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ORIGINAL ARTICLE



Anti-inflammatory effects of embelin in A549 cells and human asthmatic airway epithelial tissues

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ABSTRACT

Objective: Allergic asthma is the most common type in asthma, which is defined as a chronic inflammatory disease of the lung. In this study, we investigated whether embelin (Emb), the major component of *Ardisia japonica* BL. (AJB), exhibits anti-inflammatory effects on allergic asthma via inhibition of NF- κ B activity using A549 cells and asthmatic airway epithelial tissues.

Methods: Inflammation was induced in A549 cells, a human airway epithelial cell line, by IL-1 β (10 ng/ml) treatment for 4 h. The effects of Emb on NF- κ B activity and COX-2 protein expression in inflamed airway epithelial cells and human asthmatic airway epithelial tissues were analyzed via western blot. The secretion levels of NF- κ B-mediated cytokines/chemokines, including IL-4, 6, 9, 13, TNF- α and eotaxin, were measured by a multiplex assay.

Results: Emb significantly blocked NF- κ B activity in IL-1 β -treated A549 cells and human asthmatic airway epithelial tissues. COX-2 expression was also reduced in both IL-1 β -treated A549 cells and asthmatic tissues. Emb application. Emb significantly reduced the secretion of IL-4, IL-6 and eotaxin in human asthmatic airway epithelial tissues by inhibiting activity of NF- κ B.

Conclusions: The results of this study suggest that Emb may be used as an anti-inflammatory agent via inhibition of NF- κ B and related cytokines.

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KEYWORDS

Embelin; allergic asthma; human asthmatic airway epithelial tissue; nuclear factor kappa B; COX-2

Introduction

Allergic asthma, also called as extrinsic asthma, is the most common type of asthma which is a disease that causes extra sensitivity to certain allergens, including wind-blown pollen, mold spores, animal dander, dust mite and cockroach feces^{1,2}. Allergic asthma is characterized by intermittent reversible obstruction and chronic inflammation of the external airways induced by airway hyper-reactivity (AHR)³, which leads to symptoms including dyspnea, wheezing and coughing⁴. In addition, lymphocytes and eosinophils are infiltrated within the airway submucosa in allergic asthma⁵. Inhaled allergens play a role in triggering of allergic asthma. Allergic asthma is correlated with abnormally increased immunoglobulin E (IgE), which responds to the presence of allergens^{6,7}. Allergic asthma affects over 50% of the 20 million patients who suffer from asthma. Furthermore, over 2.5 million children under the age of 18 suffer from allergic asthma. However, the reason for the increased prevalence of allergic asthma is not clearly understood. Indoor allergens, caused by changes in the residential environment, air pollution, indoor pollution due to smoking and gas fuels and even dietary changes have been hypothesized to be responsible for the increased frequency of allergic asthma.

Type-2 helper T (Th2) cells are considered to play an important role in AHR^{5,8}. Th2 cells, which secrete interleukin (IL)-4, IL-5, IL-9 and IL-13, are thought to contribute to allergic asthma⁹⁻¹². In mice models, Th2 cytokines play a critical role in the pathophysiology of asthma, including AHR and abnormal mucus secretion⁹. Moreover, IL-4 and IL-13 are important in airway dysfunction and remodeling in response to chronic allergen exposure^{13,14}.

NF- κ B is highly related to various inflammation diseases, such as rheumatoid arthritis, atherosclerosis, multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy, inflammatory bowel disease and asthma¹⁵⁻¹⁷. It induces the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, MMPs, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which are all important factors in the development of allergic asthma. Thus, transcription factor NF- κ B is a major modulator of inflammation in the pathogenesis of lung diseases, especially allergic asthma^{18,19}. A mouse model of allergic asthma that lacks airway epithelial NF- κ B activation exhibits a diminished inflammatory phenotype, including decreased production of mucus, Th2 cytokines and antigen-specific serum antibodies²⁰.

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Complementary-alternative medicines (CAMs) have been extensively used to treat allergic diseases; however, evidence to support their use is lacking^{21,22}. In traditional Korean medicine, *Ardisia japonica* BL. (AJB) in the Myrsinaceae family of plants has been prescribed as a herbal medicinal plant for treating cough and inflammation in the lung or liver²³. Embelin (Emb) is the primary active compound in AJB^{24,25}. Emb is a well-studied compound that activates the apoptotic pathway in Jurkat cells²⁶, and it also reduces inflammation and improves organ injuries in septic rats by downregulating the STAT3 and NF- κ B pathways^{27,28}. However, no clear studies have been published on the mechanism by which Emb functions in inflammation of the lung²⁹. Therefore, we investigated whether Emb influences NF- κ B protein expression in inflammation-induced human airway epithelial cells using western blot analysis. We also determined the effects of Emb on the protein expression level of NF- κ B by western blot analysis and subsequently the effects of Emb on NF- κ B-mediated pro-inflammatory cytokines/chemokines using a multiplex assay in human asthmatic airway epithelial tissues. Our results on the effects of Emb in IL-1 β -treated A549 cells and human asthmatic airway epithelial tissues show that Emb exhibits an anti-inflammatory mechanism in allergic asthma, implying its potential use as a remedial agent for allergic asthma.

Materials and methods

Materials

Emb was provided by the laboratory of Prof. Kwang-Seok Ahn in the Department of Pathology at the College of Korean Medicine at Kyung Hee University. Dexamethasone (Dex) was obtained from Sigma-Aldrich (St. Louis, MO). IL-1 β was purchased from R&D Systems, Inc. (Minneapolis, MN). Sterile assay medium was purchased from MatTek Corp. (Ashland, MA). Protease inhibitors (complete mini EDTA-free) were obtained from Roche Diagnostics (Mannheim, Germany). Human Cytokine 6-plex Express Immunoassay kit (TNF- α , Eotaxin, IL-4, IL-6, IL-9 and IL-13) and the protein concentration assay reagent were purchased from Bio-Rad (Hercules, CA). Mouse anti- β -actin, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Dallas, TX).

Cell line and culture condition

All *in vitro* experiments were performed in A549 cells (American Type Culture Collection, Rockville, MD). The cells were cultured in RPMI 1640 medium (Corning Inc., NY) containing 10% FBS (Gibco, NY) and 1x antibiotic-antimycotic solution (ABAM, Corning Inc., NY) and were incubated at 37°C and 5% CO₂. To evaluate the cytotoxicity of Emb, A549 cells were seeded in a 96-well plate (1 × 10⁴ cells/well) and were incubated at 37°C and 5% CO₂ without serum overnight. After serum starvation, the cells were incubated with RPMI medium containing IL-1 β (10 ng/ml) for 4 h. Then, the cells were treated with different doses of Emb (1 to 10 μ M) for 12 h. Thirty microlitres of MTT solution (5 mg/ml) was

Table 1. Description of asthmatic human airway epithelial tissue donors.

Age	Sex	Race	Smoking	Disease	Cause of death	Medication
7	F	C	N	Asthma		Albuterol
9	F	B	N	Asthma since birth	Possible asthma attack	Albuterol
16	M	B	N	Asthma		Advair

B: Black; C: Caucasian; F: female; M: male; N: none.

added to each well, and the cells were incubated for 2 h. The MTT-containing medium was removed, and 50 μ l of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was added. The optical density of formazan was measured using an automated spectrophotometric plate reader at 570 nm.

Human asthmatic airway epithelial tissue culture

Human asthmatic airway epithelial tissues grown in 24-well plates on collagen-coated transwell membranes at an air-liquid interface were provided by MatTek Corp. (Ashland, MA), and donor information is shown in Table 1. According to the manufacturer's guidelines, the tissues were equilibrated. Briefly, on arrival, the *ex vivo* epithelial tissues were washed with Dulbecco's phosphate-buffered saline (D-PBS) to remove coated collagen and were carefully transferred to 6-well plates with 1 ml of sterile assay media (MatTek Corp.) per well. At this time, the state of the tissue is in the state that one side is air and the other side is in the media. The tissues were equilibrated at 37°C and 5% CO₂ for 16 h. After equilibration, dissolve each appropriate concentration of drug (5 μ M Dex and 25 μ M Emb) in the media provided by the company and replace it with the current media. Then, the tissues were incubated for 24 h at 37°C and 5% CO₂.

Western blot analysis

For the *in vitro* experiments, A549 cells were seeded in 6-well plates (5 × 10⁵ cells/well) and incubated at 37°C and 5% CO₂ overnight. After serum starvation and inflammation induction using IL-1 β , the cells were treated with Dex (1 μ M) or Emb (5 μ M) for 12 h, and the cells were lysed with cell lysis buffer (Cell Signalling Technology; Danvers, MS). The tissues were homogenized using a pestle. Each sample lysate from cells or tissues was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatants were collected and transferred to a fresh tube. Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) was used to measure protein concentrations. Each sample was separated in a 10% SDS-PAGE gel at 120 V for 120 min and then electro-transferred to a nitrocellulose membrane for 100 min at 25 V. The membranes were blocked with 5% BSA in TBST (Tris-buffered saline with 0.1% Tween 20 and 0.04% Na₃). Primary antibodies, that is, rabbit anti-phospho-NF- κ B p65, rabbit anti-NF- κ B p65, rabbit anti-COX-2 (all 1:1000) and mouse anti- β -actin (1:3000), were incubated with the membrane overnight at 4°C. Membranes were extensively washed and then incubated with the following secondary antibodies: goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP. The protein expression levels were measured using a chemiluminescence imaging system (Davinci-Chemi, CoreBio Systems, South Korea)³⁰.

Analysis of pro-inflammatory cytokines

One day after the indicated drug treatment, the medium from each treatment was collected and centrifuged at $1000 \times g$ for 10 min, and each centrifuged supernatant was transferred to a fresh tube. The Human Cytokine 6-plex Express (TNF- α , Eotaxin, IL-4, IL-6, IL-9 and IL-13; Bio-Rad, Hercules, CA) immunoassay was performed with a 1×96 -well flat bottom plate according to the manufacturer's guidelines.

Statistical analysis

All data are presented as the mean $\pm$ SEM. The GraphPad Prism 5 software package (GraphPad Software, San Diego, CA) was used to conduct the statistical analysis of the results. An unpaired *t*-test (one-tailed) was conducted to analyze

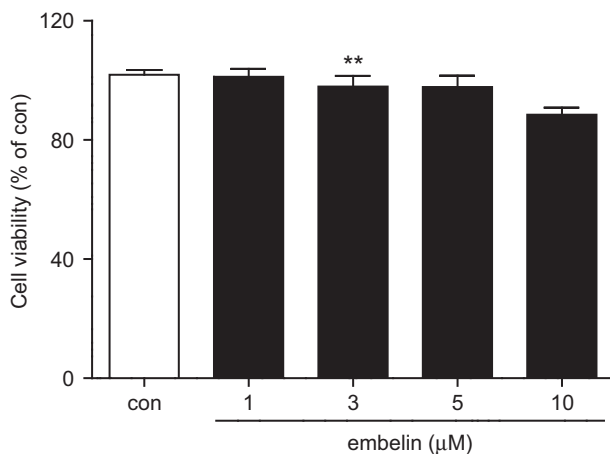


Figure 1. Effect of Emb on cell viability in IL-1 β -treated A549 cells. After treatment of IL-1 β (10 ng/ml), the cells were treated with different doses of Emb (1 to 10 μ M) for 12 h. The MTT assay was performed afterward. Cytotoxicity was shown at 10 μ M of Emb but was not detected at other concentrations. Statistical significance was determined by unpaired *t*-test (one-tailed) compared to control group. ***p* < .01.

western blot bands and multiplex assay results. A *p* values less than .05 was considered statistically significant.

Results

Cytotoxic effects of Emb in IL-1 β -treated A549 cells

We examined the cytotoxic effects of Emb on IL-1 β -treated A549 cells. Cell viability was assessed using an MTT assay. We observed no cytotoxicity induced by Emb up to 5 μ M in IL-1 β -treated A549 cells (Figure 1).

Inhibitory effects of Emb on NF- κ B activation in inflamed A549 cells

The NF- κ B pathway plays a central role in immune and inflammatory responses as the nuclear translocation of the NF- κ B p65 subunit stimulates the transcription of various pro-inflammatory genes, including iNOS and COX-2. Therefore, after inflammation was induced in A549 cells with IL-1 β (10 ng/ml) for 4 h, we observed the changes in NF- κ B by Emb treatment in inflamed human lung epithelial cells. Fully inflamed A549 cells were treated with saline for a control group, the anti-inflammatory molecule Dex (1 μ M) was used as a positive control. Emb at 5 μ M concentration, based on our MTT assay results, was treated in the IL-1 β -induced inflammatory A549 cells. As shown in Figure 2, Emb significantly decreased the ratio of phospho-NF- κ B p65 to total NF- κ B p65 in inflamed A549 cells.

Inhibitory effects of Emb on COX-2 expression in inflamed A549 cells

Considering the effects of Emb on NF- κ B activation, we then investigated whether Emb suppressed expression of COX-2, a downstream mediator of NF- κ B activation by western blot analysis. COX-2 is an enzyme that produces prostaglandins

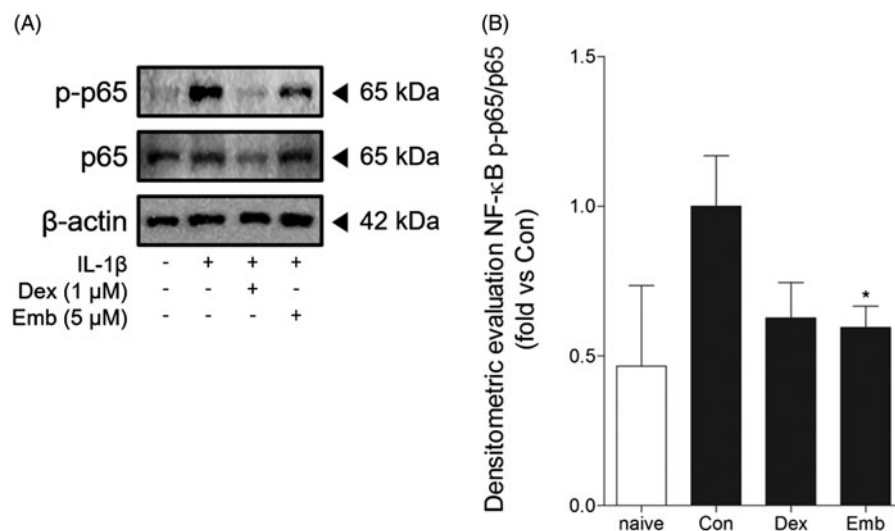


Figure 2. Suppression of IL-1 β -induced NF- κ B activity by Emb in A549 cells. Cells were pretreated with IL-1 β (10 ng/ml) for 4 h, and fully inflamed A549 cells were treated with the indicated drugs for 12 h. (A) The western blot results are representative of three separate experiments. Anti- β -actin antibody was used as an internal control. (B) Ratio of phospho-p65 to p65 expression was measured by ImageJ. Statistical significance was determined by unpaired *t*-test (one-tailed) compared to IL-1 β -induced inflamed A549 cells. Values are expressed as the mean $\pm$ SEM; **p* < .05.

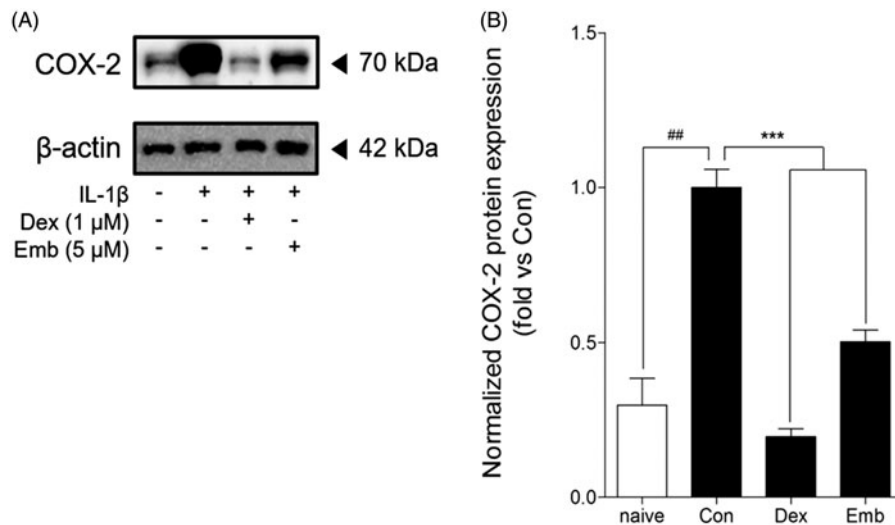


Figure 3. Effects of Emb on COX-2 expression in IL-1 β -treated A549 cells. (A) The western blot results are representative of three separate experiments. (B) COX-2 expression level normalized to β -actin was measured by densitometry analysis and is represented as a bar chart. Statistical significance was determined by unpaired *t*-test (one-tailed) compared to IL-1 β -treated A549 cells. Values are expressed as the mean $\pm$ SEM; ##*p* < .01; ****p* < .001.

which increase inflammation, pain and fever. As shown in Figure 3, the non-treated control cells expressed normally low but detectable levels of COX-2 protein. However, COX-2 protein was significantly elevated in response to IL-1 β (10 ng/ml), while the treatment with Emb (5 μ M) for 12 h resulted in a decrease in the IL-1 β -stimulated COX-2 (Figure 3).

Effects of Emb on NF- κ B activation in human asthmatic airway epithelial tissues

Based on our *in vitro* results, we hypothesized that Emb is able to inhibit NF- κ B activation in asthmatic airway tissues as well. We thus investigated the effect of Emb on NF- κ B activation in human asthmatic airway epithelial tissues. Human asthmatic airway epithelial tissues were treated with saline, 5 μ M Dex or 25 μ M Emb. Western blot analysis was used to evaluate the changes in NF- κ B activation. We observed a significantly reduced ratio of phospho-NF- κ B p65 to total NF- κ B p65 in the Emb-treated tissues compared with saline-treated tissues (Figure 4).

Inhibitory effects of Emb on COX-2 protein expression in human asthmatic airway epithelial tissues

Because we found that Emb reduced COX-2 protein expression in inflamed cells, we investigated the effects of Emb on COX-2 in human asthmatic airway epithelial tissues by the western blot method. Emb treatment reduced the COX-2 expression in human airway tissues, with no statistical significance (Figure 5).

Effects of Emb on NF- κ B-mediated pro-inflammatory cytokines secretion in human asthmatic airway epithelial tissues

The cytokine and chemokine levels in media were determined by a multiplex assay as described in the Materials and

Methods section. The Emb-treated tissues showed significantly decreased NF- κ B-mediated pro-inflammatory cytokines/chemokines, including IL-4, IL-6 and eotaxin (Figure 6(A–C)). No significant decrease was observed in IL-9, IL-13 or TNF- α , but a decreasing tendency was found in Emb-treated tissues compared to control tissues (Figure 6(D–F)).

Discussion

Allergic asthma is a chronic inflammatory disease of the lung²⁰, which is characterized by reversible obstruction of AHR, infiltration of inflammatory cells into lung tissues, and mucus overproduction. The over-expressed Th2-mediated cytokines including IL-4, IL-5, IL-9, IL-13 and TNF- α , and chemokines such as eotaxin and RANTES, in the airways is another feature of allergic asthma³¹. Because allergic asthma is one of the most common diseases and the mortality has increased worldwide, understanding the pathogenesis of allergic asthma is important. However, the mechanisms involved in allergic asthma have not been clearly elucidated^{20,29}. In addition, the limited understanding of its molecular pathogenesis makes it difficult to develop effective drugs for allergic asthma. Current treatments for allergic asthma contain adrenocortical steroids or β 2-agonists. Adrenocortical steroids reduce inflammation in the airways. Although they are an effective treatment for asthmatic airway disease, prolonged use can lead to metabolic disorders such as hyperglycemia, hypertension, obesity and depression^{32,33}. On the other hand, β 2-agonists which mitigate the symptoms in bronchial tubes are divided into three categories based on treatment method: oral administration, inhalation or injection. Despite their effectiveness on relieving asthmatic symptoms, both adrenocortical steroids and β 2-agonists exhibit crucial side effects, such as adrenal suppression, nausea and loss of mineral density in bone structure^{34–36}.

NF- κ B is normally present in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κ B (I κ B). When NF- κ B translocates into the nucleus, it acts as

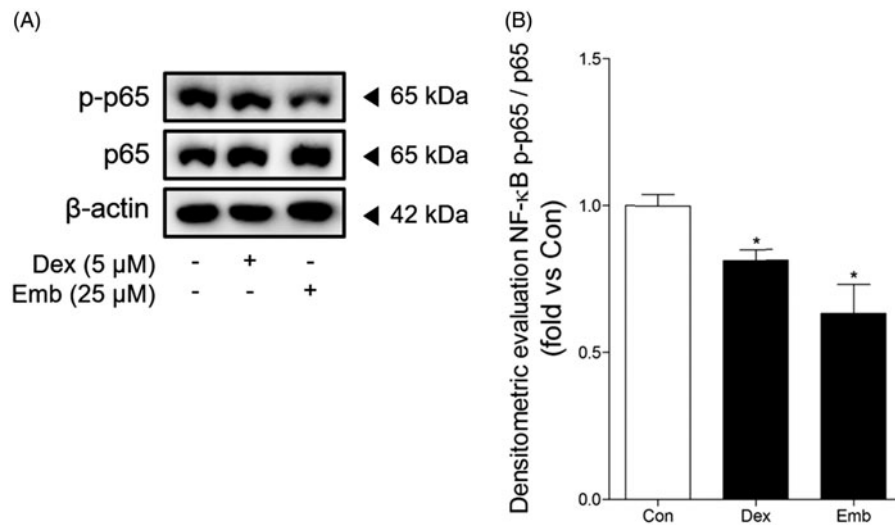


Figure 4. Effect of Emb on NF-κB activation in human asthmatic airway epithelial tissues. (A) Human asthmatic lung tissues were treated with Dex as a positive control or Emb, and whole-tissue lysates were prepared and then subjected to western blot analysis with anti-NF-κB phospho-p65 or anti-NF-κB p65 antibody. Anti-β-actin antibody was used as an internal control. (B) Ratios of phospho-p65 to p65 expression were measured by ImageJ and are represented as a bar chart. Statistical significance was determined by unpaired *t*-test (one-tailed) compared to control. Values are expressed as the mean ± SEM; **p* < .05.

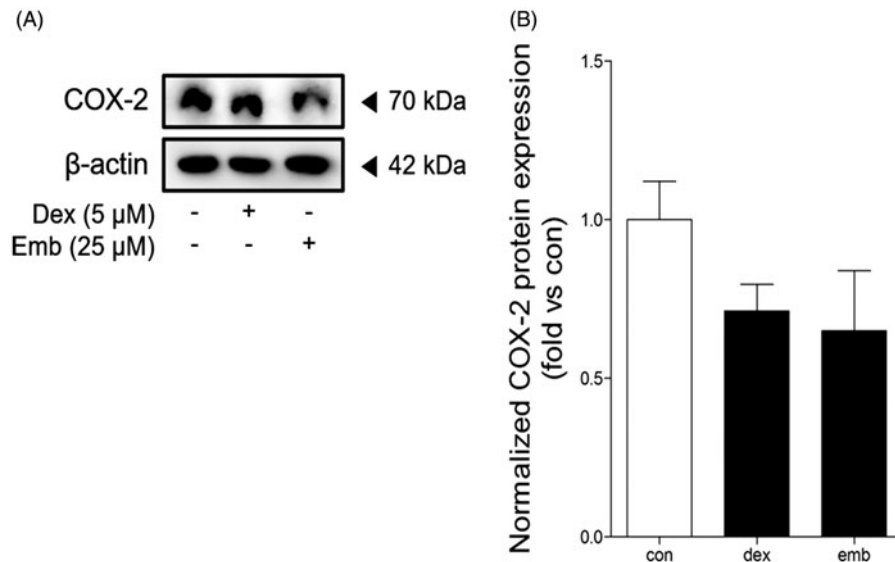


Figure 5. Effect of Emb on COX-2 protein expression inhibition in human asthmatic airway epithelial tissues. (A) Human asthmatic lung tissues were treated with Dex as a positive control or Emb, and whole-tissue lysates were prepared and then subjected to western blot analysis with anti-COX-2 antibodies. Anti-β-actin antibody was used as an internal control. The blots were normalized to β-actin expression levels and compared to the saline-treated tissue. Normalized COX-2 expression was measured by ImageJ and is represented as a bar chart (B). Statistical significance was determined by unpaired *t*-test (one-tail) compared to control. Values are expressed as the mean ± SEM.

a key player in the pathogenesis of inflammation by regulating the expression of multiple genes involved in immune responses^{15,37}. In human and animal lung tissues, increased NF-κB activity correlates with asthmatic inflammation³⁸. Moreover, distinct profiles of inflammatory cytokines, lymphocyte cell surface receptors and adhesion molecules are up-regulated in asthmatic airways³⁹. Due to the crucial role of NF-κB in inflammation, NF-κB has been targeted for directed therapy studies. In the present study, we demonstrated that Emb significantly blocked NF-κB activity in IL-1β-induced inflammatory A549 cells, a human airway epithelial cell line (Figure 2) and in human asthmatic airway epithelial tissues (Figure 4). This result on NF-κB suggested that the

possible reducing-effect of Emb on the genes regulated by NF-κB activation, such as COX-2.

Our results on the effects of Emb treatment in IL-1β-treated A549 cells and human asthmatic airway epithelial tissues supported the hypothesis that Emb may affect proteins downstream of NF-κB, such as COX-2. COX-2 has been identified in epithelial cells, macrophages, fibroblasts, smooth muscle cells and mast cells⁴⁰ and has critical functions in immunity, renal physiology, neurotransmission, bone reabsorption and pancreatic secretion^{41,42}. Using western blot analysis, we observed that Emb treatment significantly reduced the IL-1β-induced up-regulation of COX-2 expression in A549 cells (Figure 3). Emb-treated human asthmatic airway

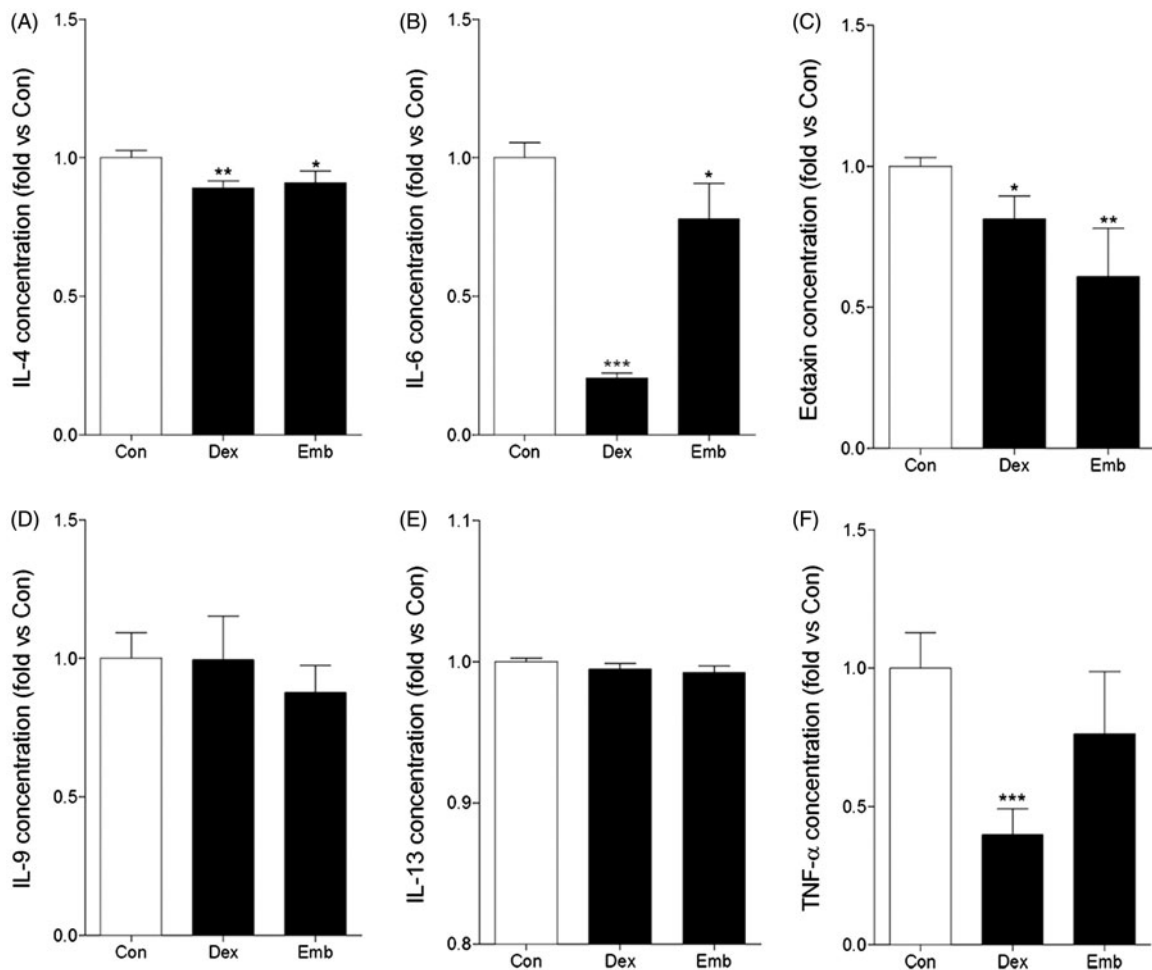


Figure 6. Effect of Emb on cytokine/chemokine levels as measured by multiplex array analysis. (A) to (C) Emb suppressed secretion of NF- κ B-mediated pro-inflammatory cytokines IL-4, IL-6 and eotaxin. Statistical significance was determined by unpaired t-test (one-tailed) compared to control, and the values are expressed as the mean $\pm$ SEM; * p < .05; ** p < .01; *** p < .001.

epithelial tissues also showed a decreasing trend in COX-2 expression compared to nontreated tissues, although these results were not statistically significant (Figure 5). The reduction of COX-2 expression by Emb may lead to inhibition of prostaglandin E2 (PGE2), an important mast cell mediator and a powerful modulator of immune responses that promotes inflammation, swelling, pain and fever⁴³.

Various cytokines and chemokines participate in the pathophysiology of allergic asthma. Some of these cytokines, including IL-1, IL-6 and TNF- α , are involved in many inflammation-related diseases, such as chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA) and inflammatory bowel disease. Other cytokines are more involved in allergic inflammation^{9,44}. Th2 cytokines, such as IL-4, IL-5, IL-9 and IL-13, all derived from Th2 cells⁴⁵, play important roles in the pathophysiology of allergic diseases including allergic asthma⁹. Thus, many studies on respiratory research focus on the action of Th2 cytokines. IL-4 regulates allergic inflammation in the pathogenesis of allergic asthma by encouraging the differentiation of naïve T cells into Th2 cells and IgE synthesis^{46,47}. IL-9 appears to increase Th2-cell-mediated responses and stimulate cell proliferation while inhibiting apoptosis. IL-13 acts similarly to IL-4, causing IgE

production under the conditions of allergic asthma. The role of IL-4 and IL-13 differ, as IL-13 does not regulate the differentiation of naïve T cells into Th2 cells. TNF- α , primarily produced by activated macrophages, is a cytokine involved in systemic inflammation and also one of the most important cytokine that comprises the acute phase reaction. It is produced in response to bacterial products, such as LPS or IL-1, and signals immune cells to induce inflammation along with IL-6. Moreover, TNF- α stimulates the production of PGE2 through the activation of COX-2. Peculiarly, IL-6 is a pro- and anti-inflammatory cytokine. It is secreted by T cells and stimulates immune responses to burns or other tissue damage, leading to inflammation. Moreover, IL-6 is secreted by macrophages in response to specific microbial molecules and acts as an anti-inflammatory cytokine by inhibiting TNF- α and IL-1⁴⁸. Eotaxin is implicated in allergen-induced eosinophil responses in the airways³. In this study, we observed that Emb significantly reduced the secretion of IL-4, IL-6 and eotaxin in human asthmatic airway epithelial tissues by inhibiting NF- κ B activity. Emb suppressed the secretion of eotaxin even more than Dex, which is widely used as a treatment for allergic asthma (Figure 6). Reduced cytokine secretion due to Emb treatment indicates that Emb may regulate the synthesis

of IgE, the differentiation of naïve T cells into Th2 cells by blocking IL-4 secretion, and the eosinophil responses via inhibitory effects on eotaxin secretion.

Conclusions

Our data suggest that Emb regulates inflammatory reactions in the airways by inhibiting NF- κ B activity, which was shown in both A549 cells exhibiting IL-1 β -induced inflammation and in human asthmatic airway epithelial tissues. We demonstrated the inhibitory effect of Emb on NF- κ B activity, COX-2 levels and the secretion of NF- κ B-mediated pro-inflammatory cytokines, including IL-4, IL-6 and eotaxin, in human asthmatic airway epithelial tissues. The results of this study are expected to provide direction for future research on the pathogenesis of allergic asthma and mechanisms of therapeutic agents in humans, and eventually improve safety in patients with allergic asthma and other inflammation-related diseases.

Disclosure statement

There is no conflict of interest in our study.

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