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ORIGINAL ARTICLE

Caffeic acid phenethyl ester suppresses eotaxin secretion and nuclear p-STAT6 in human lung fibroblast cells

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Background: Caffeic acid phenethyl ester (CAPE), an active component of propolis, has been proven to have anti-inflammatory and antiallergic properties. We have investigated the activity of CAPE in regulating cytokine-induced eotaxin production and its related signal protein, signal transducer and activator of transcription 6 (STAT6), in human lung fibroblast.
Methods: The CCD-11Lu human lung fibroblast cell line was used as an in vitro model. Cells were pretreated with CAPE followed by stimulation with interleukin-4 and tumor necrosis factor alpha. The levels of eotaxin in cultured supernatants were measured by enzyme-linked immunosorbent assay. The amounts of STAT6 and phosphorylated STAT6 in cellular nuclear protein extracts were determined by Western blot analysis. STAT6 DNA binding activities were detected by electrophoretic mobility shift assay.
Results: Pretreated CCD-11Lu cells with noncytotoxic doses (0.1—10 μM) of CAPE inhibited the production of eotaxin under stimulation of interleukin-4 (10 ng/mL) and tumor necrosis factor alpha (10 ng/mL). CAPE pretreatment also decreased the amount of phosphorylated STAT6 and the STAT6 DNA binding complexes in nuclear extracts.
Conclusion: CAPE inhibited the production of eotaxin protein in stimulated human lung fibroblast cells in a dose-dependent manner. This activity is, at least, through STAT6 inhibition. We suggest that CAPE is a promising agent in controlling eotaxin secretion and subsequent

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Introduction

Asthma is a chronic inflammatory condition of the airways resulting in episodic airflow obstruction, airway hyper-responsiveness, and airway remodeling. Asthmatic airway tissues have increased numbers of activated eosinophils, neutrophils, mast cells, and activated lymphocytes, especially Type 2 T helper (Th2) cells.[1–3] Massive accumulation of eosinophils in airways is a fundamental trait of bronchial asthma.[4] Eosinophils and their products, including cytotoxic granule proteins and *de novo* synthesized leukotrienes, are important effectors in the pathophysiology of airway allergic inflammation, causing destruction of airway epithelium, sensitization of airway nerve terminals, vascular leakage, and other pathological changes.[5] Th2 lymphocytes and their products, such as interleukin (IL)-4, IL-5, and IL-13, mediated the airway eosinophil infiltration and IgE production,[6] and are known to stimulate the expression of several chemokines and mediators, such as eotaxin.[7,8] These chemokines may favor the recruitment of Th2 cells, eosinophils, basophils, and mast cells into the airway,[9] which lead to chronic airway inflammation.

Eotaxin, a potent chemokine for eosinophil, was originally discovered in 1994, has an important local role in the recruitment of eosinophils from the microvasculature into the tissue at sites of local allergic inflammation.[10–12] Eotaxin can be produced by many cell types, including lung fibroblast, smooth muscle cell, endothelial cell, alveolar macrophage, eosinophil, lymphocyte, and bronchial epithelial cell.[13] Among them, fibroblast is the major cellular source of eotaxin.[14,15] Several studies revealed that the combination of IL-4 and tumor necrosis factor alpha (TNF-α) could amplify the production of eotaxin in human lung fibroblast, airway epithelial cell, smooth muscle cell, and dermal fibroblast.[7,9,16–19] The mechanism operative in regulating IL-4 and TNF-α-stimulated eotaxin expression in epithelial cell and human lung fibroblast was mediated by activation of transcription factors, NF-kappa B (NF-κB) and signal transducer and activator of transcription 6 (STAT6).[16,17]

Caffeic acid phenethyl ester (CAPE) is a bioactive component of propolis (honeybee resin), which has several biological properties including anti-inflammatory, antioxidant, antimicrobial, and antitumor activities.[20] More recently, in murine model of ovalbumin-induced asthma, CAPE had significant effects on inhibition of characteristic asthmatic reactions (inflammatory cells; cytokines IL-4, IL-5, and TNF-α; mucus hypersecretion; and serum ovalbumin-specific IgE), diminished reactive oxygen species in bronchoalveolar fluid, and NF-κB DNA binding activity.[21] Although the eotaxin promoter contains overlapping consensus bindings sites for transcription factors, NF-κB and STAT6 are known to mediate responses to IL-4 and TNF-α.[17] Data showed that both IL-4 and TNF-α require STAT6 as mediator to activate eotaxin gene expression in fibroblast cells.[19]

In our previous study,[22] we demonstrated that CAPE can inhibit eotaxin secretion in human lung fibroblast (CCD-11Lu cell line) stimulated synergistically by IL-13 and TNF-α. We also showed that CAPE was able to inhibit NF-κB activation. The aim of present study is to check if CAPE possesses inhibitory effect on STAT6 activation.

Methods

Reagents

CAPE was purchased from Calbiochem (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (Merck, Darmstadt, Germany) in a stock concentration of 10 mM. Recombinant human IL-4 and TNF-α were purchased from Peprotech (Rocky Hill, NJ, USA). Eotaxin sandwich ELISA kits were purchased from BD biosciences (San Jose, CA, USA).

Cells and culture medium

The human lung fibroblast cell line, CCD-11Lu, was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in minimum essential medium (Gibco-BRL, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified 5% carbon dioxide/95% air atmosphere at 37°C.

Cell culture and study design

We cultured cells under the following conditions:

1. Cells (1×10^5 cells/well) were seeded in each well of 12-well plates (BD biosciences, San Jose, CA, USA) to settle and adhere for 24 hours. The culture medium was removed and replaced by serum-free medium, then the confluent monolayer were incubated with recombinant human IL-4 (10 ng/mL, 50 ng/mL), TNF-α (10 ng/mL, 50 ng/mL), or the combination of different concentration in the dose range 10 ng/mL and 50 ng/mL for 24 hours. Cells without stimulation were used as negative control. The supernatants were harvested and conserved at −80°C.
2. We pretreated cell under condition (1) with CAPE of various concentration (0.1 μM, 1 μM, 10 μM) for 3 hours (which is the earliest effective time of CAPE on eotaxin production in our preliminary study), followed by the stimulation with recombinant human IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for another 24 hours. The supernatants were harvested and conserved at −80°C.

3. Cells (5×10^6 cells/flask) were seeded in each 75-cm^2 T flask (Nunc, Rockilde, Denmark) to settle and adhere for 24 hours. The culture medium was removed and replaced by serum-free medium, then the confluent monolayer were pretreated with CAPE of various concentration (0.01 μM, 0.1 μM, 1 μM) for 3 hours, followed by the stimulation with recombinant human IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for another 45 minutes. The cells were washed with ice-cold Dulbecco's phosphate buffered saline (Gibco-BRL, USA) to stop the biological activity, and then the cells were collected for nuclear/cytoplasmic extraction.

Cell survival

Cell survival was measured based on the ability of mitochondrial enzymes in living cells to chemically reduce a tetrazolium salt (MTT) into a colored formazan dye as previously described.[23] The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany).

Chemokine assays

The concentration of eotaxin in the previous prepared supernatants in condition (1) and (2) were quantified with the use of a commercially available ELISA kit (BD Biosciences) according to the manufacturer's instructions. Absorbance was read at 450 nm with ELISA reader (Thermo Labsystems, Waltham, MA, USA).

Preparation of nuclear/cytoplasmic extracts

The nuclear and cytoplasmic extracts were prepared by using an NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA). We followed the experimental protocol offered by the manufacturer. The CCD-11Lu cells prepared in condition (3) were resuspended in hypotonic buffer (provided in NE-PER kit). The homogenates were centrifuged and the supernatants were collected as cytoplasmic extracts. The nuclear pellets were resuspended in extraction buffer (provided in NE-PER kit). After been incubated in ice for 40 minutes, the nuclear extracts were collected. All fractions of proteins mentioned previously were identified and quantified by Bradford method (Dc Protein Assay, Bio-Rad, Hercules, CA, USA), and stored at −80°C.

Western blot analysis

We followed the standard method.[22] All membranes were incubated with the following antibody overnight at 4°C with continuous shaking: mouse monoclonal antihuman STAT6 antibody (1:250 BD biosciences) and mouse monoclonal antihuman phosphorylated STAT6 (p-STAT6) (pY641) antibody (1:500 Abcam, Cambridge, UK). We detected proliferating cell nuclear antigen by mouse monoclonal antihuman antibody (1:5,000 Abcam) as internal control. The intensities of the pixels within each immunoreactive band were quantitated on an odyssey infrared (IR) imaging system associated with Odyssey software (version 1.2) according to the manufacturer (Li-Cor Biosciences, Lincoln, NE).

Electrophoresis mobility shift assay

Nuclear extracts (20μg protein) were mixed with Odyssey IR electrophoresis mobility shift assay Kit (LI-COR, Inc., Lincoln, NE) according to the manufacturer's instructions. The IR dye-labeled STAT6 oligonucleotide (5′-IRDye800-GCTCTTCTTCCCAGGAACTCAATG; 3′-CGAGAAGAAGGGTCC TTGAGTTAC-IRdye 800-5′) was prepared as Ohmori's methods[24] and then added to the protein mixture and incubated at room temperature in the dark for 30 minutes. The protein-DNA complexes were separated by electrophoresis through 5% native polyacrylamide gels at 70 V for 1 hour in Tris-borate-EDTA buffer in the dark. Electrophoresis mobility shift assayA gels were analyzed using the LI-COR Odyssey IR laser imaging system.

Statistical analysis

All results were represented as the means ± standard error of the mean of at least three independent experiments. Statistical analyses were performed using Mann-Whitney U test to compare data. A probability value (p) less than 0.05 was considered statistically significant difference.

Results

Cell viability studies

The survival of CCD-11Lu cells incubated with various concentration of CAPE (0.1 μM, 1 μM, 10 μM) for 3 hours with and without costimulation with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for another 24 hours were all more than 90% compared with negative control. Drug concentrations
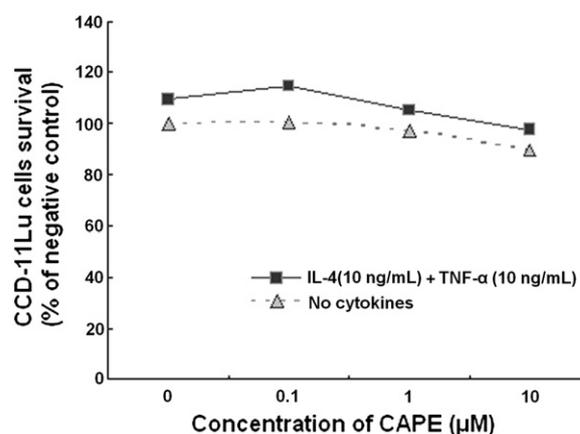

Figure 1. The survival curves of CCD-11Lu cells. CCD-11Lu human lung fibroblast cells were incubated with CAPE (0.1 μM, 1 μM, 10 μM) for 3 hours with or without followed by IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for additional 24 hours. Then, the survival assay was performed. The survival of CCD-11Lu cells were all more than 90% when comparing with the negative control group. CAPE = caffeic acid phenethyl ester; IL-4 = interleukin-4; TNF-α = tumor necrosis factor alpha.
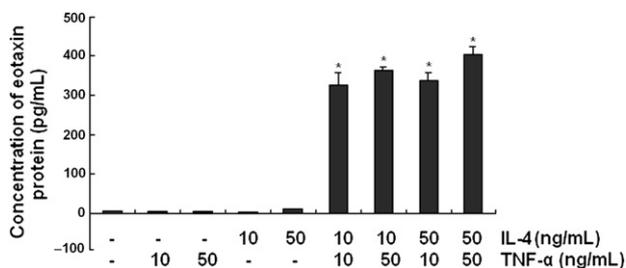
Figure 2. The effects of TNF-α and IL-4 on the induction of eotaxin. CCD-11Lu human lung fibroblast cells were cultured with IL-4 (10 ng/mL, 50 ng/mL) or TNF-α (10 ng/mL, 50 ng/mL), or the combination of different concentration. Control was represented by CCD-11Lu cells with no cytokine stimulated. (*$p < 0.05$, compared with control experiment). CAPE = caffeic acid phenethyl ester; IL-4 = interleukin-4; TNF-α = tumor necrosis factor alpha.

used in our study revealed no toxicity to CCD-11Lu cells. The data was shown in Fig. 1.

Synergistic induction of eotaxin production by IL-4 and TNF-α

As shown in Fig. 2, the concentrations of eotaxin in cultured supernatants were not significantly increased when CCD-11Lu cells were stimulated by either IL-4 or TNF-α alone. In accordance with published data, our data showed the cytokine combination had a clear synergistic effect. We chose the concentration of IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for later experiments.

The effects of CAPE on eotaxin production in IL-4 and TNF-α stimulated CCD-11Lu cell

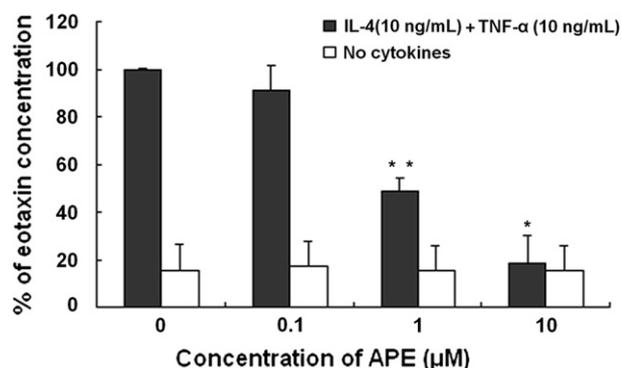To evaluate the effect of CAPE on eotaxin secretion from CCD-11Lu cells stimulated by IL-4 (10 ng/mL) and TNF-α

Figure 3. The effects of CAPE on the induction of eotaxin. CCD-11Lu human lung fibroblast cells were pretreated with various concentration of CAPE (0.1 μM, 1 μM, 10 μM) for 3 hours, followed by costimulation with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for additional 24 hours. Control was represented by stimulated CCD-11Lu cells with no CAPE pretreatment. (*$p < 0.05$, **$p < 0.01$; compared with control experiment). CAPE = caffeic acid phenethyl ester; IL-4 = interleukin-4; TNF-α = tumor necrosis factor alpha.

(10 ng/mL), we pretreated CCD-11Lu with CAPE (0.1 μM, 1 μM, 10 μM) 3 hours before stimulation. The amount of eotaxin production by cells stimulated by IL-4 (10 ng/mL) and TNF-α (10 ng/mL) was calculated to be 100%, and others were compared with it. Our results showed that cytokine-induced eotaxin production was inhibited by CAPE in a concentration-dependent manner. CAPE, 1 μM and 10 μM, suppressed eotaxin production by 48.13% and 18.88%, respectively ($p < 0.01$ and $p < 0.05$, respectively).
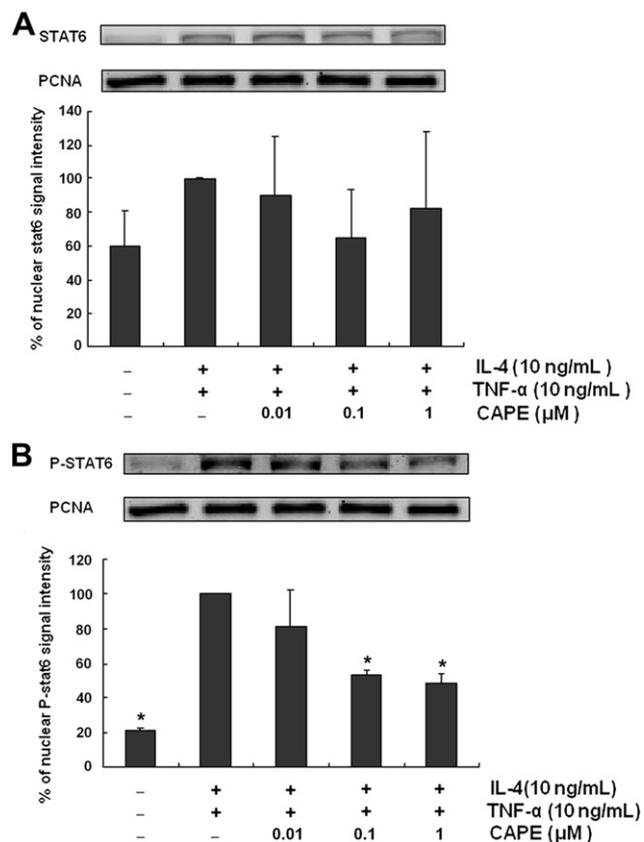

Figure 4. (A) The effects of CAPE on the level of STAT6 in nuclear extracts. CCD-11Lu lung fibroblast cells were pretreated with various concentration of CAPE (0.01 μM, 0.1 μM, 1 μM) for 3 hours, followed by costimulation with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for additional 45 minutes. Control was represented by stimulated CCD-11Lu cells with no CAPE treatment. (*$p < 0.05$, compared with control experiment). (B) The effects of CAPE on the level of p-STAT6 in nuclear extracts. CCD-11Lu lung fibroblast cells were pretreated with various concentration of CAPE (0.01 μM, 0.1 μM, 1 μM) for 3 hours, followed by costimulation with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for additional 45 minutes. Control was represented by stimulated CCD-11Lu cells with no CAPE treatment. (*$p < 0.05$, compared with control experiment). The signal intensities were analyzed by Western blotting. Western blotting (30 μg protein/lane) were performed using anti-STAT6 (1:250), anti-phosphorylated STAT6 (1:500), and anti-PCNA (1:5000) Abs, respectively. CAPE = caffeic acid phenethyl ester; IL-4 = interleukin-4; PCNA = proliferating cell nuclear antigen; STAT6 = signal transducer and activator of transcription 6; TNF-α = tumor necrosis factor alpha.

As data shown in Fig. 3, the CAPE (0.1 μM, 1 μM, 10 μM) alone have no effect on eotaxin production.

The effects of CAPE on transcriptional factor STAT6 and p-STAT6 in nucleus

We examined the signal intensity of transcriptional factor STAT6 and p-STAT6 in the nuclear extracts of CCD-11Lu cells, which were pretreated with CAPE with various concentration (0.01 μM, 0.1 μM, 1 μM) for 3 hours before stimulating with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) synergistically for 45 minutes [which is the peak time of STAT6 nuclear translocation in our preliminary study (data not shown)]. The signal intensities of nuclear STAT6 and p-STAT6 were corrected by dividing signal intensities of nuclear proliferating cell nuclear antigen in the same lane. The intensity from condition stimulated by IL-4 (10 ng/mL) and TNF-α (10 ng/mL) was calculated to be 100%, and others were compared with it. The signal intensities of nuclear STAT6 on all conditions of cells showed no statistical significance, as data shown in Fig. 4A. Our results showed that signal intensities of p-STAT6 were decreased at higher concentration of CAPE. CAPE, 0.1 μM and 1 μM, suppressed the p-STAT6 signal intensities by 53.42% and 49.07%, respectively ($p < 0.05$), the data was shown in Fig. 4B.

The effects of CAPE on STAT6 DNA binding activity

We assessed STAT6 DNA binding activity in the nuclear extracts of CCD-11Lu cells, which were pretreated with CAPE with various concentration (0.01 μM, 0.1 μM, 1 μM, 10 μM) for 3 hours before stimulating with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) synergistically for 45 minutes. The intensity from condition stimulated by IL-4 (10 ng/mL) and TNF-α (10 ng/mL) was calculated to be 100%, and others were compared with it. Our results showed that DNA binding activity of STAT6 were inhibited in the administration of CAPE before cytokine stimulation, with all experimental concentration ($p < 0.05$). The data was shown in Fig. 5.

Discussion

In the present study, we demonstrated that CAPE inhibited the secretion of eotaxin as well as the STAT-6 activation in lung fibroblast stimulated synergistically by IL-4 and TNF-α.. To the best of our knowledge, it is the first report involving the inhibitory effect of CAPE on STAT-6 activation.

We used IL-13 plus TNF-α as stimulator for eotaxin secretion in the same lung fibroblast cell line (CCD-11Lu) in our previous study.[22] IL-4 and IL-13 did share many similar
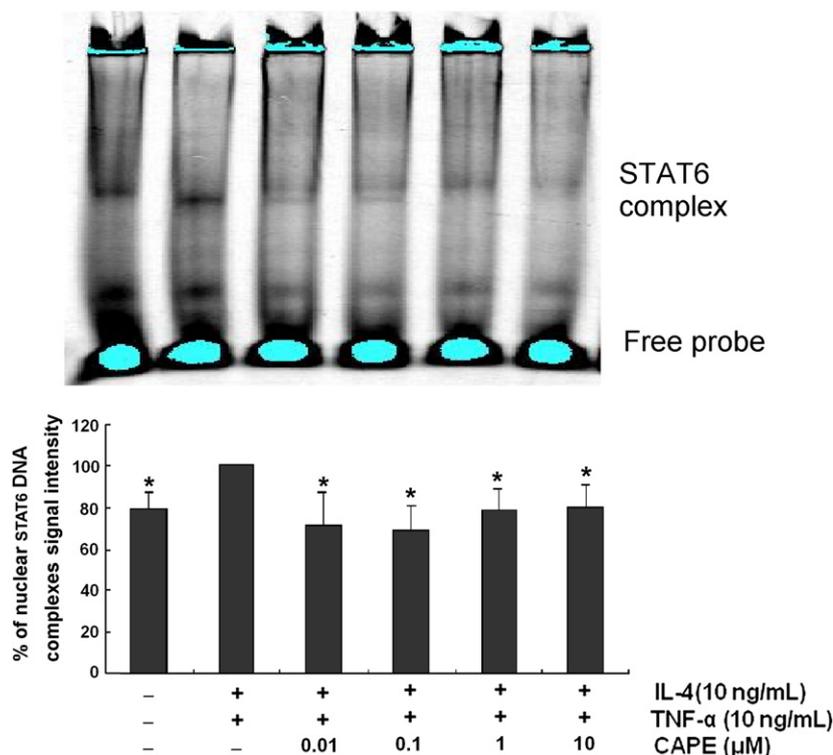

Figure 5. The effects of CAPE on the STAT6 DNA binding activity in nuclear extracts. CCD-11Lu lung fibroblast cells were pretreated with various concentration of CAPE (0.01 μM, 0.1 μM, 1 μM, 10 μM) for 3 hours, followed by costimulation with IL-4 (10 ng/mL) and TNF-α (10 ng/mL) for additional 45 minutes. The extracts was incubated with infrared dye-labeled oligonucleotide probe and analyzed for binding activity by EMSA. The signal intensity of each lane was detected by LI-COR Odyssey infrared laser imaging system (Li-Cor Biosciences, Lincoln, NB). Control was represented by stimulated CCD-11Lu cells with no CAPE pretreament. (*$p < 0.05$, compared with control experiment). CAPE = caffeic acid phenethyl ester; EMSA = electrophoretic mobility shift assay; IL-4 = interleukin-4; STAT6 = signal transducer and activator of transcription 6; TNF-α = tumor necrosis factor alpha.

activities, including being activated by STAT-6, as demonstrated before.[25] IL-4 stimulates several intracellular signaling pathways, including the recruitment of STAT6 to the IL-4Rα, where it is phosphorylated on tyrosine 641 by Jac kinases, the p-STAT6 form dimers and translocates to the nucleus to bind DNA and activate transcription. STAT6 is tightly connected to IL-4 and IL-13 signaling and plays a key role in Th2 polarization of the immune system.[26,27]

This time, we shifted IL-13 to IL-4 as part of the synergistic stimulator with TNF-α. The main concern was based on these three previous reports.[17–19] Matsukura et al[17] first disclosed that IL-4 and TNF-α stimulate expression of the eotaxin gene by activating STAT6 and NF-κB in human airway epithelial cells. Atasoy et al[18] used an actinomycin D-based assessment that demonstrated that IL-4 and TNF-α significantly increase eotaxin mRNA stability in human bronchial epithelial cells. Hoeck and Woisetschlager[19] showed that the similar synergistic fashion in human fibroblast cells were also mediated by STAT6 and NF-κB on eotaxin promoter.

Our study showed that CAPE decreased STAT6 activation and eotaxin secretion in lung fibroblast. However, our previous study[22] as well as other previous studies[28,29] did demonstrate the CAPE inhibitory effect on NF-κB activation. Recent studies revealed that eotaxin promoter contains both binding site for NF-κB and STAT6.[17,19] Moreover, a study[30] showed that blocking NF-κB activity eliminates IL-4-stimulated gene expression but not phosphorylation or nuclear localization of STAT-6. In our study, the nuclear localization of STAT6 was not changed as shown in Fig. 4A. But the STAT6 phosphorylation and STAT6 DNA binding activity in nucleus were inhibited by CAPE, as data shown in Fig. 4B and Fig. 5. The possible explanation may be that the effect of CAPE on eotaxin production could not simply be explained by inhibiting NF-κB activity.

Recent report[31] demonstrated that a novel STAT6 inhibitor, AS1517499, was able to inhibit IL-13 secretion in cultured human bronchial smooth muscle as well as to decrease airway hyperactivity in balb/c mice induced by albumin sensitization. CAPE, an extract from propolis, is a natural product used widely for many years. Based on our preliminary result that CAPE processes inhibitory characteristics on eotaxin production in human lung fibroblasts and on phosphorylated STAT6, further studies to test its inhibition on STAT6 and NF-κB activation is worthy.

Acknowledgments

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