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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.

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 NADP(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-κB, nuclear factor-κB; NMR, nuclear magnetic resonance; NQO-1, NADP(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, polyvinyldiene 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).

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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 azaphilonoid 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)-κB 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 sative*) 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 $30 \times 20 \times 5$ 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% CO_2 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 μl of cytosol extraction buffer containing DTT and protease inhibitors. After vortex and 10 min incubation on ice, the extracts were centrifuged at 16,000*g* 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 μl nuclei extraction buffer, vortexed briefly, and set on ice every 10 min for a total 40 min. After centrifuging at 16,000*g* 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 polyvinyldiene 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).