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Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-κB

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ABSTRACT

Persistent activation of nuclear factor-κB (NF-κB) has been associated with the development of asthma. Fisetin (3,7,3′,4′-tetrahydroxyflavone), a naturally occurring bioactive flavonol, has been shown to inhibit NF-κB activity. We hypothesized that fisetin may attenuate allergic asthma via negative regulation of the NF-κB activity. Female BALB/c mice sensitized and challenged with ovalbumin developed airway inflammation. Bronchoalveolar lavage fluid was assessed for total and differential cell counts, and cytokine and chemokine levels. Lung tissues were examined for cell infiltration and mucus hypersecretion, and the expression of inflammatory biomarkers. Airway hyperresponsiveness was monitored by direct airway resistance analysis. Fisetin dose-dependently inhibited ovalbumin-induced increases in total cell count, eosinophil count, and IL-4, IL-5 and IL-13 levels recovered in bronchoalveolar lavage fluid. It attenuated ovalbumin-induced lung tissue eosinophilia and airway mucus production, mRNA expression of adhesion molecules, chitinase, IL-17, IL-33, Muc5ac and inducible nitric oxide synthase in lung tissues, and airway hyperresponsiveness to methacholine. Fisetin blocked NF-κB subunit p65 nuclear translocation and DNA-binding activity in the nuclear extracts from lung tissues of ovalbumin-challenged mice. In normal human bronchial epithelial cells, fisetin repressed TNF-α-induced NF-κB-dependent reporter gene expression. Our findings implicate a potential therapeutic value of fisetin in the treatment of asthma through negative regulation of NF-κB pathway.

1. Introduction

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (Galli et al., 2008b). Cumulative evidence revealed that these inflammatory responses are mediated by T-helper type 2 (Th2) cells together with mast cells, B cells and eosinophils, as well as a number of inflammatory cytokines and chemokines (Galli et al., 2008b; Medoff et al., 2008). IL-4 is imperative for B cell isotype switching for the synthesis of IgE. Allergen-induced crosslinking of IgE-bound high-affinity IgE receptors (FcεRI) on the surface of mast cells leads to degranulation and activation of mast cells, and the release of inflammatory mediators like histamine, leukotrienes and cytokines, and immediate bronchoconstriction (Galli et al., 2008a). IL-5 is vital for the growth, differentiation, recruitment, and survival of eosinophils which contribute to inflammation and even airway remodeling in asthma (Takatsu and Nakajima, 2008). IL-13 plays a pivotal role in the effector phase of Th2 responses such as eosinophilic inflammation, mucus hypersecretion, airway hyperresponsiveness and airway remodeling (Tliba et al., 2003; Wills-Karp, 2004). In addition, chemokines such as RANTES and eotaxin are crucial to the delivery of eosinophils to the airways (Hogan et al., 2008). Airway eosinophilia, and Th2 cytokines IL-4, IL-5 and IL-13, may ultimately contribute to airway hyperresponsiveness in asthma (Berend et al., 2008; Cockcroft and Davis, 2006).

Fisetin (3,7,3′,4′-tetrahydroxyflavone) (Fig. 1A) is a bioactive flavonol molecule that can be isolated from plants like the smoke tree (*Cotinus coggygria*), and fruits and vegetables such as strawberry, apple, persimmon, grape, onion and cucumber at concentrations ranging from 2 to 160 μg/g. Of note, strawberry contains the highest concentration of fisetin per gram (Sung et al., 2007). Fisetin has been shown to possess neurotrophic (Maher, 2006), anti-cancer (Suh et al., 2009; Sung et al., 2007), anti-oxidative (Hanneken et al., 2006) and anti-inflammatory activities (Geraets et al., 2009; Lee et al., 2009b). More specifically, fisetin was found to suppress the level of tumor necrosis factor-α (TNF-α), IL-6, IL-8 and monocyte

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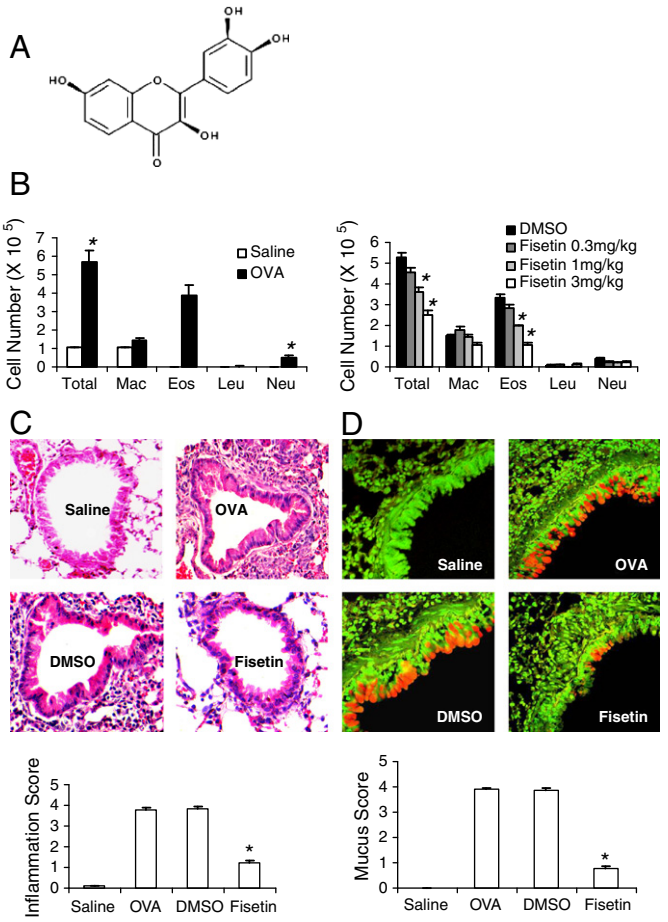

Fig. 1. Effects of fisetin on ovalbumin-induced inflammatory cell recruitment and mucus hypersecretion. (A) Structure of fisetin (3,7,3′,4′-tetrahydroxyflavone). (B) Inflammatory cell counts in bronchoalveolar lavage fluid obtained from sensitized mice 24 h after the last saline aerosol (n = 7 mice) or ovalbumin aerosol (n = 6 mice) challenge. Fisetin dose-dependently reduced ovalbumin-induced inflammatory cell counts in bronchoalveolar lavage fluid from sensitized mice 24 h after the last ovalbumin aerosol challenge (DMSO, n = 9; 0.3 mg/kg, n = 6; 1 mg/kg, n = 6; and 3 mg/kg, n = 11). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Histological examination of (C) lung tissue eosinophilia, magnification ×200 and (D) mucus secretion, magnification ×1000, 24 h after the last challenge of saline aerosol, ovalbumin aerosol, ovalbumin aerosol plus DMSO, or ovalbumin aerosol plus 3 mg/kg fisetin. Quantitative analyses of inflammatory cell infiltration (H&E staining) and mucus production (PAFS staining) in lung sections were performed as previously described (Bao et al., 2009). Scoring of inflammatory cells and goblet cells was performed in at least 3 different fields for each lung section. Mean scores were obtained from 4 animals. OVA aerosol challenge significantly ($P<0.05$) increased inflammatory cell infiltration score and mucus production score as compared with saline control. *Significant difference from DMSO control, $P<0.05$.

chemotactic protein-1 (MCP-1) in a murine rheumatoid arthritis model and in human synovial membrane samples from patients with rheumatoid arthritis (Lee et al., 2009b). In addition, fisetin could attenuate lipopolysaccharide-induced lung inflammation through reduction of inducible nitric oxide synthase (iNOS) and TNF-α levels in mice (Geraets et al., 2009). The exact molecular mechanism that mediates these anti-inflammatory effects of fisetin has not been unequivocally determined. Nevertheless, there is evidence pointing to the inhibition of NF-κB transcriptional activity (Suh et al., 2009; Sung et al., 2007). It has been shown that fisetin may inhibit the upstream kinase transforming growth factor (TGF)-β-activated kinase 1 (TAK1), which is vital for the activation of the NF-κB canonical pathway (Sung et al., 2007).

As asthma is associated with persistent NF-κB activation (Gagliardo et al., 2003; Hart et al., 1998; Pantano et al., 2008), we investigated the effects of fisetin on various aspects of ovalbumin-induced Th2-mediated allergic airway inflammation in an in vivo mouse asthma model. Our results clearly indicate that fisetin attenuates allergic airway inflammation by potentially inhibiting NF-κB pathway.

2. Materials and methods

2.1. Animals

Female BALB/c mice, 6 to 8 weeks old (Interfauna, East Yorkshire, UK), were sensitized and challenged with ovalbumin as described (Bao et al., 2009). Briefly, mice were sensitized by i.p. injections of 20 μg ovalbumin and 4 mg $Al(OH)_3$ suspended in 0.1 ml saline on days 0 and 14. On days 22, 23 and 24, mice were challenged with 1% ovalbumin aerosol for 30 min. Fisetin (0.3, 1, and 3 mg/kg; Sigma, St. Louis, MO, USA) or vehicle (8 μl DMSO in a total of 20 μl saline) was given by i.v. injection 2 h before each ovalbumin aerosol challenge. Saline aerosol was used as a negative control. Animal experiments were performed according to the Institutional guidelines for Animal Care and Use Committee of the National University of Singapore.

2.2. Bronchoalveolar lavage

Mice were anesthetized 24 h after the last aerosol challenge and bronchoalveolar lavage was performed as described (Bao et al., 2009). Briefly, tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold PBS (0.5 ml × 3) was instilled into the lungs, and bronchoalveolar lavage fluid was collected. Total cell counts were performed using a hemocytometer. For cytological examination, cytospin preparations were prepared using a Shandon Cytospin 3 (Thermo Electron Corporation, Pittsburgh, PA, USA), fixed, and stained with a modified Wright stain. Differential cell count was then performed on a minimum of 500 cells in each cytospin slide. Bronchoalveolar lavage fluid levels of cytokine and chemokine were determined using ELISA. IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA, USA). Eotaxin, IL-13 and IFN-γ ELISA were purchased from R&D Systems (Minneapolis, MN, USA).

2.3. Histologic analysis

Lungs were collected 24 h after last ovalbumin challenge, fixed in 10% neutral formalin, paraffinized, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) for examining cell infiltration and periodic acid-fluorescence Schiff stain (PAFS) for mucus production. PAFS allows visualization of mucus through covalent bonding of sulfited acriflavine to mucin glycoconjugates. Mucin granules emit red fluorescence when excited at 380–580 nm and observed at 600–650 nm using a confocal microscope (Leica TCS SP5, Leica Microsystems, Deerfield, IL, USA). Noncovalent linkage of acriflavine to nucleic acid, nuclei and cytoplasm results in green fluorescence when excited at 380–500 nm and observed at 450–475 nm (Evans et al., 2004). Quantitative analyses of cell infiltration and mucus production were performed blind as previously described (Bao et al., 2009).

2.4. Measurements of airway hyperresponsiveness

Mice were anesthetized, and tracheotomy and intubation were performed (Bao et al., 2009). The trachea was intubated with a cannula that was connected to the pneumotach, ventilator and nebulizer. Lung resistance (Rl) and dynamic compliance (Cdyn) in response to nebulized methacholine (0.5–8.0 mg/ml) were recorded using FinePointe™ data acquisition and analysis software (Buxco, Wilmington,