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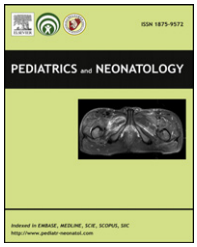

ORIGINAL ARTICLE

The Immunoregulatory Effects of Caffeic Acid Phenethyl Ester on the Cytokine Secretion of Peripheral Blood Mononuclear Cells From Asthmatic Children

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Background: Asthma is a chronic inflammatory disease of the airways for which current treatments are mainly based on pharmacological interventions, such as glucocorticoid therapy. Our objective was to study the immunoregulatory effects of caffeic acid phenethyl ester (CAPE, a phytochemical synthesized from propolis) on cytokine secretion of peripheral blood mononuclear cells (PBMCs) from asthmatic children.

Methods: PBMCs from asthmatic children (5.5 ± 3.3 years old, $n = 28$) and healthy children (5.6 ± 2.8 years old, $n = 23$) were co-cultured with CAPE *in vitro* with and without phorbol-12-myristate-13-acetate-ionomycin.

Results: Our results show that predominant interleukin 4 (IL-4) and interferon-gamma secretion of cultured supernatant were detected in healthy donors compared with asthmatics. In the presence of phorbol-12-myristate-13-acetate-ionomycin, with or without CAPE treatment, the asthmatic children showed significantly decreased levels of IL-10 secretion compared with the healthy controls. However, CAPE significantly decreased IL-10 and interferon-gamma in healthy donors. There was a slight but not statistically significant reduction of IL-4 secretion in CAPE-treated PBMCs compared with untreated control PBMCs from the healthy children. Our data also shows that CAPE significantly enhanced transforming growth factor-beta 1 production from PBMCs from asthmatic children.

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Conclusion: The immunoregulatory effects of CAPE on human PBMCs may be through the induction of regulatory T cells, as evidenced by the enhanced transforming growth factor-beta 1 production from PBMCs from asthmatic children in our study.

1. Introduction

Caffeic acid phenethyl ester (CAPE) is a phytochemical synthesized from honeybee propolis,[1] a naturally occurring bee product. Several studies have shown that CAPE has antitumor,[2–6] anti-inflammatory,[7,8] and antioxidant properties.[9–12] CAPE downregulates mitogen-induced T-cell proliferation and lymphokine production[13] by inhibition of DNA binding and transcription of nuclear factor-κappa B and the nuclear factor of activated T cells in stimulated T-cells.[14] *In vivo,* the immunomodulatory effects of CAPE have also been observed in CAPE-administered mice, with increased $CD4^+$ T cell subsets and enhanced anti-CD3-induced cytokine production in splenocytes.[15] However, there are no studies to date on how CAPE might affect the immune response in asthma.

Asthma is a chronic inflammatory disease of the airways caused by aberrant T-helper 2 (TH2) immune responses. Activation of TH2 cells mediate the synthesis of immunoglobulin E via interleukin-4 (IL-4) and eosinophilic inflammation via IL-5, which, together with IL-13, contributes to airway hyper-responsiveness and other clinical features of allergic disease.[16] Current pharmacotherapy for asthma includes glucocorticoids for relief of wheezing in preschool children.[17] Although glucocorticoids can effectively control attacks of asthma, many patients develop side effects as a result of long-term steroid therapy.[18] Even inhaled glucocorticoids have been shown to suppress bone growth in pediatric patients.[19] Thus, there is a need for novel therapeutic strategies, which are equally effective but with minimal side effects. This study investigated the immunoregulatory effects of CAPE *in vitro* on cytokine secretion of the peripheral blood mononuclear cells (PBMCs) isolated from both asthmatic and healthy children.

2. Materials and Methods

2.1. Participants

Heparinized blood was obtained from 28 untreated asthmatic patients (11 girls and 17 boys) and 23 healthy nonallergic controls (9 girls and 14 boys). Average age was 5.5 ± 3.3 years for asthmatic patients and 5.6 ± 2.8 years for healthy controls. Our asthmatic patients, children with mild intermittent asthma who were not yet treated with corticosteroids and/or bronchodilators, were recruited from the Department of Pediatrics, Cardinal Tien Hospital (Taipei, Taiwan). Healthy children, defined as those without symptoms of allergic or infectious diseases, were enlisted from participants at a promotional program hosted by Cardinal Tien Hospital on December 18, 2004. Total serum immunoglobulin E concentrations (analyzed by chemiluminescence method) in asthmatic and healthy children were 659.6 ± 648.2 kU/L and 32.0 ± 22.8 kU/L, respectively. The study protocol was reviewed and approved by the Committee on Human Experiments of Catholic Cardinal Tien Hospital, Taipei, Taiwan. Written informed consent was obtained from each participant's parents.

2.2. Reagents

CAPE was purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA)-ionomycin was all purchased from Sigma (Germany).

2.3. Preparation of PBMCs

Human PBMCs were isolated from heparinized blood after centrifugation in Ficoll-Hypaque (Pharmacia, Sweden) density gradient. The PBMC layers were collected and washed twice with cold Hanks' buffer saline solution (HyClone, USA). The cells were then resuspended in complete medium (Roswell Park Memorial Institute 1640 media supplemented with 10% fetal bovine serum, 2mM L-glutamine, 10mM HEPES, 100 U/mL penicillin, 100 U/mL streptomycin, and 5.78×10^{-5}M β-mercaptoethanol, all purchased from HyClone, USA).

2.4. Quantification of cytokine secretion by enzyme-linked immunosorbent assay

PBMC (2×10^6 cells/mL) were stimulated with 20 ng/mL PMA, Sigma (Germany) and 1μM ionomycin (Sigma, Germany) in the presence or absence of 2.5 μg/mL CAPE, and supernatants were collected after 24 hours. Culture supernatants were spun free of cells and aliquots were frozen at −80°C. Levels of transforming growth factor beta 1 (TGF-β1) in acidified supernatants were determined by capture enzyme-linked immunosorbent assay according to manufacturer's instructions (R&D Systems, Germany). Levels of IL-4, IL-5, IL-10, and interferon-gamma (IFN-γ) were measured by capture ELISA (BD PharMingen, USA) according to manufacturer's instructions.

2.5. Statistical analysis

Two-way analysis of variance was used to test for statistically significant differences; p values less than 0.05 were considered significant. All cultures were done in triplicate, and standard deviations are represented by error bars.

3. Results

To assess the effect of CAPE on TH1/TH2 cytokine secretion in both healthy and asthmatic children, PBMCs were stimulated using PMA-ionomycin in the presence or absence of CAPE. As shown in Table 1, the levels of IL-4 secretion in the untreated healthy controls were significantly higher compared with the levels observed in the asthmatic children. By contrast, PBMC production of IL-10 in untreated asthmatics was found to be significantly higher than in the healthy children (Table 1).

When PBMCs were stimulated with PMA-ionomycin, both IL-4 and IFN-γ secretion in asthmatics were lower than those in healthy children, and a significant lowering of IFN-γ production was observed in the group of asthmatics compared with that seen in healthy participants (2.6 ± 0.5 ng/mL vs. 49.4 ± 16.1 ng/mL) (Table 2). CAPE inhibited both IL-10 and IFN-γ production in PMA-ionomycin-stimulated PBMCs taken from healthy children. There was a slight reduction of IL-4 secretion in CAPE-treated PBMCs compared with the nontreated (control) group in healthy children, but this difference did not reach significance. Interestingly, CAPE reduced the production of IFN-γ and significantly enhanced the TGF-β1 in the asthmatic PBMC culture supernatants. We also checked the effects of CAPE on Th1/Th2 ratio of PMA-ionomycin-activated PBMC isolated from asthmatic patients or healthy donors. Figure 1 demonstrates that the presence of CAPE did not change the trends of Th1/Th2 in both asthmatic patients and healthy donors.

4. Discussion

Several studies have demonstrated the immunomodulatory effects of CAPE. Cytokines such as IL-2, IL-4, and IFN-γ were significantly increased in BALB/c mice treated with CAPE.[15] Likewise, Gremy et al[20] showed that ileal mucosa treated with CAPE shifted from an irradiation-induced Th2-like pattern of gene expression toward Th1 gene expression by an apoptotic sensitization pathway. It has been recently shown that CAPE suppresses phytohemagglutinin-induced cytokine production of PBMCs taken from healthy adult volunteers.[13] Thus, we studied the effects and mechanisms of CAPE on Th1/Th2 cytokines regulation of PBMC isolated from asthmatic and healthy children. Our study showed that spontaneous IL-4 secretion was higher in healthy children in contrast to significantly higher production of IL-10 in asthmatic children (Table 1). There was a slight reduction of IL-4 secretion in CAPE-treated PBMCs compared with the nontreated (control) group in healthy children, but this difference did not reach statistically significant result. But when PBMCs were stimulated with PMA-ionomycin, both IL-4 and IFN-γ secretion in asthmatics were lower than those in healthy children, and a significantly lowered IFN-γ production was observed in the group of asthmatic compared with that seen in healthy participants (Table 2). We surmise that these asthmatic patients (before treatment with glucocorticoids or bronchodilators) had ongoing acute attacks of asthma when they were included in this study, and PBMCs taken during this time failed to show any further increase of cytokine secretion with PMA stimulation as compared with baseline levels as noted by Kuo et al.[21] The lower IFN-γ production observed in the group of asthmatics compared with that seen in healthy participants is explained by the fact that IFN-γ is primarily produced by Th1 cells[22] and asthma is caused by aberrant Th2 immune responses. CAPE inhibited both IL-10 and IFN-γ production in PMA-ionomycin-stimulated PBMC separated from healthy children. It can be seen that CAPE suppresses spontaneous and PMA-ionomycin-induced cytokine production of PBMCs isolated from both healthy and asthmatic children.

As already suggested in previous studies, CAPE inhibits phytohemagglutinin-stimulated cytokine production of, but induces TGF-β1 production in healthy adult PBMCs.[13] Our

Table 1 Effects of CAPE on cytokine secretion by peripheral blood mononuclear cells from asthmatic and healthy children.*,†

Cytokine	Treatment	Healthy‡	Asthmatic‡
IL-4 (pg/mL)	Control	37.0 ± 38.7^{X}	0.0 ± 0.0^{Y}
	CAPE 2.5 μg/mL	26.6 ± 39.7	4.4 ± 14.6
IL-5 (pg/mL)	Control	1.9 ± 3.2	5.9 ± 7.7
	CAPE 2.5 μg/mL	0.0 ± 0.2	1.5 ± 4.6
IL-10 (pg/mL)	Control	350.5 ± 249.0^{X}	1283.5 ± 597.3^{Y}
	CAPE 2.5 μg/mL	255.9 ± 236.5^{X}	1029.6 ± 606.0^{Y}
IFN-γ (pg/mL)	Control	56.3 ± 87.0	30.7 ± 49.2
	CAPE 2.5 μg/mL	18.3 ± 36.4	13.7 ± 12.0
TGF-β1 (ng/mL)	Control	4.2 ± 2.3	2.8 ± 1.4
	CAPE 2.5 μg/mL	3.5 ± 2.3	3.3 ± 1.3

* Peripheral blood mononuclear cells from asthmatic patients or healthy donors were cultured with CAPE (2.5 μg/mL) or medium alone. Cultured supernatants from all treatment groups were collected following 24 hours of incubation and then analyzed by enzyme-linked immunosorbent assay.

† Values (mean ± standard deviation) with different superscript letters in the same row (X, Y) were significantly different ($p < 0.05$) via two-way analysis of variance.

‡ Patient number: IL-4, asthmatics: 11, healthy: 12; IL-5, asthmatics: 10, healthy: 12; IL-10, asthmatics: 8, healthy: 9; IFN-γ, asthmatics: 11, healthy: 12; TGF-β1, asthmatics: 11, healthy: 12.

CAPE = caffeic acid phenethyl ester; IFN-γ = interferon-γ gamma; IL = interleukin; TGF-β1 = transforming growth factor beta 1.

Table 2 Effects of CAPE on cytokines secretions by phorbol-12-myristate-13-acetate-ionomycin-activated peripheral blood mononuclear cells.[*,†]

Cytokine	Treatment	Healthy[‡]	Asthmatic[‡]
IL-4 (pg/mL)	Control	183.8 ± 137.2^{X}	76.6 ± 36.2^{Y}
	CAPE 2.5 μg/mL	126.3 ± 68.9^{X}	65.3 ± 22.7^{Y}
IL-5 (pg/mL)	Control	568.4 ± 321.0	522.2 ± 260.4
	CAPE 2.5 μg/mL	459.5 ± 248.1	459.4 ± 400.7
IL-10 (pg/mL)	Control	$591.9 \pm 217.3^{A,X}$	229.5 ± 236.4^{Y}
	CAPE 2.5 μg/mL	$288.7 \pm 145.2^{B,X}$	91.1 ± 94.9^{Y}
IFN-γ (ng/mL)	Control	$49.2 \pm 16.1^{A,X}$	$2.6 \pm 0.5^{A,Y}$
	CAPE 2.5 μg/mL	$28.7 \pm 10.4^{B,X}$	$2.2 \pm 0.6^{B,Y}$
TGF-β1 (ng/mL)	Control	5.9 ± 3.3	4.1 ± 1.9^{B}
	CAPE 2.5 μg/mL	6.3 ± 4.0	6.0 ± 2.1^{A}

* Peripheral blood mononuclear cells from asthmatic patients or healthy donors were cultured with CAPE (2.5 μg/mL) or medium alone. Cultured supernatants from all treatment groups were collected following 24 hours of incubation and then analyzed by enzyme-linked immunosorbent assay.

† Values (mean ± SD) with different superscript letters in the same column (A, B) or between asthmatics and healthy participants (X, Y) were significantly different ($p < 0.05$) via two-way analysis of variance.

‡ Patient number: IL-4, asthmatics: 11, healthy: 12; IL-5, asthmatics: 10, healthy: 12; IL-10, asthmatics: 8, healthy: 9; IFN-γ, asthmatics: 11, healthy: 12; TGF-β1, asthmatics: 11, healthy: 12.

CAPE = caffeic acid phenethyl ester; IFN-γ = interferon-γ gamma; IL = interleukin; TGF-β1 = transforming growth factor beta 1.

study showed that CAPE reduced the production of IFN-γ and significantly enhanced the TGF-β1 in the asthmatic PBMC culture supernatants (Table 2). Moreover, CAPE inhibits cytokine synthesis in stimulated T-cells, a result that correlated with its ability to inhibit PMA-ionomycin-induced DNA binding and transcriptional activity of nuclear factor of activated T cells.[14] Our study demonstrated that the possible immunoregulatory effect of CAPE is to induce the expression of regulatory T (Treg)-like cells in activated PBMC from asthmatic and healthy participants,

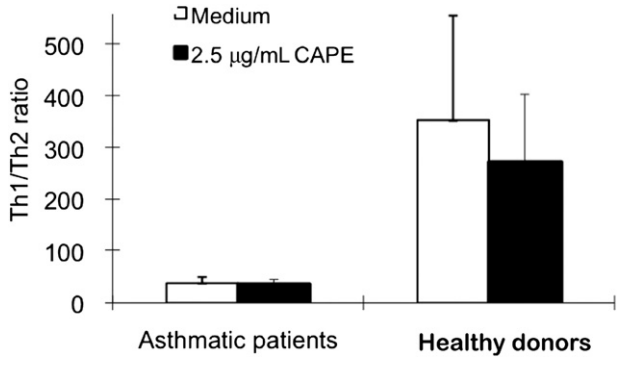

Figure 1 Effects of CAPE on Th1/Th2 ratio of phorbol-12-myristate-13-acetate (PMA)-ionomycin-activated peripheral blood mononuclear cells (PBMCs) isolated from asthmatic patients or healthy donors.[1,2] PBMCs were isolated from asthmatic patients ($n = 11$) or healthy donors ($n = 12$) and stimulated with PMA plus ionomycin in the presence or absence of CAPE. After 24 hours of incubation, cultured supernatants were collected and analyzed by enzyme-linked immunosorbent assay. The Th1/Th2 ratio was calculated as the ratio of interferon-gamma and interleukin-4. *A p value < 0.001 for comparison between asthmatic group and healthy group. CAPE = caffeic acid phenethyl ester.

which can promote TGF-β secretions from T cells and further inhibit Th1/Th2 cytokine secretions (Figure 1).

Recently, Treg cells have emerged as master regulators of immunity and a new target for asthmatic intervention. Several types of Treg cells exist, including $CD4^+$ T-cells that express $CD25^+$ constitutively ($CD4^+$ $CD25^+$ cells). Regulatory T-cells suppress immune responses via direct cell-to-cell interactions, and Type 1 T regulatory cells function via secretion of IL-10 and TGF-β1 cells.[23] When activated, Treg cells produce IL-10, TGF-β1, and lower levels of IFN-γ, but do not affect levels of IL-2 or IL-4, a profile of cytokine production, which is remarkably similar to that of Type 1 T-regulatory cells.[24]

There is evidence that the function of Treg cells appears to be compromised in those with allergic diseases, including rhinitis, atopic dermatitis, and asthma.[25] The exact mechanisms of suppression used by Treg cells remain controversial. Our study shows that in the presence of PMA-ionomycin, asthmatic children had significantly lower levels of IL-10 secretions than healthy children, which might suggest a compromised function in Treg cells in allergic diseases. Our results also shows CAPE significantly upregulated levels of TGF-β1 levels, which is also a marker of Treg cells. The immunoregulatory effects of CAPE on human PBMC may be through the induction of Treg-like cells as evidenced by the enhanced TGF-β1 production in PBMCs from asthmatic children in our study.

The conclusion of our *in vitro* study is that CAPE could increase TGF-β1 secretion by PBMCs of asthmatic children, and CAPE could inhibit IL-10 secretion, too. Although the PMA-ionomycin-stimulated PBMCs treated with CAPE could attenuate IFN-γ secretion, the ratios of Th1 and Th2 cytokines were not changed as compared with PBMCs stimulated with or without CAPE. To verify the therapeutic effect of CAPE in asthma, an *in vivo* study of the effect of CAPE on animal model of airway inflammation will be needed in the future.

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