

Kaempferol Suppresses Eosinophil Infiltration and Airway Inflammation in Airway Epithelial Cells and in Mice with Allergic Asthma^{1,2}

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Abstract

The airway epithelium is thought to play an important role in the pathogenesis of asthma. Airway epithelial activation may contribute to inflammatory and airway-remodeling events characteristic of asthma. Kaempferol, a flavonoid with antioxidative and antitumor properties, has been studied as an antiinflammatory agent. However, little is known regarding its effects on allergic asthma. Human airway epithelial BEAS-2B cells and eosinophils were used to investigate the effects of kaempferol on endotoxin- or cytokine-associated airway inflammation. Kaempferol, nontoxic at 1–20 $\mu\text{mol/L}$, suppressed LPS-induced eotaxin-1 protein expression that may be mediated, likely via Janus kinase 2 (JAK2) JAK2 signaling. Additionally, 1–20 $\mu\text{mol/L}$ kaempferol dose-dependently attenuated TNF α -induced expression of epithelial intracellular cell adhesion molecule-1 and eosinophil integrin $\beta 2$, thus encumbering the eosinophil-airway epithelium interaction. Kaempferol blunted TNF α -induced airway inflammation by attenuating monocyte chemoattractant protein-1 transcription, possibly by disturbing NF- κB signaling. This study further investigated antiallergic activity of kaempferol in BALB/c mice sensitized with ovalbumin (OVA) and challenged with a single dose of OVA. Oral administration of kaempferol attenuated OVA challenge-elevated expression of eotaxin-1 and eosinophil major basic protein via the blockade of NF- κB transactivation, thereby blunting eosinophil accumulation in airway and lung tissue. Therefore, dietary kaempferol is effective in ameliorating allergic and inflammatory airway diseases through disturbing NF- κB signaling. *J. Nutr.* 142: 47–56, 2012.

Introduction

Asthma is an allergic and inflammatory disease in the airway and lung and is characterized by airway eosinophilia, AHR⁴, mucus hyper-secretion, and elevated IgE levels (1–3). Allergen inhalation leads Th2 cells and mast cells to produce inflammatory IL or TNF α (4,5). In the allergic airway disease, increased leukocytes, eosinophils, and lymphocytes are observed in BALF and Th2-type cytokines of IL-4, IL-5, and IL-13 are produced (6). These Th2 cytokines stimulate epithelial cells to produce other chemokines and growth factors such as granulocyte/macrophage colony-stimulating factor, IL-8, and TGF β (7,8). In addition, overexpression of IL-4 and IL-13 in murine airways elicits inflammation accompanying subepithelial fibrosis and mucous

cell metaplasia (9). Persistent airway epithelial injury occurs in the asthmatic airway remodeling process. The airway epithelium is both a target of inflammatory and physical insults and an effector of ongoing airway inflammation (10,11). The interplay between airway epithelial cells and eosinophils is an essential feature of allergic asthma attacks (12) in which the recruitment of eosinophils is induced by a specific chemoattractant eotaxin (13).

Several studies have shown that eosinophil recruitment is induced by the application of OVA in asthmatic mouse models (14,15). OVA induces allergic airway disease in mice and has proven useful in investigating the mechanisms underlying airway inflammation. Eotaxin accelerates eosinophilia in cooperation with IL-5 and transmigrates eosinophils to cellular regions of lung tissues (16). Accordingly, the lung tissue damage appears to result from eosinophil trafficking and granule protein release in the airway epithelium. The eosinophil attachment and infiltration into the airway epithelium entail binding of eotaxin to C-C chemokine receptor 3 expressed on the eosinophils, basophils, and Th2 cells (17,18). There is growing interest in eotaxin as a possible target for drug development. The regulation of eotaxin production may be a useful therapeutic strategy influencing the recruitment of eosinophils and its enhancing of allergic disorders. The eosinophil major basic protein (EMBP), a constituent of the eosinophil secondary granule proteins, is implicated in mediation of allergic disorders such as asthma (19). EMBP was

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⁴ Abbreviations used: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; ICAM-1, intracellular cell adhesion molecule-1; I κ B, inhibitory κB ; MTT, 3-(4, 5-dimethylthiazol-yl)-diphenyl tetrazolium bromide; OVA, ovalbumin; Th2, T-helper 2.

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shown to induce eosinophil degranulation and stimulate production of IL-8 and leukotriene C₄ from eosinophils, in which it may act as an autocrine mediator in the pathogenesis of eosinophil-associated diseases such as bronchial asthma (20).

Flavonoids are common compounds derived from plants and are known to have antiallergic and antiinflammatory potentials as well as antioxidant property both in animal and human models (21,22). Kaempferol is a natural flavonol-type flavonoid that has been isolated from citrus fruits, Brussels sprouts, broccoli, apples, and other plant sources. Epidemiological studies have found a positive association between kaempferol consumption and a reduced risk of cardiovascular diseases (23). Some preclinical studies have shown that kaempferol has a wide range of pharmacological activities, including antioxidant, antiinflammatory, and antiallergic activities (24,25). A recent report showed that kaempferol treatments attenuated the Th2-driven allergic airway disease in an experimental model of asthma, impairing production of IL-5 and IL-13 and ameliorating AHR induced by OVA challenge (25). Flavonols including kaempferol inhibited IgE-mediated release of proinflammatory mediator from human mast cells, which may be due to inhibition of intracellular calcium influx and protein kinase C- θ signaling (26). In addition, another flavonol, fisetin, suppressed IgE-mediated induction of Th2-type cytokines by basophils (27). Similarly, blackcurrant polyphenolics reduced eosinophil recruitment and alleviated eosinophilic-driven airway inflammation (28). However, the effects of kaempferol on the induction of eotaxin and proinflammatory mediators and its possible mechanism of action in airway allergic diseases were not well defined.

Based on possible antiallergic functions of kaempferol as described in the literature (24–26), this study investigated whether kaempferol ameliorated allergic responses in LPS and TNF α -exposed human lung bronchus epithelial BEAS-2B cells by measuring eotaxin-1 production and eosinophil recruitment. For the in vivo study, OVA-sensitized BALB/c mice were used to determine the effects of administration of 10 and 20 mg/kg kaempferol by gavage on induction of eotaxin-1 and EMBP in lung tissues. The recruitment of eosinophils into airway epithelium was evaluated. Blockade of NF- κ B signaling by kaempferol was elucidated in TNF α -exposed BEAS-2B cells and OVA-challenged mice.

Materials and Methods

Chemicals. For in vitro study, M199, human epidermal growth factor, hydrocortisone, gelatin, human insulin, LPS, and apo-transferrin were obtained from Sigma-Aldrich Chemical as were all other reagents, unless specifically stated elsewhere. FBS, penicillin-streptomycin, and trypsin-EDTA were purchased from Lonza. The human bronchial airway epithelial cell line, BEAS-2B, was acquired from the American Type Culture Collection and TNF α was obtained from Roche Molecular Biochemicals. MTT was obtained from Duchefa Biochemie. To conduct an in vivo study, chicken egg white albumin was purchased from Sigma-Aldrich Chemical and Inject alum was obtained from Thermo Fisher Scientific.

For Western-blot analysis and immunocytochemical and immunohistochemical assay, antibodies against human ICAM-1, human integrin β 2, human/mouse NF- κ B, or mouse EMBP were purchased from Santa Cruz Biotechnology. Anti-phospho-I κ B α was obtained from Cell Signaling Technology. Anti-human/mouse eotaxin-1 (CCL11) was purchased from R&D Systems, human JAK2 inhibitor provided by Calbiochem, and human β -actin antibody was obtained from Sigma-Aldrich Chemicals. HRP-conjugated goat anti-rabbit IgG, donkey anti-goat IgG, and goat anti-mouse IgG were acquired from Jackson Immuno-Research Laboratories. For the RT-PCR analysis, reverse transcriptase and Taq DNA polymerase were purchased from Promega.

BEAS-2B cell culture and cell viability. The human lung bronchus epithelial BEAS-2B cells were cultured in 25 mmol/L HEPES-buffered M199 containing 10% FBS, 2 mmol/L L-glutamine, 100 kU/L penicillin, 100 mg/L streptomycin supplemented with 2.5 mg/L insulin, 0.361 mg/L hydrocortisone, 2.5 mg/L apo-transferrin, and 20 μ g/L human epidermal growth factor. BEAS-2B cells at 90–95% confluence were sustained at 37°C in an atmosphere of 5% CO₂. LPS or TNF α was applied for various times to BEAS-2B cells to induce expression of gene proteins, including eotaxin-1 and ICAM-1. The cytotoxicity of $\leq$ 20 μ mol/L kaempferol was determined using MTT assay after 48-h culture of BEAS-2B cells (29).

Western-blot analysis. Whole BEAS-2B cell lysates or BALB/c lung tissue extracts were prepared in 1 mol/L Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1%-glycerophosphate, 0.1 mol/L Na₃VO₄, 0.5 mol/L NaF, and protease inhibitor cocktail. Each cell lysate or tissue extract containing an equal amount of proteins was electrophoresed on 8–15% SDS-PAGE and transferred onto a nitrocellulose membrane. Blocking to avoid a nonspecific binding was performed using either 3% fatty acid-free BSA buffer or 5% nonfat dry milk for 3 h. The membrane was incubated overnight at 4°C with a specific primary antibody. The membrane was then applied to a secondary antibody conjugated to HRP for 1 h. Following another triple washing, the target protein was determined using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology) and Agfa medical X-ray film blue (Agfa HealthCare).

RT-PCR analysis. Total RNA was obtained from BEAS-2B cells (a density of 6×10^5 /60-mm dish) using a commercial Trizol reagent kit (Invitrogen), and cDNA was synthesized using 5 μ g total RNA with 0.5 μ g/L oligo-(dT)₁₅ primer (Bioneer) and 200 units of reverse transcriptase. The PCR (Bio-Rad Laboratories) was accomplished using primers including human MCP-1 (forward primer: 5'-AACTGAAGCTCGCACTCTCG-3', reverse primer: 5'-TCAGCACAGATCTCCTTGGC-3', 258 bp) and β -actin (forward primer: 5'-GACTACCTCATGAAGATC-3', reverse primer: 5'-GATCCACATCTGCTGGAA-3', 500 bp) with an addition of 25 μ L of 10 mmol/L Tris-HCl (pH 9.0) containing 25 mmol/L MgCl₂, 10 mmol/L dNTP, and five units of Taq DNA polymerase. The PCR reaction was performed under the condition of 60-s denaturation at 94°C, 60-s annealing at 55°C, and 60-s elongation at 72°C as a cycle. After thermocycling, electrophoresis on 1% agarose-formaldehyde gel containing 0.5 mg/L ethidium bromide was achieved and bands were visualized, taken, and quantified.

Nuclear extract preparation. Cell protein fraction to cytosolic or nuclear protein was achieved by a procedure conducted by Choi et al. (30). Collected cell lysates were centrifuged at $794 \times g$ for 20 min (Hanil Science). Proteins extracted from nuclear pellets were incubated in a high-salt buffer composed of 420 mmol/L NaCl and 20 mmol/L HEPES (pH 7.9) at 4°C with vigorous shaking. Another centrifugation at $19,872 \times g$ for 30 min was conducted to remove nuclear debris. The supernatants were collected, stored, and used to determine the translocation of NF- κ B.

Immunocytochemistry. After challenging 20 μ g/L TNF α to BEAS-2B cells grown on a glass-chamber slide, a brief washing with PBS-0.2% Tween 20 was performed, and BEAS-2B cells were fixed with 4% formaldehyde for 15 min. Cells were blocked using a 4% FBS for 1 h, and polyclonal rabbit anti-human NF- κ B p65 was applied to cells. After overnight incubation, triple washing was conducted and incubation with FITC-conjugated goat anti-rabbit was achieved for 1 h. Images were taken using a fluorescence microscope (Axiomager, Zeiss).

Induction of chronic allergic airway inflammation in murine models. Six-week-old male BALB/c mice (Hallym University Breeding Center for Laboratory Animals) were used in the present study. Mice were kept on a 12-h-light/dark cycle at $23 \pm 1^\circ\text{C}$ with $50 \pm 10\%$ relative humidity under specific pathogen-free conditions, fed a non-purified diet (RodFeed, DBL), and consumed water ad libitum at the animal facility of Hallym University. The nonpurified diet composition was $\geq 20.5\%$ crude protein, $\geq 3.5\%$ crude fat, $\leq 8.0\%$ crude fiber, $\leq 8.0\%$

crude ash, $\geq 0.5\%$ calcium, and $\geq 0.5\%$ phosphorus. Mice were allowed to acclimatize for 1 wk before beginning the experiments. Mice were divided into four subgroups ($n = 6$ for each subgroup). Mice were sensitized with $20 \mu\text{g}$ OVA dissolved in a solution of $30 \mu\text{L}$ PBS and $50 \mu\text{L}$ Imject Alum by s.c. injection twice on d 0 and 14. For dietary interventions, 0.1 mL kaempferol solution (10 or 20 mg/kg body weight) was orally administered to OVA-sensitized mice 1 h before challenge. On d 28, 29, and 30, 5% OVA inhalation to mice was performed for 20 min in a plastic chamber linked to an ultrasonic nebulizer (Clenny² Aerosol). Control mice were sensitized and challenged with PBS as the OVA vehicle. Twenty-four hours after the latest provocation (d 30), all mice were killed with an anesthetic ($2 \mu\text{L/kg}$ rompun and $8 \mu\text{L/kg}$ zoletil, i.p.). The trachea was cannulated and both lungs and airways were rinsed in 1 mL PBS for the collection of BALF. The numbers of neutrophils, eosinophils, and basophils in BALF were determined using a Hemavet HV950 Multispecies Hematologic Analyzer (Drew Scientific). The right lungs were collected, frozen in liquid nitrogen, and kept at -80°C until used for Western blotting. Left lungs were preserved and fixed in 4% paraformaldehyde and then used for immunohistochemical analyses.

All experiments were approved by the Committee on Animal Experimentation of Hallym University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals. No mice were dead and no apparent signs of exhaustion were observed during the experimental period.

Lung histology and immunohistochemistry. All paraffin-embedded lung tissues were sliced in $5\text{-}\mu\text{m}$ thickness and tissue sections were stained using a Modified Harris Hematoxylin and Shandon Instant Eosin (Thermo Fisher Scientific) to confirm cellular penetration. For the immunohistochemical analysis of lung tissues against anti-mouse eotaxin-1 and EMBP, all sections were subject to a series of immunohistochemical procedures, including the Ag retrieval followed by quenching of endogenous peroxidase activity. For the detection, a 3,3'-

diaminobenzidine chromogenic substrate detection kit (Dako) was used. Counter staining was conducted with hematoxylin and each slide was mounted in VectaMount mounting medium (Vector Laboratories). Images of each slide were taken using an optical microscope system. Lung inflammatory cells and protein levels of eotaxin-1 and EMBP were quantified by image analysis program of the microscope system.

To elucidate the impact of kaempferol on NF- κB transactivation in OVA-challenged lung tissues, an immunofluorescent histochemical analysis was conducted using mouse NF- κB antibody and Cy3-conjugated anti-goat IgG. Nuclear staining was done with 4',6-diamidino-2-phenylindole. Images of each slide were taken using a fluorescent microscope system.

Statistical analysis. The data are presented as mean $\pm$ SEM for each treatment group in in vitro and in vivo experiments. Statistical analyses were conducted using the SAS program (SAS Institute). Significance was determined by 1-way ANOVA, followed by the Duncan Multiple Range Test for multiple comparisons. $P < 0.05$ was considered significant.

Results

Suppression of eotaxin-1 expression by kaempferol in LPS-stimulated BEAS-2B cells. To evaluate the cytotoxicity of kaempferol, the viability of BEAS-2B cells incubated with $1\text{--}20 \mu\text{mol/L}$ kaempferol for 24 h was determined by using MTT assay. The viability of BEAS-2B cells was not influenced by treating with $\leq 20 \mu\text{mol/L}$ kaempferol (data not shown).

BEAS-2B cells were incubated with 2 mg/L LPS and eotaxin-1 release was determined based on 2-h intervals up to 24 h. The eotaxin-1 expression was strikingly elevated up to 8 h and thereafter diminished (Fig. 1A). Thus, BEAS-2B cells were

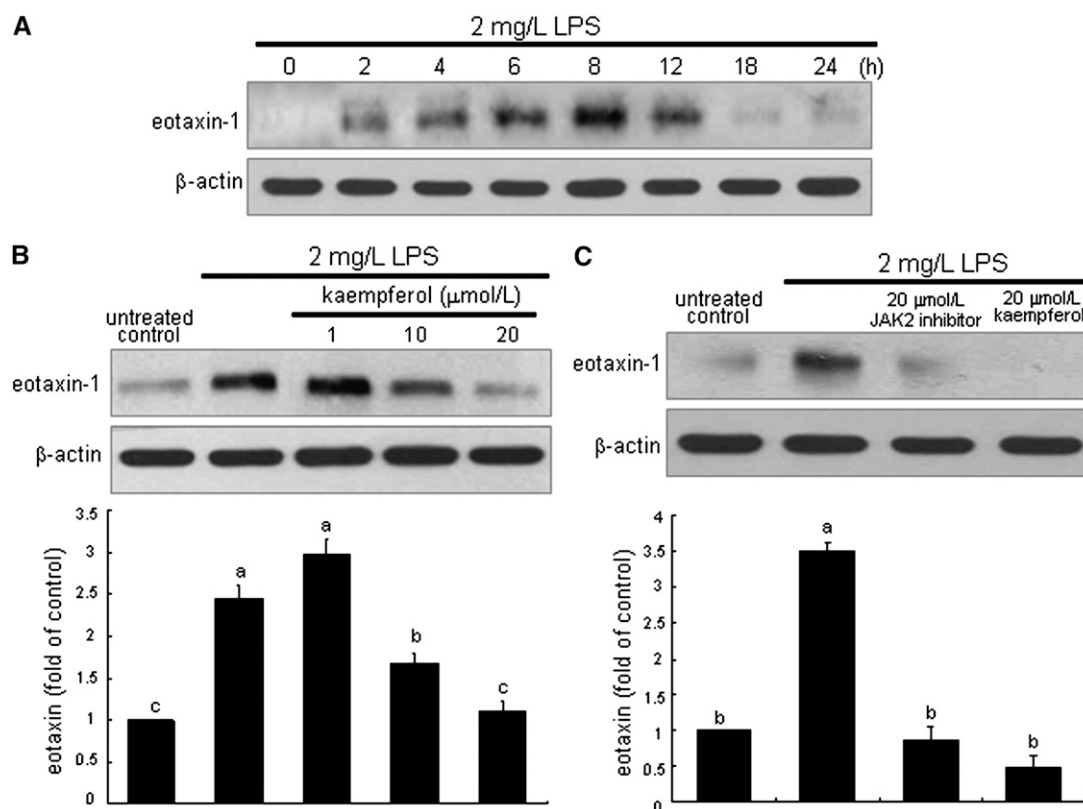


FIGURE 1 Temporal response (A) and inhibition (B) of eotaxin-1 expression by kaempferol (B) and JAK2 inhibitor (C) in LPS-activated BEAS-2B cells. After cell culture protocols with 2 mg/L LPS for 24 h (A), cell lysates were prepared for Western blotting. BEAS-2B cells were treated with kaempferol or JAK2 inhibitor and stimulated with 2 mg/L LPS for 8 h. Representative blot data were obtained from separate experiments and β -actin protein was used as an internal control. Quantitative densitometric data in B and C are mean $\pm$ SEM, $n = 3$. Means without a common letter differ, $P < 0.05$.

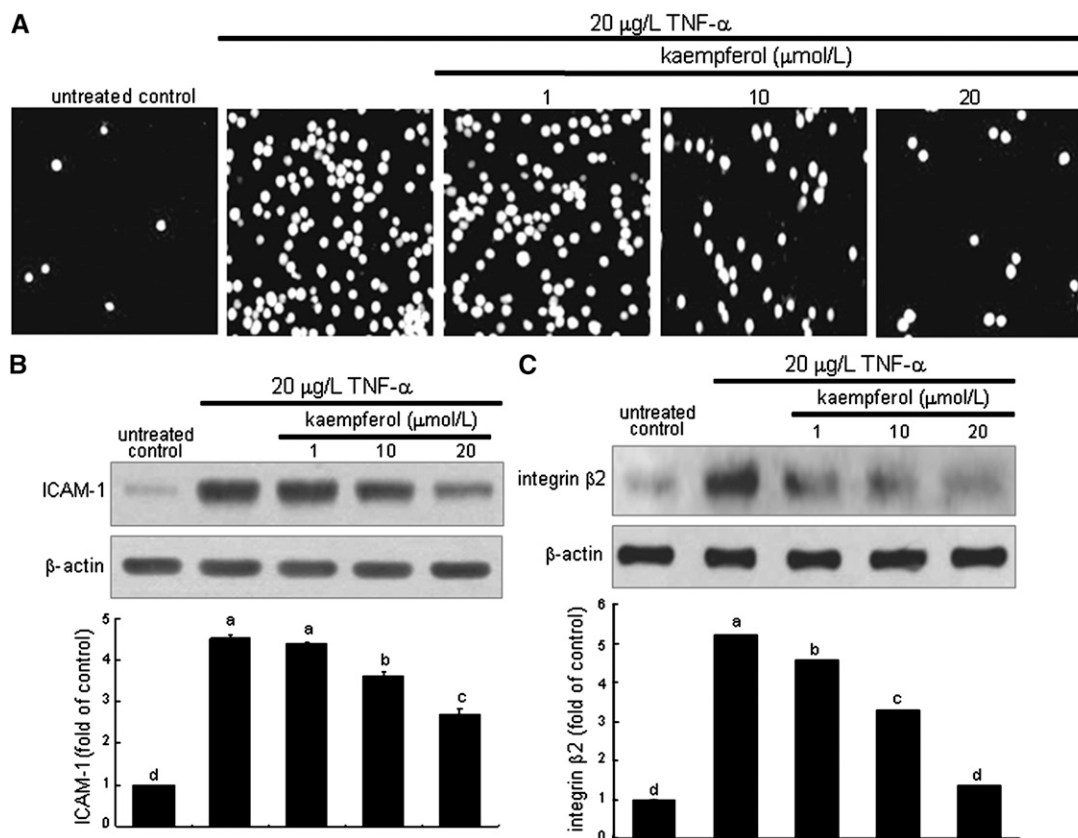


FIGURE 2 Inhibition of human eosinophil adhesion (A) to and suppression of TNF α -induced ICAM-1 expression (B) of TNF α -activated BEAS-2B cells by kaempferol. Inhibitory effects of kaempferol on expression of integrin β 2 in human eosinophils (C). β -Actin protein was used as an internal control. Quantitative densitometric data in B and C are mean $\pm$ SEM, $n = 3$. Means without a common letter differ, $P < 0.05$. ICAM-1, intracellular cell adhesion molecule-1.

incubated for 8 h to determine the effect of kaempferol on the eotaxin-1 expression induced by 2 mg/L LPS addition. The eotaxin-1 expression was very weak in untreated quiescent BEAS-2B cells, whereas its marked increase was observed in LPS-exposed BEAS-2B cells, suggesting that LPS acts as the prototypical endotoxin recruiting eosinophils onto the airway epithelial cells. However, BEAS-2B cells treated with ≥ 10 μ mol/L kaempferol significantly declined in eotaxin-1 expression (Fig. 1B). Eotaxin-1 expression was further examined by using 20 μ mol/L JAK2 inhibitor. Like 20 μ mol/L kaempferol, the JAK2 inhibitor dampened eotaxin-1 expression highly enhanced in LPS-stimulated BEAS-2B cells. This finding showed that LPS-induced epithelial eotaxin-1 expression occurred by activating JAK2 signaling, which was blunted by kaempferol (Fig. 1C).

Inhibition of eosinophil recruitment on TNF α -activated BEAS-2B cells by kaempferol. This study elucidated that kaempferol treatment might inhibit eosinophil recruitment on the TNF α -induced airway epithelium. It has been shown that LPS is a potent stimulus for TNF α synthesis. The in vitro adhesion assay of Clone 15 HL-60 eosinophils to 20 μ g/L TNF α -activated BEAS-2B cells supported this hypothesis. A small number of eosinophils adhered to quiescent BEAS-2B cells free of TNF α (Fig. 2A). There was a heavy eosinophil staining on the epithelial cells exposed to TNF α alone for 6 h, indicative of a marked increase in eosinophil adherence to the activated BEAS-2B cells. However, adding kaempferol at nontoxic doses of 1–20 μ mol/L to TNF α -exposed cells for 2 h diminished the number of eosinophils adhered to the activated epithelial cells in a dose-dependent manner. It should be noted that ≥ 10 μ mol/L

kaempferol was effective in inhibiting eosinophil adhesion to TNF α -activated airway epithelium.

Western-blot analysis was performed to address whether kaempferol inhibited the induction of ICAM-1 and integrin β 2 triggered by 20 μ g/L TNF α . There was no or relatively weak expression of ICAM-1 in quiescent cells (Fig. 2B). In contrast, the expression of ICAM-1 protein was greatly elevated in TNF α -stimulated epithelial cells by ~ 2 -fold. When 1–20 μ mol/L kaempferol was added, the enhanced expression of ICAM-1 was substantially attenuated but yet not fully in a dose-dependent manner (Fig. 2B). These results imply that kaempferol may block eosinophil trafficking in response to an allergic inflammatory mediator by inhibiting induction of epithelial ICAM-1 adhesion molecule.

Additional inhibitory effects of kaempferol on the expression of integrin β 2 were determined in eosinophils inflamed by 20 μ g/L TNF α . The supplementation of kaempferol lessened the TNF α -upregulated expression of integrin β 2. Surprisingly, adding 20 μ mol/L kaempferol to Clone 15 HL-60 cells abolished the expression of integrin β 2. These results imply that kaempferol may block the tight binding of eosinophils to activated epithelial cells in response to the inflammatory mediator of TNF α . The tight adhesion of eosinophils was mediated by integrin β 2 and its epithelial cell counter-receptor of ICAM-1. Accordingly, the Western-blot data (Fig. 2B,C) supported the in vitro adhesion results (Fig. 2A).

Kaempferol attenuation of TNF α -triggered MCP-1 transcription and NF- κ B activation. When 20 μ g/L TNF α was added to BEAS-2B cells, the MCP-1 mRNA level was elevated

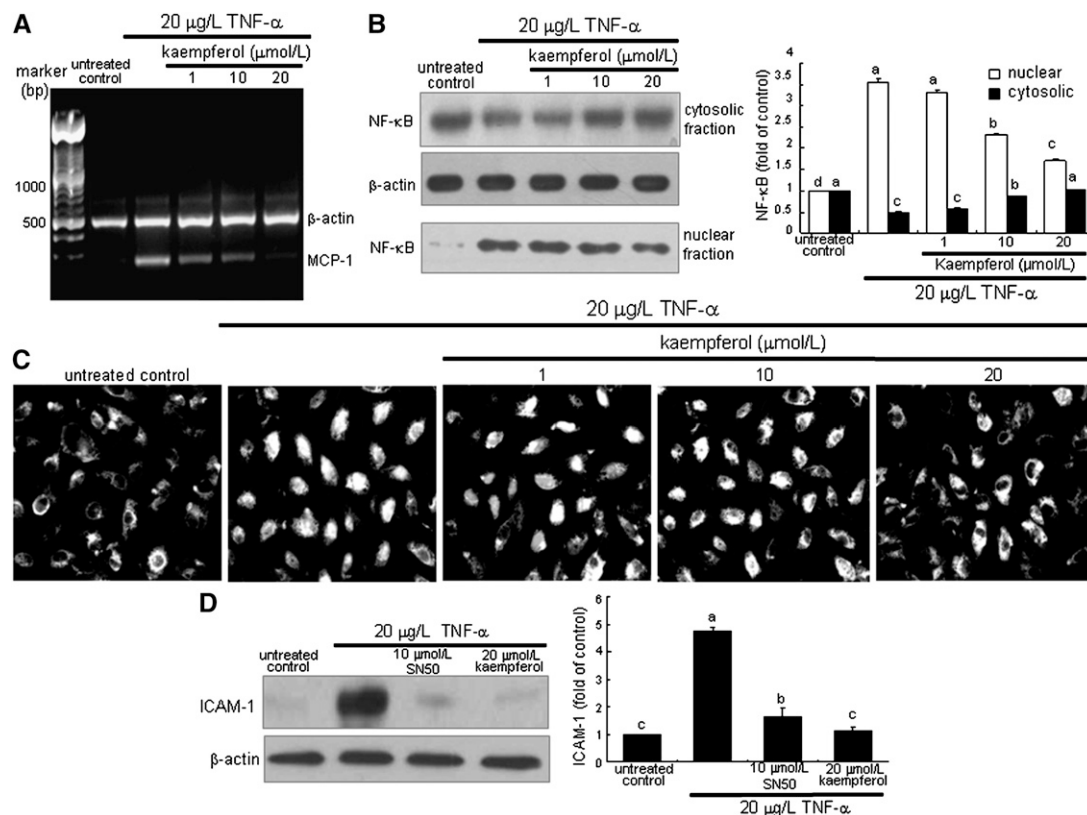


FIGURE 3 Suppression of transcription of MCP-1 (A) and inhibition of nuclear translocation of NF-κB (B,C) by kaempferol in TNF α -stimulated BEAS-2B cells. RT-PCR (A) showing mRNA levels of MCP-1 in kaempferol-treated and TNF α -stimulated epithelial cells. β -Actin genes were used as an internal control for the coamplification with MCP-1 ($n = 3$). Western-blot (B) and immunocytochemical analyses (C) showing inhibition of TNF α -induced NF-κB translocation by kaempferol. The blockade of TNF α -induced ICAM-1 expression by the NF-κB inhibitor SN50 (D). β -Actin was used as an internal control. Quantitative densitometric data in the right panels are mean $\pm$ SEM, $n = 4$. Means without a common letter differ, $P < 0.05$. NF-κB localization was visualized with a FITC-conjugated secondary antibody (C). Original magnification of microscopic images ($n = 3$), 200 $\times$. ICAM-1, intracellular cell adhesion molecule-1.

(Fig. 3A). However, the MCP-1 transcription was reduced when 1–20 μ mol/L kaempferol was applied to BEAS-2B cells. Accordingly, this study found that kaempferol encumbered airway epithelial inflammation instigated by TNF α .

To determine whether the airway inflammation was mediated via NF-κB-responsive mechanism(s), this study investigated nuclear transactivation of NF-κB in epithelial inflammation. Following exposure to 20 μ g/L TNF α , nuclear p65 increased with a decrease in cytosolic NF-κB p65 protein (Fig. 3B). When TNF α -exposed BEAS-2B cells were treated with 1–20 μ mol/L kaempferol, a diminution in the nuclear p65 protein occurred. In contrast, the NF-κB p65 protein in the cytosolic fraction substantially increased following treatment with kaempferol (Fig. 3B). In addition, intracellular localization of NF-κB p65 in BEAS-2B cells was evaluated by fluorescent microscopy using specific NF-κB p65 antibody (Fig. 3C). Cytoplasmic immunofluorescence staining was observed in untreated epithelial cells,

and a heavy nuclear staining in cells exposed to TNF α alone occurred, indicative of nuclear localization of activated NF-κB p65 at the single cell level. However, 20 μ mol/L kaempferol- and TNF α -treated cells clearly demonstrated the diminished staining level of nuclear p65 (Fig. 3C).

As shown in Figure 3D, as with 20 μ mol/L kaempferol, the NF-κB inhibitor SN50 suppressed ICAM-1 expression induced by TNF α . Accordingly, kaempferol may limit an interaction between epithelial cells and eosinophils via disturbing a NF-κB-responsive mechanism.

Blockade of eosinophil airway infiltration by kaempferol administration. The total cell number in the BALF of mice exposed to OVA increased by ~ 2.3 -fold with developing a predominance of eosinophils (4-fold increase) (Table 1). The administration of kaempferol prevented the increase of eosinophil numbers, recovering to eosinophil counts observed in the

TABLE 1 Inhibition of eosinophil airway infiltration by kaempferol in OVA-sensitized mice¹

Mice	Neutrophils	Eosinophils	Basophils	Total cells
Cells, $n \times 10^6/L$				
Control	193 $\pm$ 35 ^b	47 $\pm$ 7 ^b	27 $\pm$ 7 ^b	267 $\pm$ 29 ^b
OVA-challenged	367 $\pm$ 58 ^a	160 $\pm$ 52 ^a	60 $\pm$ 20 ^a	587 $\pm$ 109 ^a
10 mg/kg kaempferol-treated	227 $\pm$ 41 ^b	107 $\pm$ 24 ^a	27 $\pm$ 7 ^b	360 $\pm$ 60 ^b
20 mg/kg kaempferol-treated	200 $\pm$ 42 ^b	53 $\pm$ 13 ^b	27 $\pm$ 7 ^b	280 $\pm$ 61 ^b

¹ Values are mean $\pm$ SEM, $n = 6$. Means in a column without a common letter differ, $P < 0.05$. OVA, ovalbumin.

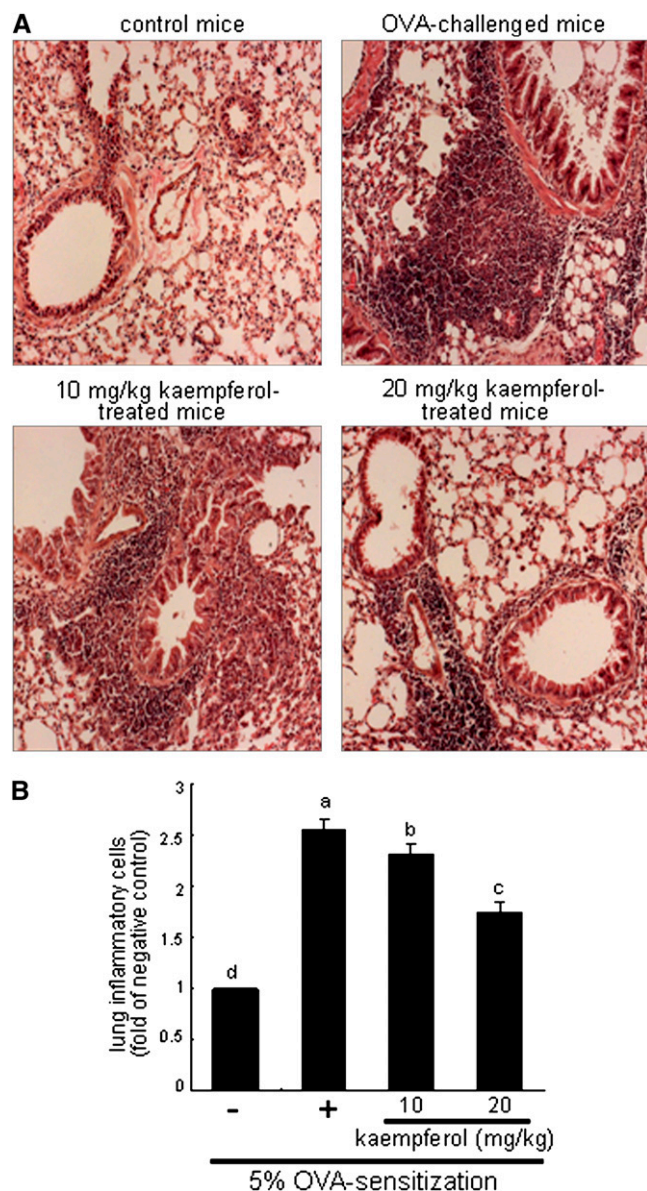


FIGURE 4 Inhibition of eosinophil airway infiltration by treatment with kaempferol in OVA-sensitized mice. Histological examination was performed in lung tissue sections stained with Modified Harris Hematoxylin and Shandon Instant Eosin (A). Cellular infiltration was identified as blue-stained cells. Representative tissue sections from 6 mice are shown. Magnification: 200-fold. Blue-stained inflammatory cells infiltrated were quantified (B). Means without a common letter differ, $P < 0.05$. OVA, ovalbumin.

BALF of corresponding control PBS-treated mice. Interestingly, the mice in the OVA group had a higher number of neutrophils and basophils in the BALF compared with the control group, indicating that OVA evoked airway inflammation entailing leukocyte infiltration (Table 1). Kaempferol reduced the numbers of these cells in the BALF. Furthermore, the histological examination was assessed in paraformaldehyde-fixed lung tissues stained with Modified Harris Hematoxylin and Shandon Instant Eosin. Sensitization and subsequent OVA challenge resulted in significant increases in the numbers of peribronchial and perivascular cells (Fig. 4A,B). The treatments with kaempferol noticeably attenuated the inflammatory eosinophil accumulation in lung tissues of OVA-challenged mice. Few infiltrated eosinophils were detected in the negative control mice.

Diminution of eotaxin-1 and EMBP production by kaempferol. There was a significant increase in eotaxin-1 secretion in the lung tissues of OVA-challenged mice compared with control mice, which were not stimulated by 5% OVA (Fig. 5A). When 10 and 20 mg/kg body weight of kaempferol was administered to OVA-challenged mice, the enhanced eotaxin-1 secretion was abrogated. This study further investigated eotaxin-1 induction in airway and lung tissues during an asthmatic episode. The OVA provocation increased eotaxin-1 secretion in the lung tissues, as evidenced by immunohistochemical staining using an eotaxin-1 antibody (Fig. 5B,C). Conversely, the gavage application of kaempferol to the OVA-challenged airway substantially retarded the eotaxin-1 induction, which was consistent with Western-blot data.

The OVA challenge enhanced EMBP secretion in lung tissues by ~3-fold, which was attenuated by administration of ≥ 10 mg/kg kaempferol (Fig. 6A). In addition, kaempferol inhibited the OVA-elevated EMBP production from lung tissues in a similar manner (Fig. 6B,C).

Disturbance of NF- κ B transactivation by kaempferol. The transcription factor NF- κ B was used as a definitive marker for airway inflammation. Consistent with TNF α -induced nuclear NF- κ B activation in airway epithelial cells (Fig. 3B,C), OVA challenge increased I κ B phosphorylation, leading to nuclear translocation of NF- κ B (Fig. 7A). The OVA-promoted I κ B phosphorylation was diminished in ≥ 10 μ g/kg kaempferol-treated mice. As expected, the OVA challenge increased reddish pink staining of nuclear NF- κ B, indicating that the OVA provocation inflamed nuclear activation of NF- κ B (Fig. 7B). Such activation was disrupted by the gavage administration of kaempferol to sensitized mice.

Discussion

Eight major findings were observed from this study: 1) LPS increased eotaxin-1 expression in a temporal fashion from airway epithelial BEAS-2B cells. Nontoxic kaempferol at ≤ 20 μ mol/L suppressed LPS-induced epithelial eotaxin-1 expression, likely via blunting JAK2 signaling; 2) the kaempferol treatment at doses of ≥ 10 μ mol/L inhibited the adherence of eosinophils onto TNF α -exposed epithelial cells; 3) the induction of epithelial ICAM-1 and its eosinophil counter-receptor integrin $\beta 2$ by TNF α was dose-dependently attenuated by adding kaempferol; 4) kaempferol markedly suppressed MCP-1 transcription of BEAS-2B cells upregulated by TNF α , indicative of blunting epithelial inflammation; 5) nuclear transactivation of NF- κ B promoted by TNF α was blocked by the presence of ≥ 10 μ mol/L kaempferol in BEAS-2B cells; 6) in OVA-challenged BALB/c mice the kaempferol administration attenuated cellular infiltration in peribronchial and perivascular airway; 7) treatment of mice with ≥ 10 mg/kg body weight of kaempferol retarded the secretion of eotaxin-1 and EMBP upregulated by OVA challenge; and 8) OVA challenge-enhanced nuclear translocation of NF- κ B was disturbed by ≥ 10 mg/kg kaempferol, suggesting that kaempferol administration encumbered inflammatory NF- κ B signaling fired by OVA challenge. These results demonstrate that the dietary compound kaempferol may be a potent therapeutic agent for the prevention and treatment of toxin- or allergen-evoked asthma accompanying eosinophilia and airway inflammation.

Asthma is the common chronic inflammatory disease of the airways characterized by airway eosinophilia, AHR, mucus hyper-secretion, and elevated IgE levels (1–3), causing variable

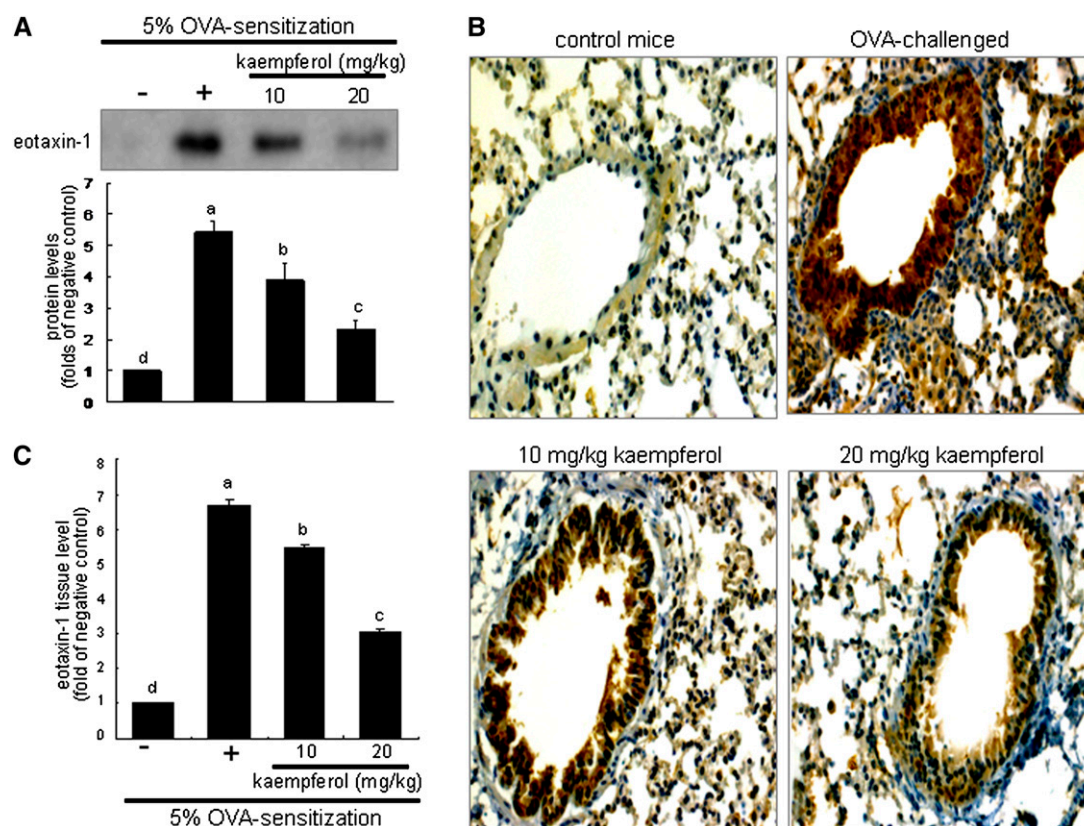


FIGURE 5 Reduction of lung tissue levels of eotaxin-1 by kaempferol administration in OVA-sensitized mice. Western-blot analysis was performed using anti-eotaxin-1 (A). Quantitative densitometric data in A are mean $\pm$ SEM, $n = 3$. For immunohistochemical staining (B), lung tissue was immunostained using anti-eotaxin-1 and 3,3'-diaminobenzidine-conjugated IgG. Eotaxin-1 was identified as brown staining and quantified (C). Each photograph is representative of 4 mice. Magnification: 200-fold. Means without a common letter differ, $P < 0.05$. OVA, ovalbumin.

and recurring symptoms, reversible airflow obstruction, and bronchospasm. In the allergic airway diseases, eosinophils as well as lymphocytes are elevated in BALF and airway tissue. The airway epithelium is both a target of inflammatory and physical insults and an effector of ongoing airway inflammation (10,11). Accordingly, the interplay between airway epithelial cells and eosinophils is an essential feature of allergic asthma attacks (12), and the recruitment and accumulation of eosinophils are caused by a specific chemoattractant eotaxin (13). Allergen inhalation leads Th2 cells and mast cells to produce inflammatory Th2-type cytokines and non-Th2-type TNF α (4–6). In this study, the endotoxin LPS acting as an inflammatory insult increased epithelial eotaxin-1 expression. In addition, TNF α markedly stimulated the adhesion of eosinophils onto activated epithelial cells. Several studies have shown that eosinophil recruitment is induced by the application of OVA in asthmatic animal models (14,15). As expected, the OVA challenge enhanced the production of eotaxin-1 and EMBP in lung tissues, facilitating eosinophil infiltration. Histological examination showed that the OVA challenge appeared to elicit subepithelial fibrosis and mucous cell metaplasia. Therefore, the regulation of eotaxin-1 production may be a useful therapeutic strategy influencing the recruitment of eosinophils and its enhancing of allergic disorders.

Kaempferol is a natural flavonol-type flavonoid that has been isolated from citrus fruits, Brussels sprouts, broccoli, and other plant sources. Epidemiological studies have found that kaempferol consumption reduced the risk of cardiovascular diseases (23). Also, some preclinical studies have shown that this dietary compound has antioxidant, antiinflammatory, and antiallergic

activities (24,25). A recent report showed that kaempferol treatments attenuated the Th2-driven allergic airway disease in an experimental model of asthma, impairing production of IL-5 and IL-13 and ameliorating AHR induced by OVA challenge (25). This study revealed that kaempferol inhibited LP-induced epithelial eotaxin-1 expression and TNF α -induced eosinophil-epithelial interaction. Consistently, it was shown that kaempferol encumbered the eosinophil recruitment and accumulation observed in OVA-experienced mice. Similarly, other flavonols such as fisetin inhibited IgE-mediated release of proinflammatory mediator and Th2-type cytokines from human mast cells and basophils (26,27). In addition, a recent study showed that blackcurrant polyphenolics reduced eosinophil recruitment and alleviated eosinophil-driven airway inflammation (28). However, the possible mechanisms of kaempferol action in blunting airway allergic diseases were not well defined.

Allergic disorders cause a marked increase in nonblood tissue eosinophil count that is diagnostic for tissue eosinophilia (31). The perivascular eosinophil accumulation in airway and lung tissues entails their epithelial adhesion followed by transmigration. This study showed that the inflammatory cytokine TNF α induced both epithelial ICAM-1 and its counter-receptor eosinophil integrin β 2, promoting vascular eosinophil trafficking to nonblood airway tissues. Such induction abrogated by treating kaempferol appeared to be one possible mechanism of eosinophil extravasation. Cell adhesion molecules including ICAM-1 are essentially involved in the induction of the immune responses and the pathogenesis of inflammation. This study revealed that kaempferol diminished the increased epithelial ICAM-1 expres-

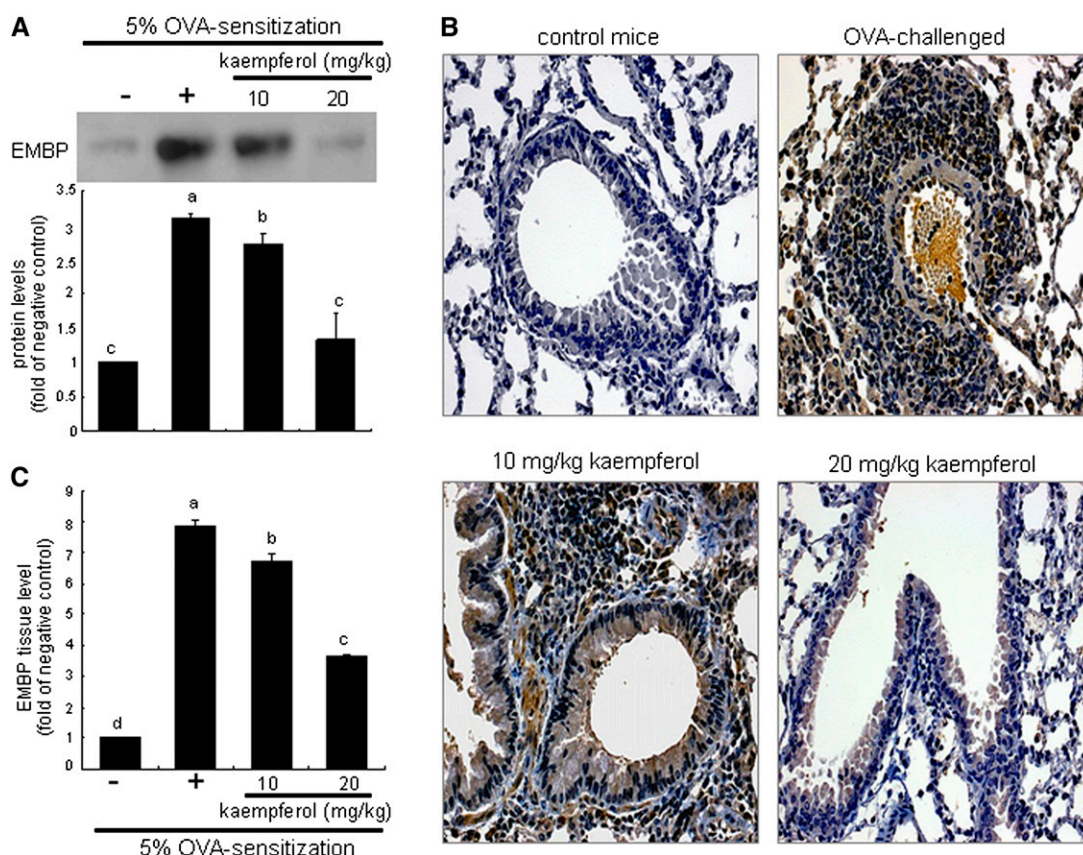


FIGURE 6 Impacts of kaempferol administration on lung tissue levels of EMBP in OVA-sensitized mice. Tissue extracts were subject to Western-blot analysis with anti-EMBP (A). Quantitative densitometric data in A are mean $\pm$ SEM, $n = 3$. Histological sections of lung tissues were immunostained with using anti-EMBP and 3,3'-diaminobenzidine-conjugated IgG (B). EMBP secretion was identified as brown staining and quantified (C). Each photograph is representative of 4 mice. Magnification: 200-fold. Means without a common letter differ, $P < 0.05$. OVA, ovalbumin.

sion and MCP-1 transcription caused by $\text{TNF}\alpha$, indicating that this compound ameliorated epithelial inflammation (32). Accordingly, kaempferol appeared to be effective in relieving the clinical symptoms of asthma with AHR. The present *in vivo* observations suggest that kaempferol administration may prevent OVA-induced airway disorders associated with immune suppression. Following activation by an immune stimulus, eosinophils degranulate to release an array of cytotoxic granule cationic proteins that are capable of inducing tissue damage and dysfunction (33). EMBP induces mast cell and basophil degranulation, being implicated in immune responses and remodeling (34). The OVA challenge-induced deposition of toxic EMBP declined in kaempferol-treated mice. Thus, kaempferol hampered degranulation of eosinophils acting as an autocrine mediator in the pathogenesis of eosinophil-associated diseases (20).

This study attempted to elucidate the possible mechanisms of kaempferol action in blunting airway allergic diseases. Kaempferol treatment suppressed LPS-induced epithelial eotaxin-1 expression most likely via disrupting JAK2 signaling. In addition, the epithelial inflammation by $\text{TNF}\alpha$ was induced through triggering $\text{NF-}\kappa\text{B}$ -responsive signaling, which was diminished by kaempferol. Similarly, this compound suppressed OVA challenge-elicited airway inflammation by antagonizing $\text{NF-}\kappa\text{B}$ activation. Activation of $\text{NF-}\kappa\text{B}$ in local immune cells during OVA challenge is critically involved in the effector phase of allergic airway disease (35). Collectively, a specific $\text{NF-}\kappa\text{B}$

inhibition in the lungs has therapeutic potential in the control of pulmonary allergy. The polyphenol naringenin chalcone suppresses asthmatic symptoms by inhibiting Th2 cytokine production from CD4 T cells (36). Unfortunately, this study did not examine Th1/Th2 cytokine-associated allergic responses. $\text{NF-}\kappa\text{B}$ as the master switch for proinflammatory genes was shown to transactivate arachidonic acid pathway enzymes when activated (37). Plant-derived compounds exhibit antiinflammatory activity in allergy and asthma via the suppression of the arachidonic acid pathway of phospholipase A2, cyclooxygenases, and lipoxygenases. Therefore, it is speculated that kaempferol prevented airway inflammation through abrogating the arachidonic acid pathway.

In summary, this study investigated the beneficial effects of the dietary compound kaempferol on endotoxin- or cytokine-associated airway inflammation. Nontoxic kaempferol suppressed LPS-induced eotaxin-1 protein expression, which was mediated likely via JAK2 signaling. Additionally, kaempferol attenuated $\text{TNF}\alpha$ -induced expression of epithelial ICAM-1 and eosinophil integrin $\beta 2$, and MCP-1 transcription encumbering eosinophil-epithelial interaction. In further *in vivo* BALB/c mouse study, kaempferol administration blunted eosinophil deposition and degranulation in airway and lung tissues elevated by OVA challenge. Moreover, the OVA challenge inflamed nuclear $\text{NF-}\kappa\text{B}$ transactivation. Therefore, kaempferol is effective in ameliorating allergic and inflammatory airway diseases through disturbing $\text{NF-}\kappa\text{B}$ signaling.

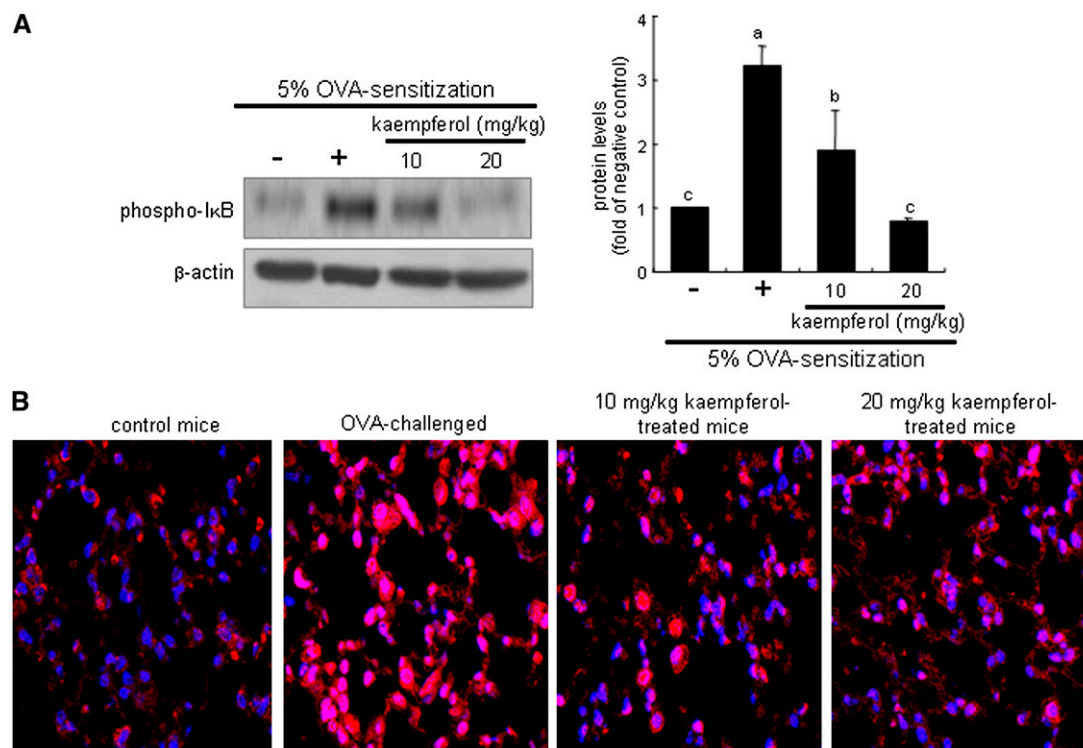


FIGURE 7 Attenuation of kaempferol administration on IκB phosphorylation (A) and NF-κB transactivation (B) in OVA-sensitized mice. Lung tissue extracts were subject to Western-blot analysis with antiphosphorylated IκB (A). β-Actin protein was used as an internal control. Quantitative densitometric data in A are mean ± SEM, *n* = 3. Means without a common letter differ, *P* < 0.05. Immunohistochemical analysis showing inhibition of NF-κB transactivation by kaempferol (B). NF-κB localization was visualized with a Cy3-conjugated secondary antibody. Nuclear staining was done with 4',6-diamidino-2-phenylindole. Original magnification of microscopic images (*n* = 3), 200×. IκB, inhibitory κB;

Acknowledgments

J.-H.G. and Y.-H.K. designed research; J.-H.G., D.S., and S.-Y.H. conducted research; D.S. and J.-L.K. analyzed data; D. S. and Y.-H.K. wrote the paper; and Y.-H.K. had primary responsibility for final content. All authors read and approved the final manuscript.

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