

Caffeic acid phenethyl ester attenuates allergic airway inflammation and hyperresponsiveness in murine model of ovalbumin-induced asthma

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Abstract

Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of propolis, which has several interesting biological properties, including antioxidant and anti-inflammatory; however, its anti-allergic effects are poorly understood. The objective of this study was to determine whether treatment with CAPE results in significant inhibition of asthmatic reactions in a mouse model. Mice sensitized and challenged with ovalbumin (OVA) had the following typical asthmatic reactions: an increase in the number of eosinophils in bronchoalveolar lavage (BAL) fluid; a marked influx of inflammatory cells into the lung around blood vessels and airways, and airway luminal narrowing; the development of airway hyperresponsiveness (AHR); the presence of tumor necrosis factor- α (TNF- α) and Th2 cytokines, including IL-4 and IL-5, in the BAL fluid; and the presence of allergen-specific IgE in the serum. Five successive intraperitoneal administrations of CAPE before the last airway OVA challenge resulted in significant inhibition of characteristic asthmatic reactions. We determined that increased generation of reactive oxygen species (ROS) by inhalation of OVA was diminished via the administration of CAPE in BAL fluid, as well as nuclear factor- κ B (NF- κ B) DNA binding activity. These findings indicate that oxidative stress may have a crucial function in the pathogenesis of bronchial asthma, and that CAPE may be useful as an adjuvant therapy for the treatment of bronchial asthma.

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Keywords: Asthma; Caffeic acid phenethyl ester; Reactive oxygen species; Airway hyperresponsiveness; Nuclear factor- κ B

Introduction

Asthma is a complex inflammatory disease of the lungs that is characterized by variable airflow obstruction, airway

hyperresponsiveness (AHR), and airway inflammation. The inflammatory response in the asthmatic lung is characterized by infiltration of the airway wall by mast cells, lymphocytes, and eosinophils, and is associated with the increased expression of several inflammatory proteins, including cytokines, enzymes, and adhesion molecules, within the airways (Barnes, 1994, 2003). Oxidative stress is induced by a large variety of free oxygen radicals, including reactive oxygen species (ROS). An increasing amount of clinical and experimental evidence suggests that ROS perform an essential

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function in the pathogenesis of airway inflammation (Kwak et al., 2003; Hamelmann et al., 1997; Lee et al., 2002). ROS secreted by eosinophils and other leukocytes infiltrating the airways is extremely relevant to the airway tissue injury and remodeling process associated with asthma. In an earlier study, we demonstrated that oxidative stress is one of the crucial determinants of asthma, and that treatment with an antioxidant may constitute a useful therapeutic strategy (Lee et al., 2004).

The induction of chronic cytokine expression, and the recruitment and activation of inflammatory cells in asthma involve the increased transcription of inflammatory cytokine genes, which is regulated via transcription factors (Barnes and Adcock, 1998). The nuclear factor (NF)- κ B is one of the transcription factors that induce a variety of genes involved in immune and inflammatory responses (Blackwell and Christman, 1997; Wulczyn et al., 1996). NF- κ B is a member of the Rel family of proteins, a novel family of ubiquitous transcription factors that share a common structural motif for both DNA binding and dimerization. The most prevalent activated form of NF- κ B is a heterodimer that consists of the p50 and p65 subunits, and has the strongest transactivation activity among NF- κ B dimers, as well as the most widely distributed NF- κ B binding activity. The NF- κ B dimers are usually present in the cytoplasm of most cells in an inactive form, due to the fact that they bind to an inhibitor protein referred to as I κ B (Baldwin, 1996). After an inflammatory stimulus, the phosphorylation of I κ B triggers their degradation and the translocation of NF- κ B to the nucleus, where it induces the expression of a broad variety of genes in inflammation, including cytokines (e.g. IL-1, IL-6, and TNF- α), enzymes (including nitric oxide synthase), adhesion molecules, and acute-phase proteins (Barnes and Karin, 1997). The previous studies have suggested that NF- κ B activity may be involved in the pathogenesis of asthma, given that its increased activation has been demonstrated in the lungs following allergen challenge (Burrows et al., 1989), and in the airway epithelial cells and macrophages of asthmatic patients (Burrows, 1995). In an earlier study, we showed that NF- κ B has a crucial function in the pathogenesis of asthma in mice (Choi et al., 2004).

Caffeic acid phenethyl ester (CAPE), a potent flavonoid antioxidant, is the active component of honeybee (*Apis mellifera*) propolis. It has profound antiviral (Fesen et al., 1993), antitumoral (Liao et al., 2003), anti-inflammatory (Orban et al., 2000), antioxidant (Hepsen et al., 1999), neuroprotective (Ilhan et al., 2004; Wei et al., 2004), antiatherosclerotic (Hishikawa et al., 2005), and immunomodulatory properties (Park and Kang, 1999) in a variety of systems. These diverse effects of CAPE may be attributed to its ability to permeate the cell membrane (Orsolic et al., 2005). CAPE inhibits certain enzyme activities, including lipooxygenase, cyclooxygenase, glutathione *S*-transferase, and xanthine oxidase (Koshihara et al., 1984; Mirzoeva et al., 1996; Michaluart et al., 1999; Chan et al., 1995), and is a potent and specific inhibitor of NF- κ B activation (Natarajan et al., 1996). CAPE may be associated with a reduced incidence of allergic asthma; however, there is no experimental evidence to support this suggestion. The objective of this study was to determine the

effect of CAPE on ovalbumin (OVA)-induced murine allergic airway reactions.

Materials and methods

Animals

Female BALB/c mice were obtained from the Charles River Laboratories (Yokohama, Japan), and were kept in our animal facility for at least 1 week before use. All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Inje University Medical School. All mice were at 6–8 weeks of age.

Immunization and challenge

Mice were immunized on days 1 and 15 by intraperitoneal injection of 20 μ g OVA emulsified in 1.0 mg aluminium hydroxide adjuvant in a total volume of 200 μ l, as described previously (Kim et al., 2004). Mice were challenged via the airway with OVA (50 mg/ml of saline) each day from days 22 to 24 on consecutive days. Control mice were exposed to aerosolized saline. Aerosolization was performed for 20 min by placing the mice in a chamber (15 $\times$ 25 $\times$ 15 cm) connected to the ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan). Mice were injected intraperitoneally with 10 mg/kg/day in 200 μ l of CAPE (Sigma, St. Louis, MO) each day from days 16 to 20 on consecutive days. In the preliminary study (Park et al., 2008; Kuo et al., 2006), the optimal dosage of CAPE with non-toxic and potent therapeutic effect was determined as 10 mg/kg/day.

BAL collection and cell counting

Mice were anaesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 0.7 ml of PBS. The BAL fluid samples collected and the number of cells in a 50 μ l aliquot were determined using a haemocytometer. The remaining sample was centrifuged, and the supernatant was stored at -70° until assay of cytokines. The pellet was resuspended in PBS and cytospin preparations of BAL cells were stained with Diff-Quik (International reagents Corp., Kobe, Japan). Two independent, blinded investigators counted the cells, using a microscope. Approximately, 400 cells were counted in each of four different random locations.

Measurement of intracellular ROS

ROS were measured by a method described previously with modification (Sundaresan et al., 1995). BAL fluids were washed with phosphate-buffered saline (PBS). To measure intracellular ROS, cells were incubated for 10 min at room temperature with PBS containing 3.3 μ M 2',7'-dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR), to label intracellular ROS. The cells were then immediately observed fluorescence-

activated cell sorting (FACS) analysis (BD Biosciences, San Jose, Ca).

Cytokine assays

The levels of cytokines in bronchoalveolar lavage (BAL) fluid were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits from R&D Systems (Minneapolis, MN) were employed for the measurement of IL-4, IL-5, IFN- γ , and TNF- α .

Determination of AHR

AHR was measured at 3 days after the last OVA challenge in mice in an unrestrained conscious state, as described previously (Hamelmann et al., 1997). Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) in increasing concentrations (from 2.5 to 50 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, readings were taken and were averaged for 3 min after each nebulization. The bronchopulmonary resistances is expressed as enhanced pause (Penh), calculated as: $[\text{expiratory time } T_e] / [\text{relaxation time } (RT) - 1] \times [\text{peak expiratory flow (PEF)} / \text{peak inspiratory flow (PIF)}]$, according to the manufacturer's protocol. Results are expressed as the percentage increase of Penh following challenge with each concentration of Mch, where the baseline Penh (after saline challenge) is expressed as 100%.

Histological studies

At 48 h after the last challenge, lungs were removed from the mice after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). An inflammation score was graded by three independent investigators who were not associated with this study.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared using NE-PER nuclear extraction reagent (Pierce). As a probe for the gel retardation assay, an oligonucleotide harboring the immunoglobulin κ -chain binding site (κB , 5'-CCGGTTAACAGAGGGGGCTTCCGAG-3') was synthesized. A non-radioactive method whereby the 3' end of the probe was labeled with biotin was employed in these experiments (Pierce). The binding reactions contained 10 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 1 mM dithiothreitol, 0.05% Nonidet P-40,

and 2.5% glycerol), 50 ng of poly (dI–dC), and 20 fM of biotin-labeled DNA. The reactions were incubated for 20 min at room temperature in a final volume of 20 μL . The competition reactions were conducted via the addition of a 100-fold excess of cold κB or irrelevant oligonucleotide (cAMP response element (CRE)) to the reaction mixture. The reaction mixture was electrophoretically analyzed on 5% polyacrylamide gel in $0.5 \times \text{Tris}$ –borate buffer. The reactions were transferred to nylon membranes. The biotin-labeled DNA was detected using a LightShift chemiluminescent electrophoretic mobility-shift assay kit (Pierce).

Western blot analysis

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. The samples were loaded to 10% SDS-PAGE gels and were electrotransferred to nitrocellulose. The blots were incubated with an anti-NF- κB p65 (Upstate Biotech, Lake Placid, NY) overnight at 4 $^{\circ}\text{C}$. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody. Following three washes with TBST, immunoreactive bands were visualized using ECL detection system (Pierce). In a parallel experiment, nuclear protein was prepared by nuclear extraction reagents (Pierce) according to the manufacturer's protocol.

Measurement of OVA-specific serum levels of IgE

OVA-specific serum IgE levels were determined by Enzyme Linked Immuno Sorbent Assay (ELISA) using samples collected 12 h after the last OVA challenge, as described previously (Ohno et al., 2005). In brief, a 96 well microtitre plate was coated with OVA (10 mg/ml), and then treated with mouse sera followed by biotin-conjugated rat anti-mouse IgE (pharmingen, San Diego, CA). Then, avidin–horseradish peroxidase (HRP) solution was added to each well. Units are optical density readings at 405 nm.

Measurement of eosinophil peroxidases (EPO)

The suspension of BAL cells and the pulmonary homogenates were frozen/thawed three times using liquid nitrogen and a water bath at 37 $^{\circ}\text{C}$ to obtain the EPO. The BAL fluid was centrifuged to 4 $^{\circ}\text{C}$ for 10 min and serially diluted in a 96-well plate (75 μL /well) followed by the addition of 150 μL of substrate (1.5 mM *o*-phenylenediamine and 6.6 mM H_2O_2 in 0.05 M Tris–HCl, pH 8.0). After 30 min at room temperature, the reaction was stopped by the addition of 75 μL of 30% H_2SO_4 , and the absorbance of the samples was determined at 492 nm on an ELISA reader.

Statistics

Data are expressed as mean $\pm$ S.E.M. Statistical comparisons were performed using one-way ANOVA followed by the Fisher's test. Significant differences between 2 groups were

determined using the unpaired Student's *t* test. Statistical significance was set at $p < 0.05$.

Results

Effect of CAPE on cellular changes in BAL fluid

In the murine asthma model, OVA sensitization and challenge markedly increased the number of inflammatory cells in the BAL fluid as compared to the control group.

The total number of cells in the BAL fluid was increased significantly, by ~4.3-fold, and the number of eosinophils was increased by ~18-fold as compared to those in the control group 2 days after the final OVA challenge. Interestingly, the number of eosinophils observed in the BAL fluid of the CAPE-treated group of mice was only 5.3-fold that of the OVA-challenged group (Fig. 1A).

Effects of CAPE on pathological changes in OVA-induced asthma

The results of histological examinations of lung tissues paralleled the results of analysis of the cell numbers in the BAL

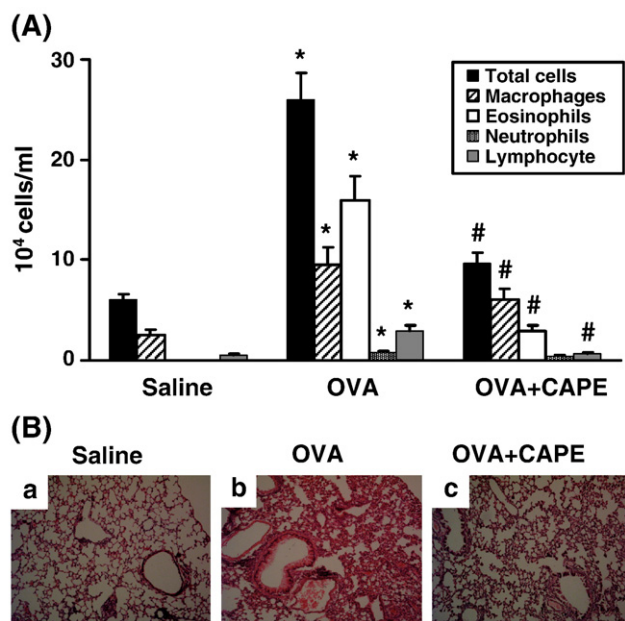


Fig. 1. Effect of CAPE on the recruitment of inflammatory cells into BAL (A) and pathological changes in lung tissues (B) in OVA-induced allergic asthmatic mice. (A) Mice were treated with the Saline, OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CAPE 10 mg/kg (OVA+CAPE), respectively. BAL cells were separated using a Cytospin, and then stained with Diff-Quik. Differential cell counting was performed using standard morphological criteria. The BAL cells were collected 2 days after the last OVA challenge. Data are expressed as the mean $\pm$ S.E.M. of six independent experiments. This experiment used 5 mice per group ($n=5$). * $p < 0.05$ vs. saline-treated mice; # $p < 0.05$ vs. OVA-treated mice. (B) Sections were obtained from the lungs of mice receiving saline, OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CAPE (10 mg/kg, OVA+CAPE), respectively. Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining ($\times 200$). Six animals were assigned to each group.

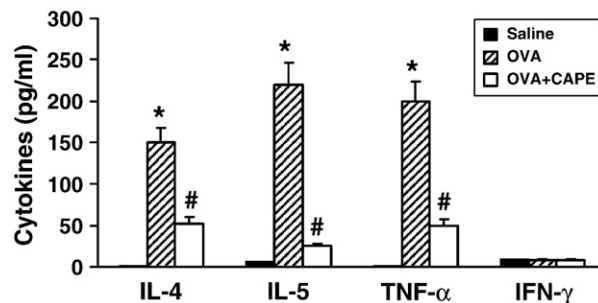


Fig. 2. The effect of CAPE (10 mg/kg) on IL-4, IL-5, and TNF- α cytokine levels. OVA-sensitized mice were treated as described in methods. Bronchoalveolar lavage (BAL) was performed 4 h after the last airway challenge. IL-4, IL-5, and TNF- α cytokine levels in the BAL fluids were measured by ELISA Kit. Data represent means $\pm$ S.E.M. from five independent experiments of the response in 5–7 animals. * $p < 0.05$ vs. saline-treated mice; # $p < 0.05$ vs. OVA-treated mice.

fluid. Marked influxes of inflammatory cells into the airway and around the blood vessels were observed in the OVA-sensitized/challenged mice (Fig. 1B, OVA), but not in the saline-treated control mice (Fig. 1B, Saline). Mice treated with CAPE showed marked reductions in the infiltration of inflammatory cells within the peribronchiolar and perivascular regions (Fig. 1B, OVA+CAPE). Mucus hypersecretion and airway occlusion were the prominent histopathological features of the murine asthmatic lung. Mucus cell hypertrophy and airway luminal narrowing induced by secreted mucus were observed in the OVA-sensitized/challenged mice (Fig. 1B, Saline). The administration of CAPE (10 mg/kg) resulted in a marked improvement of luminal narrowing by reduced mucus secretion in the airway (Fig. 1B, OVA+CAPE). These results indicate that

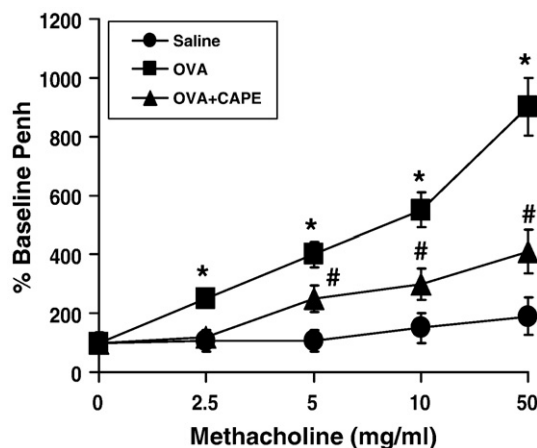


Fig. 3. Effect of CAPE on airway responsiveness in OVA-sensitized mice and OVA-challenged mice. Airway responsiveness was measured at 72 h after the last airway challenge. CAPE (10 mg/kg) was administered i.p. from last sensitization to 1 day before the first airway challenge. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with saline, then with increasing doses (2.5 to 50 mg/ml) of methacholine for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization during which Penh values were determined. Data represent mean $\pm$ S.E.M. from 6 independent experiments. This experiment used 5 mice per group ($n=5$). * $p < 0.05$ vs. saline-treated mice; # $p < 0.05$ vs. OVA-treated mice.

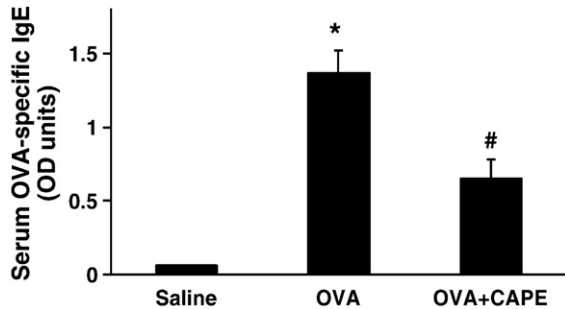


Fig. 4. Effect of CAPE on serum OVA-specific IgE antibody levels. Mice were sensitized and challenged as described in methods. The levels of OVA-specific IgE were measured at 12 h after the last airway challenge. Blood was collected by cardiac puncture for measurement of OVA-specific IgE with ELISA. CAPE (10 mg/kg) was administered i.p. from last sensitization to 1 day before the first airway challenge. Data represent means $\pm$ S.E.M. from five independent experiments of the response in 5–7 animals. * $p < 0.05$ vs. saline-treated mice; # $p < 0.05$ vs. OVA-treated mice.

CAPE is very capable of inhibiting the development of allergic status induced by OVA in mice.

Effect of CAPE on BAL fluid cytokines

We used ELISA for Th1 cytokines and Th2 cytokines to determine if treatment with CAPE could affect cytokine secretion in the BAL fluid. The Th2 cytokines IL-4 and IL-5 are important with regard to the initiation and propagation of allergic inflammatory responses. While Th2 cytokines promote airway inflammation in asthma, Th1 cytokines protect against allergic disease and asthma. Several studies have suggested that TNF- α may have an important function in the pathogenesis of asthma. TNF- α can induce lung neutrophil infiltration, and some asthma patients exhibit elevated serum levels of TNF- α . The levels of IL-4, IL-5, IFN- γ , and TNF- α were determined via ELISA to determine if treatment with CAPE affects the secretion of cytokines in the BAL fluid, which was obtained 4 h

after the final airway challenge. The levels of IL-4, IL-5, and TNF- α in the BAL fluid were increased significantly by airway challenge with OVA, but IFN- γ was detected as low levels in BAL fluid in three groups. The administration of CAPE led to a reduction in the concentration of IL-4 and IL-5 by 65% and 88%, respectively, and resulted in 75% reduction in TNF- α secretion (Fig. 2). However, CAPE failed to increase the level of Th1 cytokine. These findings suggest that CAPE could inhibit allergic airway reactions by inhibiting the recruitment of Th2 cells into the airway rather than enhancement of a Th1 response.

CAPE decreases the AHR

AHR was assessed as a percentage increase of Penh in response to increasing doses of methacholine. In the OVA-sensitized and challenged mice, the dose–response curve of percentage Penh was shifted to the left as compared to that of the control mice (Fig. 3). In addition, the percentage Penh generated as the result of methacholine administration (at doses of 2.5–50 mg/ml) was increased significantly in the OVA-sensitized and OVA-challenged mice as compared to the controls. OVA-sensitized and challenged mice treated with CAPE (10 mg/kg) had a dose–response curve of percentage Penh that was shifted to the right as compared to those of untreated mice, and the shift was dose-dependent. These results indicate that treatment with CAPE effects a reduction in OVA-induced AHR.

CAPE reduces the levels of OVA-specific serum IgE

Cross-linking of allergen-specific IgE on the mast cell surfaces upon allergen challenge is crucial to the initiation of the early asthmatic reaction. The serum levels of OVA-specific immunoglobulins were measured 12 h after the final airway challenge. Sensitization and challenge with OVA resulted in increased serum levels of OVA-specific IgE when compared to the saline-treated control mice. The treatment of sensitized mice with CAPE (10 mg/kg) resulted in a 52% reduction of OVA-specific IgE (Fig. 4).

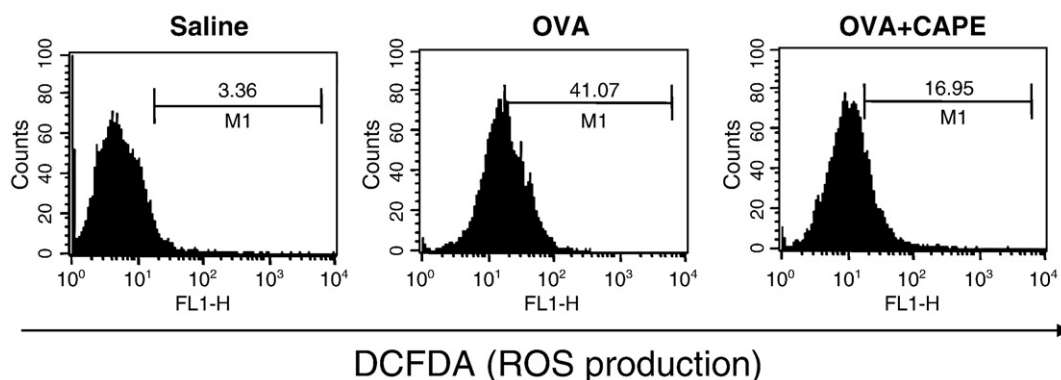


Fig. 5. Effect of CAPE administration on ROS levels in BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (Saline), OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CAPE (10 mg/kg, OVA + CAPE). The BAL fluid cells were stained with PBS containing 3.3 μ M DCFDA, and analyzed using flow cytometry. ROS levels was lower ($16.95 \pm 0.4\%$) than for OVA-induced levels of ROS ($41.07 \pm 0.8\%$), suggesting that the administration of CAPE had a reducing effect on ROS generation. The histogram shown is gated on BAL cells. Data represent means $\pm$ S.E.M. from 5 independent experiments. This experiment used 5 mice per group ($n = 5$).

CAPE diminishes the ROS generation in BAL fluid

Oxidative stress occurs in many allergic and immunologic disorders. Many studies have shown an increased production of ROS in asthma, allergic rhinitis, and atopy dermatitis, which contributes, in part, to tissue injury at sites of inflammation (Bowler and Crapo, 2002). A respiratory burst of eosinophils activated by eotaxin has been demonstrated, leading to production of ROS (Woerly et al., 2003; Elsner et al., 1998). We examined the effect of CAPE on generation of ROS, as described in Fig. 5. For this experiment, sampling was performed at 72 h after the last challenge. The level of ROS in BAL fluid was increased significantly at 72 h after inhalation of OVA compared to the levels after inhalation of saline (Fig. 5). We used DCFDA to investigate whether CAPE affects the generation of ROS. When treated with CAPE (10 mg/kg), the level of ROS was lower (16.95%) than for OVA-induced levels of ROS (41.07%), suggesting that the administration of CAPE exerts an inhibitory effect on the generation of ROS.

Effect of CAPE on the level of EPO in BAL fluid

An increased number of eosinophils are found in bronchial biopsies and in the BAL fluid of patients with bronchial asthma, and the number of eosinophils tends to correlate with the severity of the disease. Tissue levels of EPO were found to correlate well with the numbers of eosinophils at the inflammation site. In accordance with the numbers of eosinophils measured in the BAL fluid of OVA-challenged mice (Fig. 6), we determined that the levels of EPO in BAL fluid were increased significantly 24 h after inhalation of OVA as compared to the levels observed after inhalation of saline. The increased levels of EPO were reduced significantly, by ~51%, by the administration of CAPE (10 mg/kg).

CAPE inhibits NF- κ B activation in a murine asthma model

Many studies have demonstrated that NF- κ B over-activation underlines the chronicity of airway inflammation characteristic

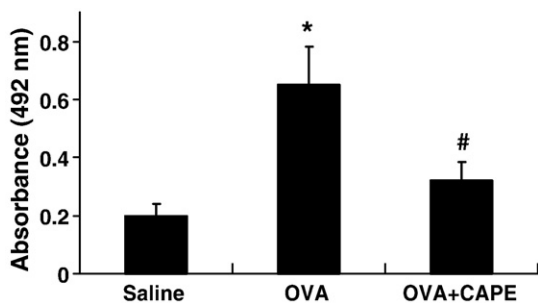


Fig. 6. Effect of CAPE on the level of EPO content in BAL fluid of OVA-sensitized mice and OVA-challenged mice. Mice were sensitized and challenged as described in methods. EPO activity was measured in BALF 24 h after the last airway challenge. CAPE (10 mg/kg) was administered i.p. from last sensitization to 1 day before the first airway challenge. Data represent mean $\pm$ S.E.M. from 5 independent experiments. This experiment used 5 mice per group ($n=5$). * $p<0.05$ vs. saline-treated mice; # $p<0.05$ vs. OVA-treated mice.

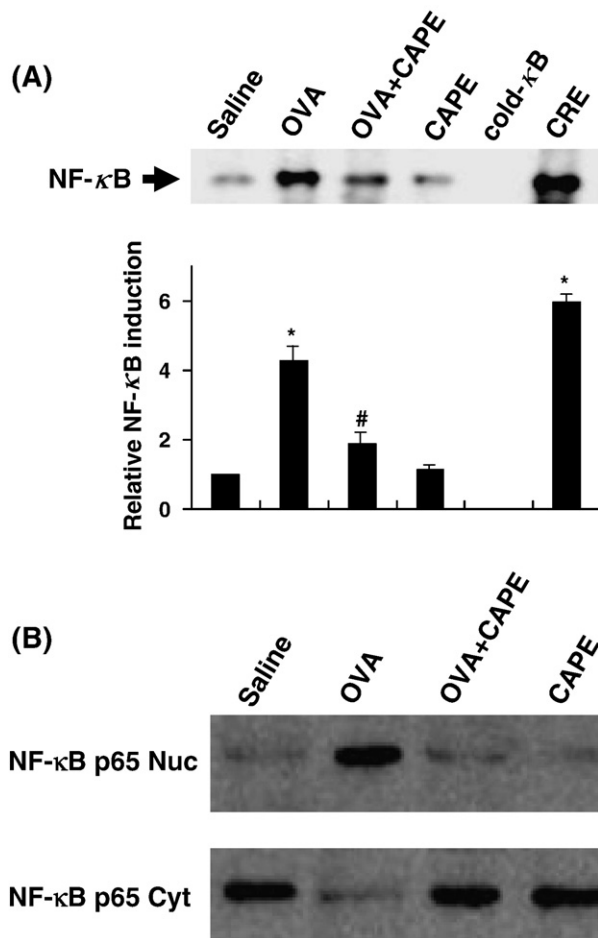


Fig. 7. Effect of CAPE on NF- κ B activation and nuclear translocation in lung tissues of OVA-sensitized and OVA-challenged mice. Representative electrophoretic-mobility-shift assay of nuclear extracts from lung tissues (A). NF- κ B expression was measured at 2 h after the last challenge in saline-inhaled mice administered saline (Saline), OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CAPE (10 mg/kg, OVA+CAPE), saline-inhaled mice administered CAPE (CAPE). Nuclear extracts were incubated with a biotin-labeled κ B oligonucleotide, and the samples were electrophoresed on a 5% polyacrylamide gel. A 100-fold excess of cold κ B or cAMP response element (CRE) oligonucleotide was added as a competitor. (B) NF- κ B p65 protein expression in nuclear and cytosolic protein extracts from lung tissues. NF- κ B p65 Nuc, in nuclear protein extracts and NF- κ B p65 Cyt, in cytosolic protein extracts from lung tissues. NF- κ B protein expression was measured at 2 h after the last challenge in saline-inhaled mice administered saline (Saline), OVA-inhaled mice administered saline (OVA), and OVA-inhaled mice administered CAPE (10 mg/kg, OVA+CAPE), saline-inhaled mice administered CAPE (CAPE). The results were similar in five independent experiments. This experiment used 5 mice per group ($n=5$). * $p<0.05$ vs. saline-treated mice; # $p<0.05$ vs. OVA-treated mice.

of asthma. The lungs were removed from the control and the CAPE-treated mice 2 h after the final airway OVA challenge, and the nuclear proteins were extracted as described in Materials and methods. We assessed NF- κ B activity via gel mobility-shift assays (Fig. 7A). The increased NF- κ B DNA binding observed in the nuclear extracts from lung tissues 2 h after inhalation of OVA was compared to the levels in the control mice. As shown in Fig. 7A, the induction of specific NF- κ B DNA binding activity by inhalation of OVA was inhibited by CAPE (10 mg/kg). We

also investigated the effect of CAPE on NF- κ B p65 nuclear translocation in a murine model of OVA-induced asthma. As shown in Fig. 7B, significant levels of NF- κ B p65 were localized to the nucleus at 2 h after inhalation of OVA. The increased levels of NF- κ B p65 in nuclear protein extracts from lung tissues at 2 h after inhalation of OVA were decreased by the administration of CAPE. In contrast, levels of NF- κ B p65 protein in cytosolic protein extracts from lung tissues were decreased at 2 h after inhalation of OVA compared with the levels in the control mice (Fig. 7B). The decreased levels of NF- κ B p65 in cytosolic protein extracts from lung tissues at 2 h after inhalation of OVA were increased by the administration of CAPE. These results indicate that CAPE inhibits NF- κ B activity by preventing translocation of this transcription factor into the nucleus.

Discussion

Bronchial asthma is characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation, and nonspecific AHR. This study used an OVA-induced murine model of allergic airway disease and showed an increase in the number of eosinophils in BAL fluid, a marked influx of inflammatory cells into the lung around blood vessels and airways, and airway luminal narrowing, the development of AHR, the detection of TNF- α and Th2 cytokines, including IL-4 and IL-5 in the BAL fluid, and the presence of allergen-specific IgE in the serum. The administration of CAPE before the final airway OVA challenge resulted in a significant inhibition of characteristic asthmatic reactions. In addition, the administration of CAPE reduced the increased levels of ROS in cells from the BAL fluid. Our results showed that CAPE could downregulate NF- κ B activity and reduce the levels of EPO, indicating that CAPE may be utilized as an adjuvant therapy for bronchial asthma patients.

In this study, we observed that administration of CAPE results in the downregulation of the Th2 cytokines IL-4 and IL-5 in BAL fluid; however, there was no significant increase in the levels of the Th1 cytokine IFN- γ in BAL fluid. Although further studies will be required to elucidate the effect of CAPE on the Th1–Th2 balance, these results suggest that treatment with CAPE could, at least partially, suppress the Th2 response without augmenting the Th1 response in our asthma model.

Oxidative stress is induced by a large variety of free oxygen radicals, referred to collectively as ROS. At sites of inflammation, multiple inflammatory cells, including eosinophils, neutrophils, and macrophages, are capable of generating ROS, which can participate in the development of a variety of diseases, including allergic asthma. Such ROS may contribute to tissue injury and to inflammatory reactions. Increased generation of ROS leads directly to the oxidation of proteins, DNA, and lipids, all of which injure the airways directly and may induce pathophysiological events in the lungs. Although the lungs possess a well-developed antioxidant system, the overproduction of ROS or the depression of the protective system results in epithelial cell damage, cell shedding, and bronchial hyperreactivity (Hulsmann et al., 1994; Cortijo et al., 1999).

Earlier reports indicated that the production of ROS is increased in the airways of OVA-challenged animals (Nishida et al., 2002; Zhang et al., 2002). These findings indicate that ROS are involved in the regulation of airway inflammation and bronchial hyperresponsiveness. Therefore, treatment of asthma with antioxidants has long been a subject of therapeutic strategy. CAPE extracted from honeybee propolis has several interesting biological properties, such as antiviral, antitumoral, anti-inflammatory, antioxidant, antiatherosclerotic, and immunomodulatory properties in a variety of systems. In this study, we have determined that the generation of ROS in BAL fluid is increased significantly following allergen challenge in an OVA-induced asthma model, and that the administration of CAPE reduced the generation of ROS. Airway inflammation and hyperreactivity in asthma are likely to involve oxidative stress to the lung. Excess production of ROS by immune cells may have an important role in airway injury in asthma (Talati et al., 2006; Dworski, 2000). In this study, CAPE markedly reduced the expression of elevated ROS generation, suggesting many anti-inflammatory effects of CAPE in an OVA-induced murine asthma model.

ROS promote the activities of proinflammatory redox-sensitive nuclear factors, including NF- κ B, thus increasing the occurrence of allergic inflammation in asthmatic patients (Rahman, 2003; Henderson et al., 2002). NF- κ B is detected in most cell types, and has been determined to have a critical function in immune and inflammatory responses, including asthma (Baldwin, 1996; Gilmore, 1999; Barnes and Adcock, 1997). In an earlier study, we demonstrated that NF- κ B has a pivotal role in the pathogenesis of asthma in mice (Choi et al., 2004). Consistent with these observations, the level of NF- κ B proteins in nuclear protein extracts obtained from lung tissues is increased substantially in the OVA-induced model of asthma used in this study. It has been determined that the activation of this transcription factor induces a variety of inflammatory genes that are expressed abnormally in asthma. These genes include cytokines (e.g., IL-4, IL-5, and TNF- α), chemokines (e.g., RANTES and eotaxin), and adhesion molecules (e.g., ICAM-1 and VCAM-1) (Stutz and Woisetschlager, 1999; Chvatchko et al., 2003; Zhou et al., 2006; Mori et al., 1999; Barnes, 1997). Thus, the development of an oxidant–antioxidant imbalance in the lungs of asthmatic patients may be the stimulus that activates redox-sensitive transcription factors, including NF- κ B. This suggests that redox-sensitive transcription factors may be a useful therapeutic target in allergic asthma. In this study, we have determined that CAPE suppressed NF- κ B activity in the lung tissues effectively via a reduction in oxidative stress.

The observed increases in oxidative stress and NF- κ B have a critical role in asthma. On the basis of these observations, we administered CAPE to OVA-challenged mice. We have demonstrated that the administration of CAPE resulted in a significant reduction of all pathophysiological symptoms examined. These results indicate that CAPE modulates intracellular generation of ROS, as well as the redox-sensitive transcription factor NF- κ B. The findings of this study indicate that CAPE may be useful as an adjuvant therapy for asthmatic patients.

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