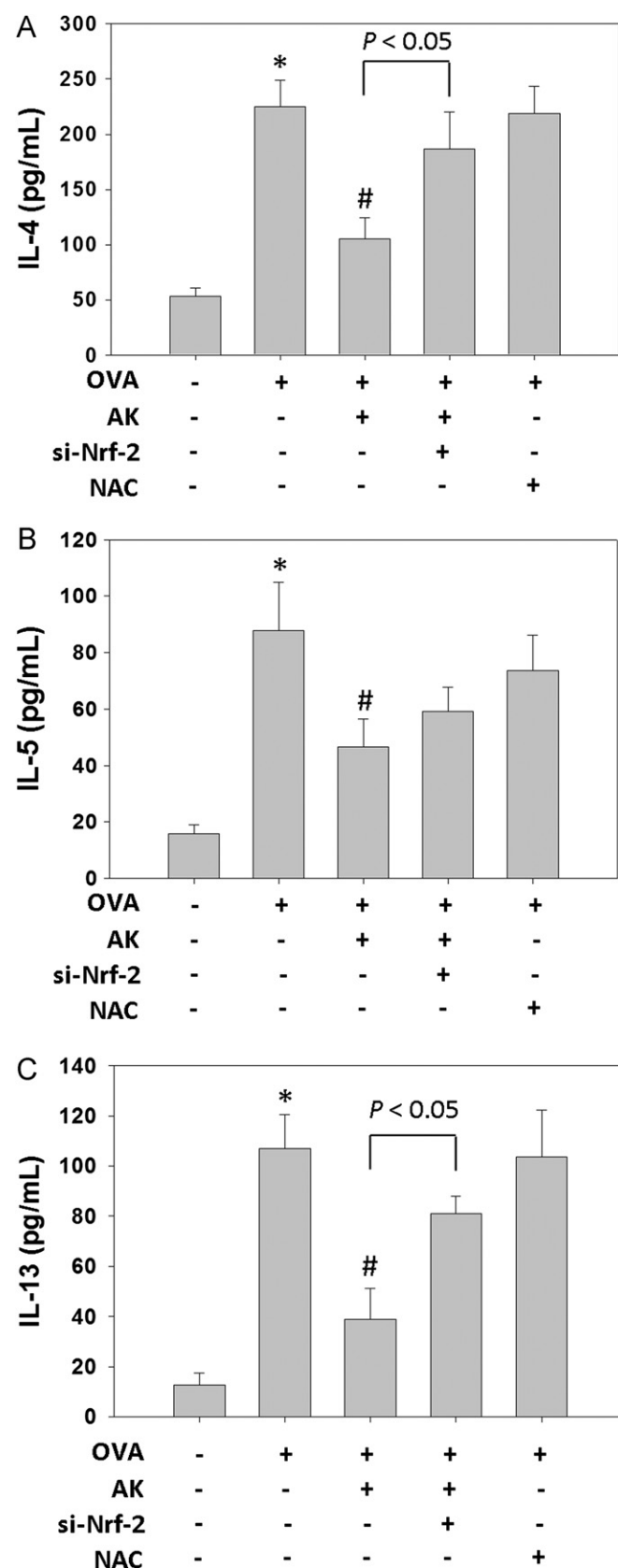


Fig. 6. AK diminished allergen-induced inflammatory cytokine production. The supernatants of BALFs were collected for measurement of (A) IL-4, (B) IL-5, and (C) IL-13 levels by ELISA. Results are expressed as mean $\pm$ SEM ($n=6$). *Significantly different from control group at $P<0.05$. #Significantly different from OVA-challenged group at $P<0.05$. si-Nrf-2, siRNA for Nrf-2.

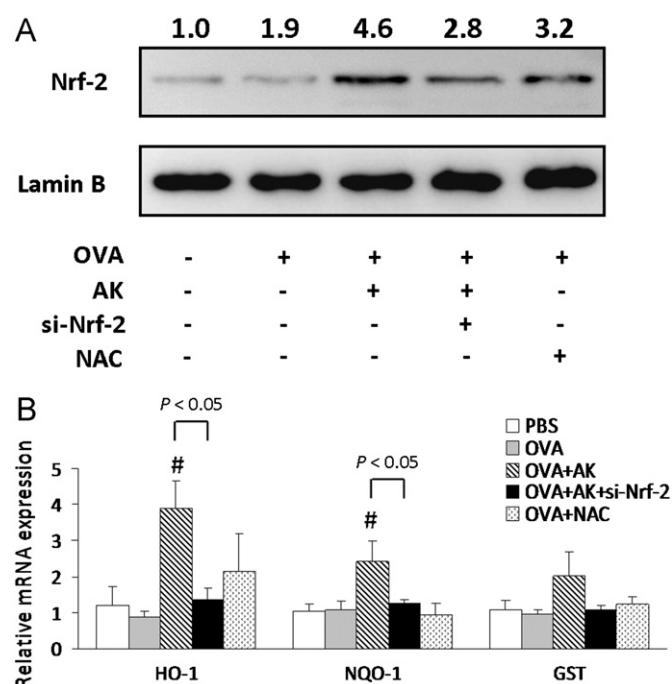


Fig. 7. Effect of AK on the expression of Nrf-2 and downstream antioxidant enzymes in lung tissues. (A) Elevation of AK on Nrf-2 expression in nuclear protein extracts from lung tissues. (B) Real-time PCR analysis of antioxidant enzymes expression in the lung tissue of mice. GAPDH was used as a control. Results are expressed as mean $\pm$ SEM ($n=6$). #Significantly different from OVA-challenged group at $P<0.05$. HO-1, heme oxygenase-1; NQO-1, NAD(PH): quinone oxidoreductase; GST, glutathione S-transferase; si-Nrf-2, siRNA for Nrf-2.

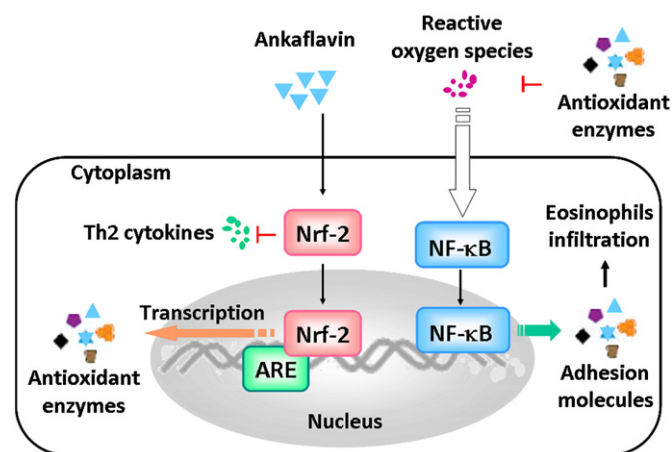


Fig. 8. The proposed mechanism of AK-mediated amelioration of airway inflammation.

activated in response to allergen challenge in the lungs of AK-treated mice, leading to the transcriptional induction of many antioxidant genes that might provide resistance against the development of airway inflammation, immune cell infiltration, and Th2 cytokine release (Fig. 8). Maintenance of a proper balance between ROS production and Nrf-2-regulated antioxidant activity is essential for an appropriate inflammatory response to allergen challenge. Conversely, defective Nrf-2 signaling may confer susceptibility to severe lung inflammation because of allergen exposure in this model. Taken together, we conclude that AK may modulate the oxidative stress pathway to ameliorate the development of airway inflammation.

Acknowledgments

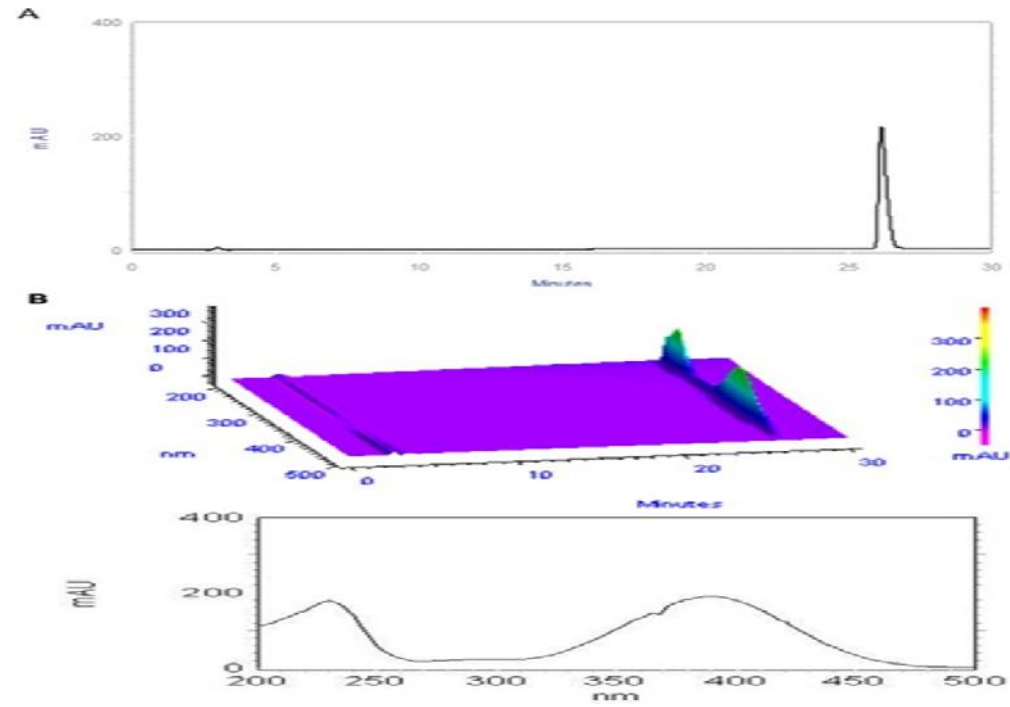
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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2012.08.587>.

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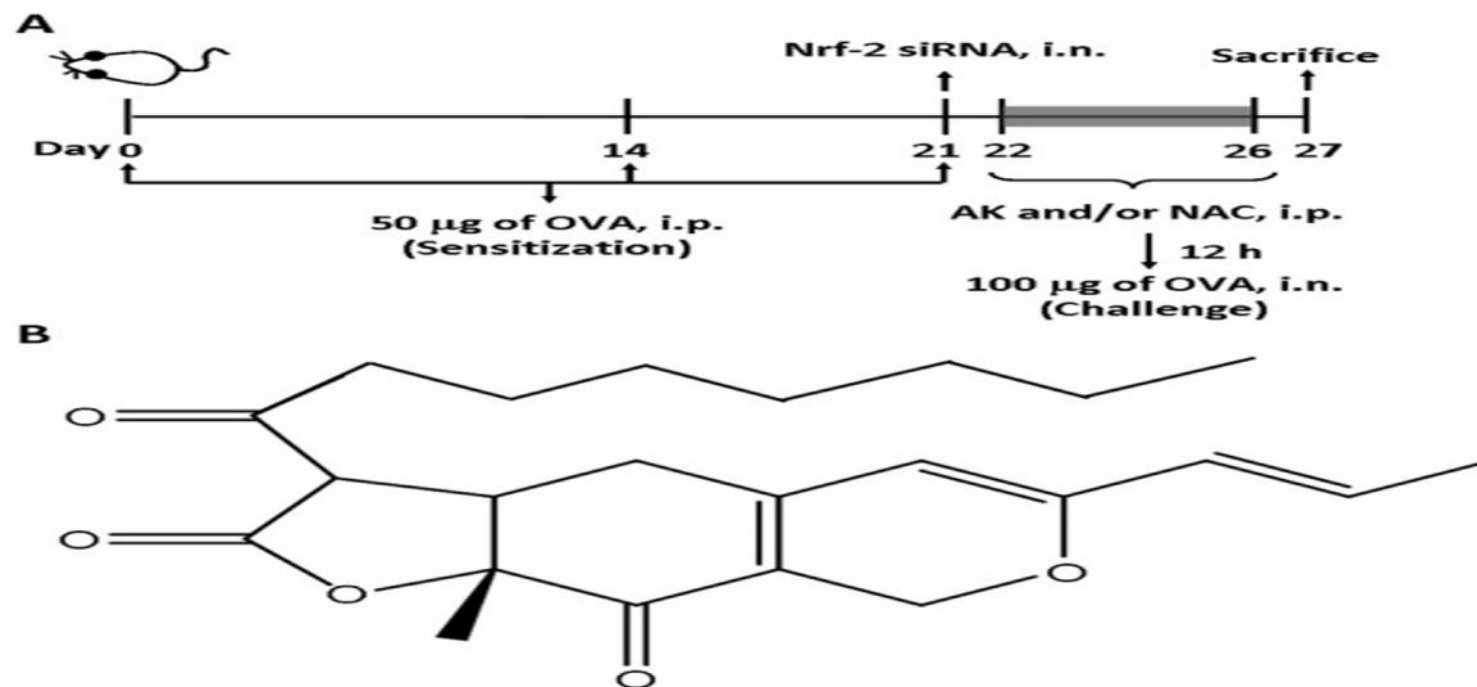


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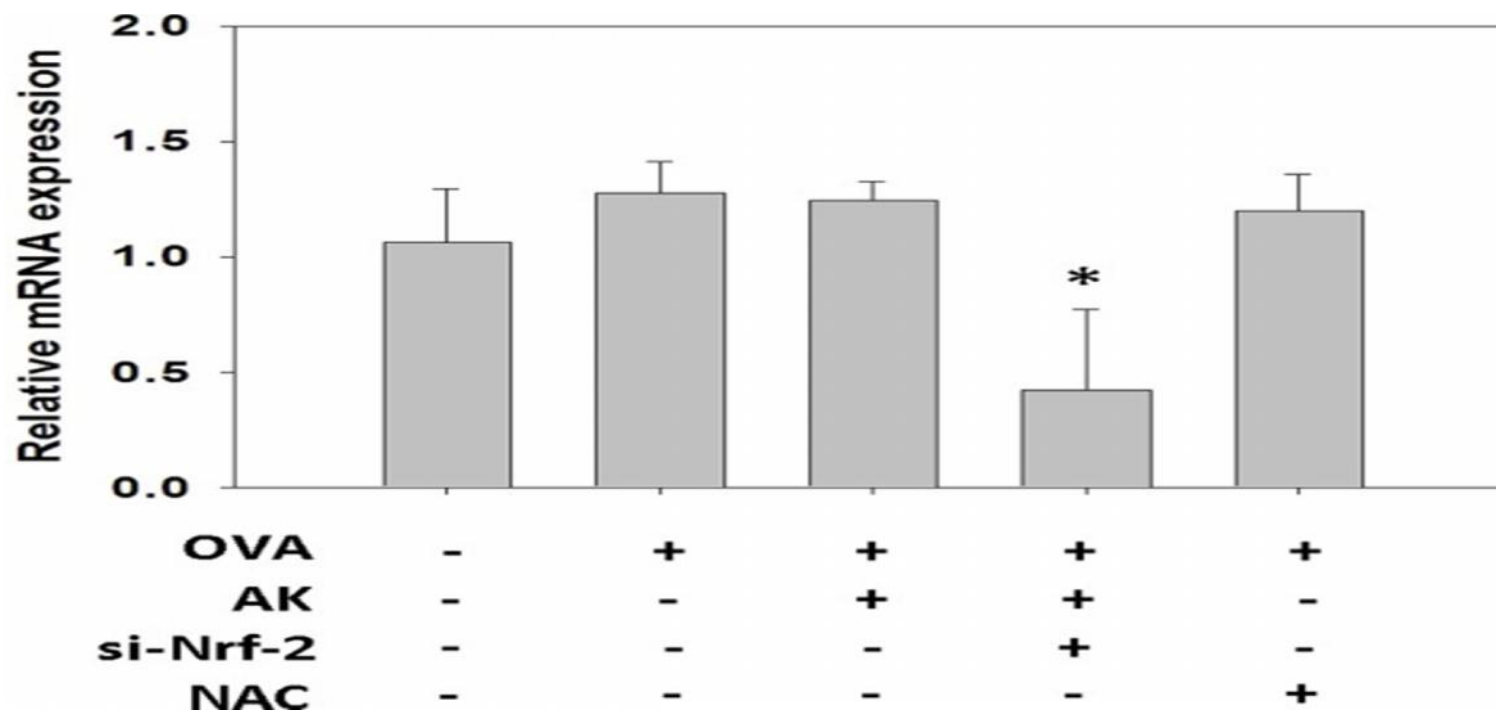


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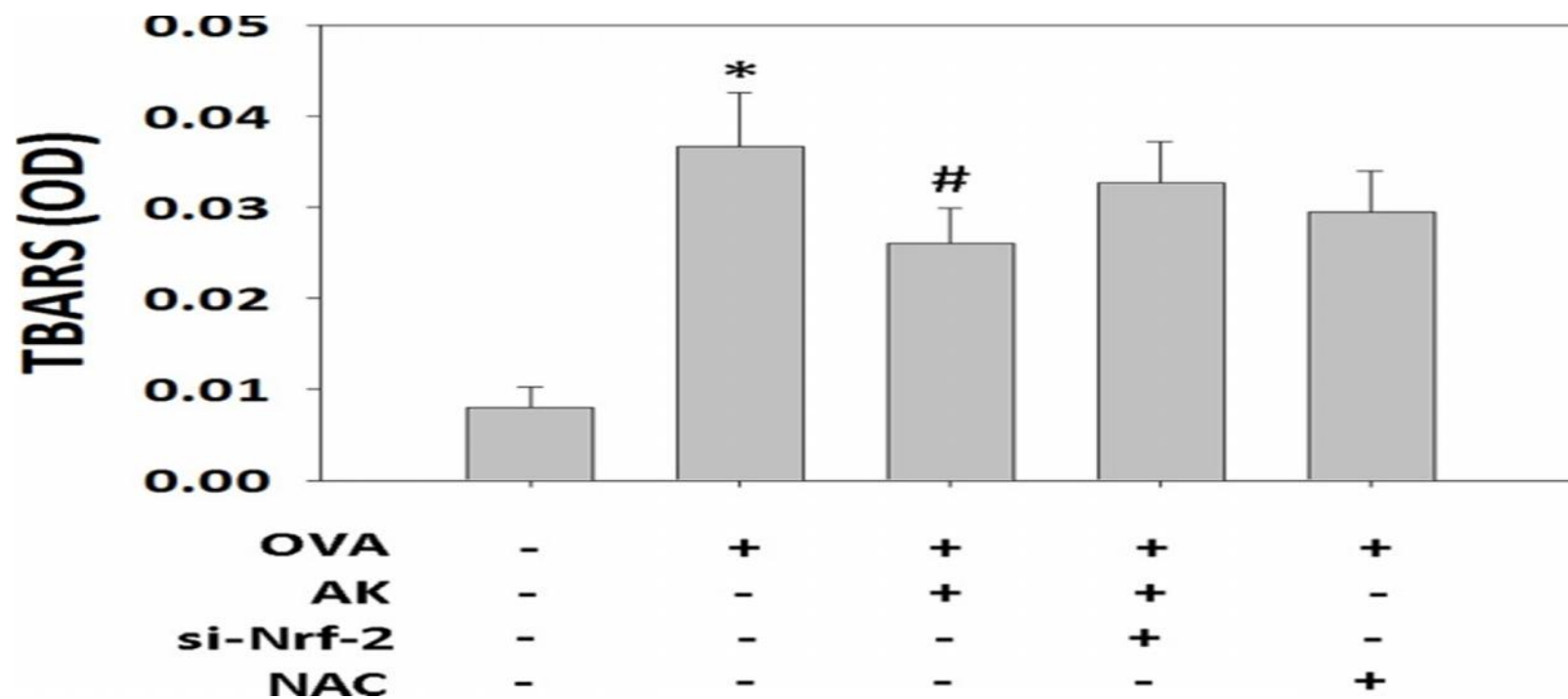


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