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Research Paper

Piperine inhibits eosinophil infiltration and airway hyperresponsiveness by suppressing T cell activity and Th2 cytokine production in the ovalbumin-induced asthma model

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Abstract

Objectives This study aimed to investigate the effect of piperine on airway hyperresponsiveness, pulmonary eosinophilic infiltration, various immune cell phenotypes, Th2 cytokine production, immunoglobulin E and histamine production in a murine model of asthma.

Methods Asthma was induced in Balb/c mice by ovalbumin sensitization and inhalation. Piperine (4.5 and 2.25 mg/kg) was orally administered 5 times a week for 8 weeks. At 1 day after the last ovalbumin exposure, airway hyperresponsiveness was determined and samples of bronchoalveolar lavage fluid, lung cells and serum were collected for further analysis.

Key findings Piperine-treated groups had suppressed eosinophil infiltration, allergic airway inflammation and airway hyperresponsiveness, and these occurred by suppression of the production of interleukin-4, interleukin-5, immunoglobulin E and histamine. Moreover, polymerase chain reaction products for thymus and activation regulated chemokine from lung cell RNA preparations were decreased in the piperine-treated group compared with control groups, although transforming growth factor-β products were increased in the piperine-treated group.

Conclusions The results suggest that the therapeutic mechanism by which piperine effectively treats asthma is based on a reduction of Th2 cytokines (interleukin-4, interleukin-5), eosinophil infiltration, and by marked reduction of thymus and activation regulated chemokine, eotaxin-2 and interleukin-13 mRNA expression (especially transcription of nuclear factor-κB dependent genes) in lung tissue, as well as reduced interleukin-4, interleukin-5 and eotaxin levels in bronchoalveolar lavage fluid, and histamine and ovalbumin-specific immunoglobulin E production in serum.

Keywords asthma; CCR3; eosinophil; interleukin-4; interleukin-5; piperine

Introduction

Asthma is an inflammatory disease caused by dysregulated immune responses in the airway mucosa, with characteristic features including airway inflammation, excessive airway mucus production due to goblet cell hyperplasia and thickening of the airway wall.[1] Airway inflammation is associated with the infiltration of eosinophils, neutrophils, and T and B lymphocytes into airway and lung tissues.

$CD4^+$ T cells producing Th2 cytokines play an important role in the lungs of asthmatic subjects, particularly because interleukin-4 (IL-4) and interleukin-13 (IL-13) enhance immunoglobulin E production, interleukin-5 (IL-5) enhances eosinophil accumulation, and IL-13 directly enhances mucus hypersecretion and airway hyperresponsiveness.[2,3] IL-5 is a central factor mediating eosinophil expansion, recruitment and prolonged tissue survival in response to allergic stimuli. It seems reasonable to assume that blockade of IL-5 would result in elimination of eosinophilia associated with allergic asthma. Cytokine receptor antagonists to IL-4, IL-5 and IL-13 have been tested for their ability to control the balance between Th1 and Th2 responses. However, these reagents are not orally active and often cause undesirable side-effects. Thus, there is a need for development of an orally active and safe immune modulator for the treatment of allergic asthma. We therefore screened for natural reagents from plants based on Korean traditional herb medicines.

Piperine, a main component of *Piper longum* Linn. is a plant alkaloid known to exhibit a variety of biological activities. Piperine was the first amide to be isolated from piper

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species and was reported to display anti-inflammatory activity.[4] Other biological activities of piperine include immunomodulatory properties,[5] a consistent immunosuppressive effect,[6] and immunomodulatory and antitumour effects.[7] However, there are no reports of the anti-asthmatic and anti-inflammatory activities of piperine *in vivo*. The aim of this study was to evaluate the ability of piperine to control Th1- and Th2-type cytokines, various immune cell phenotypes and other factors. Using a murine model of asthma, we studied the effect of piperine on airway eosinophil accumulation, Th2 cytokine production, various immune cell phenotypes and histology.

Materials and Methods

Animals

Female Balb/c mice, 7–8 weeks old, were obtained from Daehan Biolink Co. Ltd (Eumsung, Republic of Korea). The study was approved by the Committee for Animal Welfare at Daejeon University. All animal procedures were conducted in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

Ovalbumin sensitization and inhalation

According to the modified protocol previously reported,[8,9] ovalbumin (500 μg/ml) in phosphate-buffered saline (PBS) was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma-Aldrich Korea, Yongin, Korea) in distilled water, then incubated for 60 min at room temperature after adjustment to pH 6.5 using 10 N NaOH, and centrifuged at 750*g* for 5 min. The ovalbumin–alum pellet was resuspened to the original volume in distilled water. All mice were immunized at different times (0, 1 and 2 weeks before inhalational exposure) by intraperitoneal injection of 0.2 ml alum-precipitated antigen containing 100 μg of ovalbumin (Sigma-Aldrich Korea) bound to 4 mg of aluminum hydroxide (Sigma-Aldrich Korea) in PBS. At 7 days after the second sensitization (intratracheal injection with 250 μg ovalbumin on the back of the tongue), mice were exposed to aerosolized ovalbumin for 30 min/day, 3 days/week for 8 weeks (at a flow rate of 250 l/min, 2.5% ovalbumin in normal saline). Piperine (4.5 and 2.25 mg/kg; Sigma-Aldrich Korea) and ciclosporin A (CsA; 20 mg/kg) in solution form were orally administered 5 times a week for the last 8 weeks. At 1 day after the last ovalbumin exposure, airway hyperresponsiveness was determined and samples of bronchoalveolar lavage fluid (BALF), lung cells and serum were collected for further analysis.

Bronchoalveolar lavage fluid

Immediately following assessment of airway hyperresponsiveness, mice were killed with an intraperitoneal injection of sodium pentoparbitone (100 mg/kg). The trachea was cannulated and BALF obtained by washing the airway lumina. Briefly, cells in the lungs were recovered by flushing 1 ml of BALF (1 mM EDTA, 10% fetal bovine serum, PBS) into the lungs via the trachea. Total cell counts were determined and 100 μl of fluid was cytospun onto glass slides using a Cytospin centrifuge (Cellspin, Hanil, Korea) (400*g* for 4 min). Differential cell counts were performed after staining with a Diff-Quik Stain set (Baxter Healthcare Corp., Miami, FL, US). The supernatant of BALF was stored at −25°C for the determination of cytokine levels.

Digestion of pulmonary tissue and cell preparation

Single-cell suspensions from lung tissues and BALF were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 20 mM HEPES and 2% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY, US). Briefly, the lungs were removed from the thoracic cavity. After mincing using sterile scalpels, the tissue was incubated in PBS containing 1 mg/ml collgenase IV and 2 mg/ml dispase for 40 min at 37°C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to further dissolve any remaining tissue clumps and then filtered using a 70-μm cell strainer (Falcon, Le Pont de Claix, France). The total number of cells was counted manually using a Fisher haemocytometer chamber Fisher Scientific Korea, Seoul, Korea. Cells ($2–4 \times 10^3$) were spun onto glass slides (400*g* for 4 min; Cytospin centrifuge; Cellspin). Differential counts were done according to standard morphologic criteria.

Determination of airway hyperresponsiveness

Airway hyperresponsiveness in mice was estimated using a previously described method with modifications.[10,11] A Buxco system (Biosystem XA; Buxco Electronics Inc, Troy, CT, US) was used to evaluate the extent of airway constriction (expressed as penh) in different groups of mice following the protocol described previously.

Penh = pause × PEF/PIF, where pause = (Te − Tr)/Tr (PIF, peak inspiratory flow; PEF, peak expiratory flow; Te, expiratory time; Tr, relaxation time).

In this experiment, mice were aerosolized with ovalbumin for 30 min/day, 3 days/week for 12 weeks. At 24 h after the final inhalation, mice were given aerosolized normal saline, followed by 3.15, 6.25, 12.5, 25 and 50 mg/ml methacholine (Sigma) serially. Airway reactivity was then monitored for 30 min. Differences of Penh values between groups were evaluated using Student's *t*-test.

Haematoxylin and eosin and Masson trichrome staining in murine ovalbumin-induced asthma lung tissue

Balb/c mice were treated with ovalbumin 3 times a week for 8 weeks (by injection, inhalation and spraying) to induce asthma. Two experimental groups were treated with different concentrations of piperine for the next 8 weeks (5 times/week). At the end of the experiment, the lungs were removed and analysed histologically using a modified protocol described previously.[9,12]

Antibodies and flow cytometric analysis

All antibodies (such as anti-CD3, CD4, CD8, CCR3, CD69, CD25, CD11b, Gr-1) for flow cytometric analysis were

purchased from BD Biosciences PharMingen (San Diego, CA, US). Cells from lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% fetal bovine serum and 0.01% NaN_3) for 10 min on ice, and analysed by two-colour flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA, US).

Quantitative real-time polymerase chain reaction

To study the anti-asthmatic effects of piperine on cytokine gene expression from lung tissue, quantitative real-time polymerase chain reaction (PCR) was performed after quantitative normalization for each gene by densitometry using β-actin gene expression. Briefly, total cellular RNA was extracted from the lung by the phenol–chloroform method (RNAsol^B; Tel-Test Inc., Friendswood, TX, US) according to the manufacturer's instructions. cDNA was synthesized from 3 μg of total RNA using a ReverTraAce-a-cDNA synthesis kit (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster, CA, US) with the following primer sequences: mouse thymus and activation regulated chemokine (TARC), 5′-CCCATGAAGACCTTCACCTC-3′ and 5′-ACTCTCGGCCTACATTGGTG-3′; eotaxin-2, 5′-CTGTGACCATCCCCTCATCT-3′ and 5′-CTTATGGCCCTTCTTGGTGA-3′; IL-13, 5′-ATGCCCAACAAAGCAGAGAC-3′ and 5′- TGAGAGAACCAGGGAGCTGT-3′; transforming growth factor-β (TGF-β), 5′-TGGAGCAACATGTGGAACTC-3′ and 5′-CTGCCGTACAACTCCAGTGA-3′; β-actin, 5′-TGGAATCCTGTGGTCCATGAAAC-3′ and 5′-GTCACAGTCAGCTGTATAGGG-3′.

Pro-inflammatory cytokine gene expression was analysed with SYBR Green PCR Mastermix (Applied Biosystems) and a final concentration of 200 nM of primers, using β-actin as the internal standard. The following PCR parameters were used: 2 min at 50°C, 10 min at 94°C, then 40 cycles of 1 min at 94°C, and 1 min at 60°C. The amount of SYBR Green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rose above baseline was referred to as the RQ (relative quantitative) and was proportional to the target concentration. Real-time PCR was performed in duplicate and analysed by an Applied Biosystems 7500 Fast Real-Time PCR system manual (threshold: 0.05, baseline: 6–15 cycles). To generate standard curves for pro-inflammatory cytokine and β-actin, serially diluted cDNA (1/1~1/16) was prepared and real-time PCR was performed as above. RQ evaluation by real-time PCR was determined and expressed for various samples.

Enzyme-linked immunosorbent assay

Interferon-γ (IFN-γ), IL-4, IL-5, IL-13 and eotaxin production from BALF and the serum of the indicated mice ($n = 5$) was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions with a monoclonal antibody based mouse interleukin ELISA kit (R&D Systems, Minneapolis, MN, US). Ovalbumin-specific IL-4 and IFN-γ production was measured in spleen cells, which were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% fetal bovine serum. The spleen cells were then cultured for 48 h at a concentration of 1×10^5 cells/well in 96-well culture plates (Corning Inc, Cambridge, MA, US) with or without 1 μg/ml of ovalbumin in a humidified atmosphere of 5% CO_2 in air at 37°C. The culture supernatants were collected and assayed for IFN-γ and IL-4 antibodies induced by ovalbumin using ELISA. All data represent the mean ± SD from at least three separate determinations and were compared using analysis of variance.

Statistical analysis

Data were analysed by two-way analysis of variance or unpaired Student's t-test followed by Dunnett's multiple comparison test (SPSS version 14.0 software SPSS Datasolution Korea, Seoul, Korea). The difference between the normal and the control groups (ovalbumin + vehicle) was clearly distinguished and so the significant differences between the normal and the control group are not shown in the figures and tables in order to highlight the differences between the experimental and the control groups. Results (mean ± SEM) were considered statistically significant at values of $P < 0.05$.

Results

Inhibitory effect of piperine on airway hyperresponsiveness

To evaluate the effect of piperine on airway hyperresponsiveness, total pulmonary airflow in mice was estimated using a mouse model. Penh was measured using a Buxco system on Day 1 after final inhalation and samples were immediately collected. Animals exposed to aerosolized ovalbumin showed increased airway hyperresponsiveness compared with animals receiving saline only (Figure 1b). As shown in Figure 1, relative to animals sensitized with ovalbumin (control group), piperine (4.5 mg/kg) treatment resulted in a significant ($P < 0.01$, $P < 0.05$) decrease in methacholine-induced airway hyperresponsiveness. However, piperine (2.25 mg/kg) treatment did not cause a significant decrease in Penh values.

Histological analysis of lung sections

We found infiltration of leukocytes in histological sections of lungs from ovalbumin-exposed control mice, and the tissue sections showed airway inflammation and erosion. Eosinophil infiltration was mainly observed in the peribronchial regions of the lung. In contrast, histological sections from piperine-treated mice indicated reduced airway inflammation in the lung tissue (Figure 1c).

Inhibitory effect of piperine on airway eosinophil accumulation and influx of inflammatory cells into airways

The total number of leukocytes was significantly reduced in piperine-treated mice compared with control mice, and the number of total lung cells was also significantly reduced in

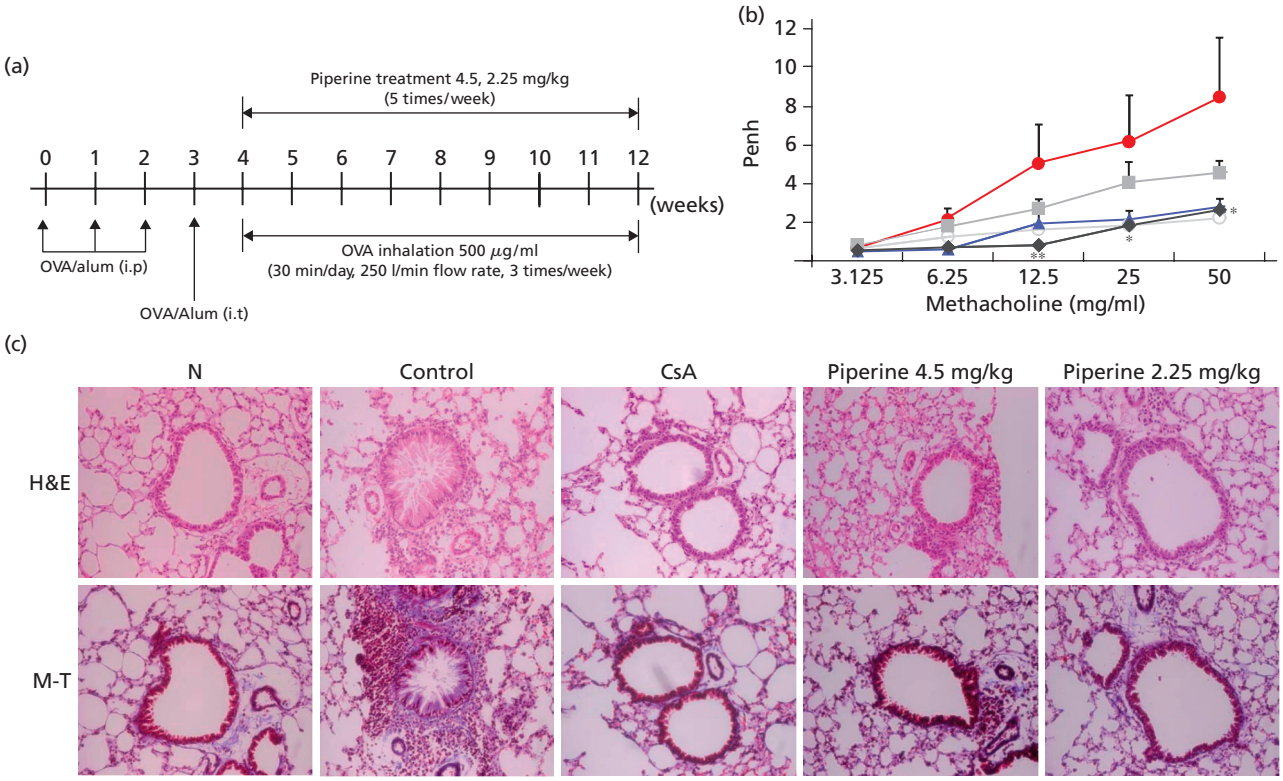

Figure 1 Effect of piperine on airway hyperresponsiveness. (a) Schematic diagram of methacholine-induced airway hyperresponsiveness in the sensitization protocol. OVA/alum, ovalbumin/aluminum potassium sulfate. (b) Penh was measured with a Buxco box. ○, Normal Balb/c mice; ●, control (ovalbumin inhalation + vehicle); ▲, ovalbumin + ciclosporin A (10 mg/kg); ■, ovalbumin + piperine (2.25 mg/kg); ◆, ovalbumin + piperine (4.5 mg/kg). $*P < 0.05$, $**P < 0.01$, significantly different compared with the control group. (c) Effect of piperine on the histology of lung tissue in lung cells in the ovalbumin-induced murine model of asthma. H&E, haematoxylin–eosin stain; M-T, Masson trichrome stain, N, normal Balb/c mice; control, ovalbumin inhalation + vehicle; CsA, ovalbumin + ciclosporin A (10 mg/kg); piperine 4.5 mg/kg, ovalbumin + piperine (4.5 mg/kg); piperine 2.25 mg/kg, ovalbumin + piperine (2.25 mg/kg).

piperine-treated mice (Table 1). Piperine (4.5 mg/kg) also decreased the absolute number of eosinophils in BALF. Moreover, in the piperine-treated group, the number of eosinophils was significantly decreased compared with the ovalbumin-exposed group.

Inhibitory effect of piperine on absolute number of immune cell subtypes in murine ovalbumin-induced asthma lung tissue

The numbers of $CD4^+$, $CD8^+$, $CCR3^+$, $Gr\text{-}1^+CD11b^+$, $CD69^+$, $CD3^+$ and $CD25^+$ cells in the lungs of ovalbumin-exposed mice were increased compared with the control group, and values from the piperine-treated mice were significantly lower compared with those of ovalbumin-exposed mice (Table 2).

Inhibition of cytokines (*in vivo* and *in vitro*), immunoglobulin E and histamine production in bronchoalveolar lavage fluid and serum

As shown in Figure 2a, IL-4, IL-5 and eotaxin levels were significantly reduced in piperine-treated mice. Otherwise, piperine enhanced IFN-γ production in BALF cells and IFN-γ production was not detected in serum (data not shown).

In our study, serum immunoglobulin E levels from ovalbumin-induced asthmatic mice were significantly increased compared with normal mice (PBS only), and piperine-treated mice had significantly reduced immunoglobulin E and histamine production (Figure 2b). We also measured IL-4 and IFN-γ in the culture supernatants by ELISA and found that piperine significantly inhibited Th2 cytokine (IL-4) production in splenocytes (Figure 2c), which was accompanied by a concurrent decrease in Th2 cytokine production in BALF.

Detection of mRNA in lung tissue by SYBR Green real-time polymerase chain reaction

As shown in Figure 2d, the mRNA for TARC, eotaxin-2, IL-13 and TGF-β was detectable in lung cells treated with PBS and ovalbumin + piperine (4.5, 2.25 mg/kg, respectively).

PCR products for TARC, eotaxin-2 and IL-13 amplified from lung cell RNA preparations were decreased in the piperine-treated mice compared with control mice (ovalbumin-induced asthma model group), but that of TGF-β was increased in the piperine-treated group. This was accompanied by changes in the eosinophil influx ($CD3^-/CCR3^+$) (Tables 1 and 2), and BALF cytokines (IL-4, IL-5 and eotaxin-1) production to some degree.

Table 1 Effect of piperine on airway inflammatory cells

Cell phenotype	Normal Balb/c mice	Ovalbumin-induced asthma model			
		Control[a]	Ciclosporin A (10 mg/kg)	Piperine (4.5 mg/kg)	Piperine (2.25 mg/kg)
Eosinophils in blood (%)	1.9 ± 0.5	5.2 ± 0.9	3.0 ± 1.5	1.85 ± 0.15**	4.65 ± 2.05
Neutrophils in blood (%)	26.5 ± 4.1	66.5 ± 6.1	31.95 ± 4.45	21.15 ± 10.45*	29.7 ± 9.7*
Basophils in blood (%)	0.3 ± 0.01	0.25 ± 0.05	0.3 ± 0.1	0.35 ± 0.05	0.2 ± 0.01
Total cells in lung ($\times 10^6$ cells)	14.7 ± 4.05	36.25 ± 3.25	25.38 ± 5.63	21.38 ± 1.88*	27.5 ± 1.5*
Total cells in BALF ($\times 10^4$ cells)	8.8 ± 3.6	30.7 ± 2.1	7.9 ± 1.7**	8.1 ± 1.3**	10.1 ± 1.1*
Eosinophils in BALF (×400)	9.0 ± 2.0	259.0 ± 65.1	56.5 ± 9.5*	41.0 ± 10.0*	133.0 ± 41.0
$CD3^-/CCR^+$ in BALF ($\times 10^4$ cells)	5.0 ± 1.0	81.1 ± 13.0	9.0 ± 2.0**	16.1 ± 5.0**	32.0 ± 11.0*

[a]Ovalbumin inhalation + vehicle. BALF, bronchoalveolar lavage fluid. Whole blood was harvested 24 h after the last ovalbumin challenge. Total inflammatory cell numbers in blood were counted and cell classification was performed on a minimum of 200 cells to classify eosinophils and lymphocytes. Results are expressed as the mean ± SE ($n = 5$). Significant differences between control and treatment groups were determined by analysis of variance ($^*P < 0.05$, $^{**}P < 0.01$).

Table 2 Quantification of immune cell subtypes in lung

Cell phenotype	Normal Balb/c mice	Ovalbumin-induced asthma model			
		Control[a]	Ciclosporin A (10 mg/kg)	Piperine (4.5 mg/kg)	Piperine (2.25 mg/kg)
$CD4^+$ ($\times 10^4$ cells)	50.9 ± 8.96	146.82 ± 3.71	68.72 ± 11.03**	66.11 ± 2.9**	99.99 ± 1.18**
$CD8^+$ ($\times 10^4$ cells)	7.11 ± 0.16	28.81 ± 2.78	17.47 ± 5.27	12.21 ± 0.23*	18.23 ± 1.47*
$Gr\text{-}1^+/CD11b^+$ ($\times 10^4$ cells)	14.03 ± 1.11	61.74 ± 4.22	19.16 ± 3.83**	16.97 ± 3.2**	39.09 ± 2.0*
$CD3^-/CCR3^+$ ($\times 10^4$ cells)	10.21 ± 3.38	63.52 ± 8.99	31.49 ± 1.18**	22.17 ± 0.24**	40.85 ± 6.87*
$CD3^+/CD69^+$ ($\times 10^4$ cells)	9.83 ± 0.96	49.53 ± 1.48	24.08 ± 5.77	17.6 ± 2.86**	36.32 ± 3.18*
$CD4^+/CD25^+$ ($\times 10^4$ cells)	6.25 ± 0.12	8.0 ± 1.6	11.74 ± 1.76	11.95 ± 0.17	11.43 ± 0.27

[a]Ovalbumin inhalation + vehicle. Absolute numbers of various immune cell subtypes in lung were counted. Results are expressed as mean ± SE ($n = 5$). Significant differences between control and treatment groups were determined by analysis of variance ($^*P < 0.05$, $^{**}P < 0.01$).

Discussion

The therapeutic agents that may be used in the treatment of asthma are numerous. For example, anti-IL-4 inhibits immunoglobulin E production and inhibits eosinophil infiltration.[13] Anti-IL-5 inhibits eosinophil adhesion, infiltration and mediator release.[14] Eosinophilia is driven by allergen-activated Th2 cells that generate large amounts of Th2 cytokines (such as IL-4, IL-5 and IL-13). IL-5 is the most critical cytokine mediating increased eosinophil differentiation, activation and survival.[15]

Modern research into the pharmacological actions of piperine was made possible by the Danish chemist Hans Christian Orsted, who was the first to isolate piperine in 1820.[16] Piperine also possesses bioavailability enhancing activity with various structurally and therapeutically diverse drugs.[17] Although the exact mechanism underlying the biological efficacy of piperine remains elusive, its antioxidant, antidepressant, hepatoprotective, antimetastatic, antithyroid, immunomodulatory and antitumour effects have been reported. Piperine also has inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*.[18] Regarding the immunomodulatory activity of piperine, an increase in the circulating antibody titre and antibody-forming cells has been demonstrated, reflecting a stimulatory effect on the humoral arm of the immune system.[19] There have been no reports on the effect of piperine in the asthma model until now.

In a previous study, it was shown that piperine at various doses (5, 10 and 20 mg/kg) once daily for 4 weeks possessed antidepressant-like activity and a cognitive-enhancing effect.[20] In our preliminary study, piperine treatment (at 4.5 and 2.25 mg/kg) did not cause toxic effects on alanine aminotransferase and aspartic acid transaminase levels (data not shown). The above doses were therefore used in the present study. CsA has been shown to inhibit single-allergen induced allergic inflammation such as eosinophilic and lymphocytic infiltration and mRNA expression for IL-4 and IL-5. In the present study, we used CsA as a positive control.

Piperine prevented the development of airway hyperresponsiveness (Figure 1b), airway eosinophilia (Table 1), lung inflammation and increased Th2 cytokine levels (Figure 2a, c) in BALF. These results demonstrate that piperine has profound negative regulatory effects on the development of lung allergic responses in the ovalbumin-induced asthma model. Moreover, the negative regulatory effects exhibited by piperine were accompanied by the production of IL-4 and IL-5.

Asthma produces immune abnormalities in a wide variety of cell populations. Thus, another goal in asthma research includes the evaluation of specific cell subpopulations. Immunophenotyping by flow cytometry showed a similar pattern as total lymphocyte numbers in BALF and lung. Our results showed that piperine downregulates CD4 and CD8 T lymphocyte subsets and $CD3^+$ and $CD69^+$ (early activated T cells). It was recently suggested that a transient activation-induced CD69

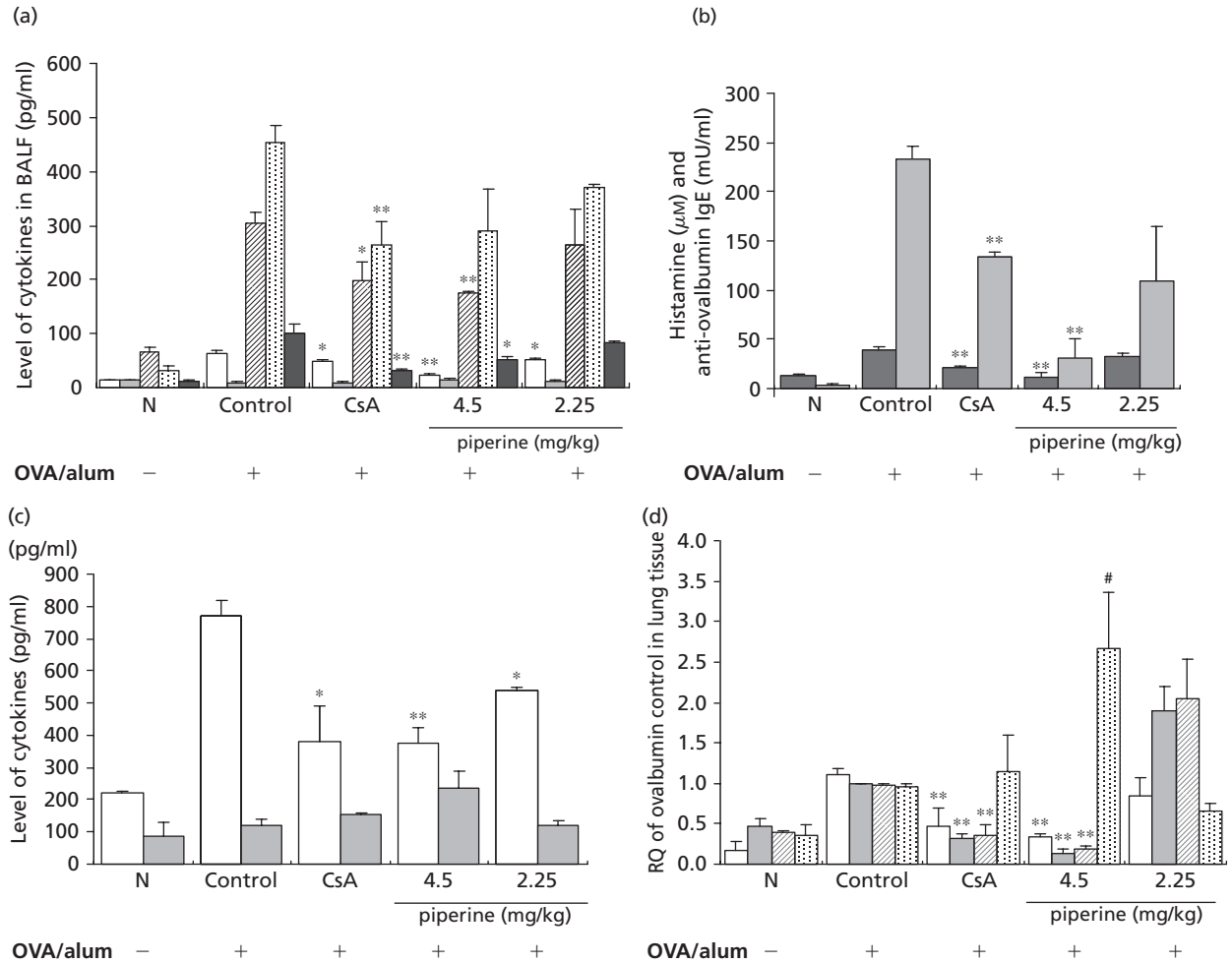

Figure 2 Inhibition of cytokines, immunoglobulin E and histamine production in bronchoalveolar lavage fluid and serum. (a, b), Effect of piperine on Th2 cytokines (□, interleukin-4; ▨, interleukin-5; ⊡, interleukin-13), Th1 cytokine (■, interferon-γ) and eotaxin (■) in bronchoalveolar lavage fluid (BALF), and immunoglobulin E (■) and histamine (■) levels in serum. (c) Immunomodulatory effects of piperine on ovalbumin-specific Th1/Th2 cytokine production in spleen cells. (□, interleukin-4; ■, interferon-γ) (d) Effect of piperine on thymus and activation regulated chemokine (□), eotaxin-2 (■), interleukin-13 (▨) and transforming growth factor-β (⊡) mRNA gene expression in lung tissue in the ovalbumin-induced murine model of asthma. Results are expressed as the mean ± SE ($n = 5$). $^{\#}P < 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences between control and treatment groups (analysis of variance). OVA/alum, ovalbumin/aluminum potassium sulfate; N, normal Balb/c mice; control, ovalbumin inhalation + vehicle; CsA, ovalbumin + ciclosporin A (10 mg/kg); ovalbumin + piperine (4.5, 2.25 mg/kg).

surface expression may be important for regulating T cell trafficking.[21] Moreover, CD69 might affect the immune response during T cell differentiation, involving immunoregulatory cytokines that include, but might not be limited to, TGF-β, which controls T cell differentiation.[22]

Eosinophils are one of the cell types known to express Gr-1, therefore eosinophil populations may constitute a substantial portion of the $Gr\text{-}1^{+}CD11b^{+}$ populations. Our results showed that $Gr\text{-}1^{+}$ cells were increased with ovalbumin challenge but this was significantly reduced in piperine-treated mice (Table 2).

The second member of the eotaxin group, eotaxin-2,[23] serves as a specific eosinophil chemoattractant, acting via chemotactic chemokine receptor (CCR3). Eotaxin-2, together with IL-5, also functions to promote lung eosinophilia.[24,25] TARC is a member of the C-C chemokine group and is predominantly expressed on Th2 lymphocytes, basophils and natural killer cells.[26] TGF-β is produced by many cells, including regulatory T cells, that play down-regulatory roles in allergic asthma.[27]

In our study, PCR products for TARC from lung cell RNA preparations were decreased in the piperine-treated group compared with control groups, but those of TGF-β were increased in the piperine-treated group. These results indicate that piperine significantly affects TARC and TGF-β mRNA expression, and these molecules attract eosinophils and Th2 cells into the airway.

Our study failed to demonstrate that low-dose piperine (2.25 mg/kg) significantly inhibited eotaxin-2 and IL-13 mRNA levels. Low-dose piperine had inconsistent effects on mRNA levels (Figure 2d). The eotaxin-2 and IL-13 mRNA levels appeared to be raised, but not significantly, whereas at the high dose they were reduced. It may be that the low dose did not affect the eotaxin and IL-13 mRNA levels other than by upregulation of eotaxin and IL-13, however the exact

explanation and the mechanism of the above result by piperine is unknown. Further work in multiple-dose and long-term studies with piperine are necessary to evaluate if a meaningful therapeutic endpoint is achieved.

Nuclear factor-κB (NF-κB) is a major family of transcription factors activated during the inflammatory response in asthma. NF-κB is a key transcriptional regulator of multiple pro-inflammatory mediators such as tumour necrosis factor α (TNFα) and interleukins, and enhanced activation of NF-κB has been implicated in asthma.[28]

A preliminary study indicated that piperine interferes with earlier steps of transcription of NF-κB dependent genes.[29] Piperine is capable of inhibiting inflammatory cytokine-induced activation of NF-κB by blocking IκBα kinase activation. Our results also showed that piperine can inhibit the neutrophil (including eosinophil) concentration in blood (Table 1). Therefore, piperine may reduce Th2 cytokine production and gene expression by inhibiting NF-κB activation by a similar mechanism as described above. Additional studies are needed to characterize the precise mechanism of the therapeutic action of piperine for the treatment of asthma.

Conclusions

Piperine had profound inhibitory effects on airway inflammation in a murine model of asthma, and this effect was caused by suppression of Th2 cytokines (IL-4, IL-5, IL-13), immunoglobulin E, eosinophil CCR3 expression, and by increased TGF-β gene expression in lung. Hence, piperine may act as a potential immunomodulator by downregulating Th2 cytokines.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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