

Low-dose benzo[a]pyrene aggravates allergic airway inflammation in mice

Rie Yanagisawa^{a*}, Eiko Koike^a, Tin-Tin Win-Shwe^a, Takamichi Ichinose^b and Hirohisa Takano^c

Abstract: Benzo[a]pyrene (BaP) reportedly has mutagenic and adjuvant activities. We aimed to determine the effects of low-dose BaP administration on allergic airway inflammation and mediastinal lymph node (MLN) cell activation/proliferation in mice. Male C3H/HeJ mice were intratracheally administered ovalbumin (OVA) every 2 weeks and/or BaP (0, 0.05, 1 and 20 pmol per animal per week) once per week for 6 weeks. The cellular profile of bronchoalveolar lavage (BAL) fluid, histological changes, inflammatory cytokines/chemokines in the lungs, OVA-specific immunoglobulin (Ig) in serum and MLN cell activation/proliferation were examined. BaP administration of 20 pmol with OVA enhanced neutrophil and macrophage accumulation in the lungs. Compared with OVA administration, BaP administration with OVA tended to enhance pulmonary eosinophilia and goblet cell hyperplasia. Furthermore, it increased the levels of interleukin (IL)-5, IL-13, IL-33, monocyte chemoattractant protein-1 and eotaxin in the lungs, and OVA-specific IgG₁ in serum, although not dose-dependently. Compared with the vehicle group, IL-6 and tumor necrosis factor- α levels were higher in the OVA + 1 pmol BaP group and IL-12 production was higher in the OVA + 20 pmol BaP group. *Ex vivo* studies showed that co-exposure to OVA and BaP activated the MHC class II and CD86 expression in MLN cells. Exposure to BaP with OVA increased IL-4, IL-5 and interferon gamma levels in culture supernatants of OVA-re-stimulated MLN cells. In conclusion, low-dose BaP can, at least in part, enhance allergic airway inflammation by facilitating Th2 responses and activating MLN cells; a high BaP dose may contribute to activating both Th1 and Th2 responses. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: benzo[a]pyrene; diesel exhaust particles; allergic airway inflammation; diesel exhaust particles; polycyclic aromatic hydrocarbons; Th2 responses

Introduction

Epidemiological studies have shown that exposure to ambient particulate matter (PM) is positively associated with an exacerbation of respiratory infections and asthmatic and allergic symptoms (Dockery *et al.*, 1993; Peterson and Saxon, 1996). PM_{2.5}, which is PM with a < 2.5- μ m diameter, is closely associated with respiratory diseases and subsequent mortality (Romieu *et al.*, 1996; Peters *et al.*, 1997). Diesel exhaust particles (DEPs) are a substantial portion of ambient PM_{2.5} in urban areas (Yoshizumi and Ishii, 1989), suggesting that DEPs affect allergic responses. Diaz Sanchez *et al.* (1997) showed that intranasal DEP administration enhances allergen-induced airway inflammation and allergen-specific IgE production in mice (Fujimaki, 1995). Intranasal instillation of DEPs with the allergen to mice enhances *in vitro* allergen-stimulated IL-4 production from cervical lymph node cells (Fujimaki *et al.*, 1995). In addition, we reported that intratracheal DEP instillation enhances airway hyper-responsiveness, Th2 cytokine secretion, eosinophil recruitment into the airways and goblet cell metaplasia (Miyabara *et al.*, 1998; Takano *et al.*, 1998).

DEPs are a complex mixture, consisting of carbonaceous nuclei and a vast number of organic chemicals such as polycyclic aromatic hydrocarbons (PAHs), aliphatic hydrocarbons, heterocycles and quinones (IARC, 1989; DeMarini *et al.*, 2004). Nel *et al.* (1998) reported that numerous experimental studies have shown that PAHs from DEPs play an important role in allergic respiratory diseases. Our previous studies demonstrated that extracted organic chemicals from DEPs, rather than residual carbonaceous nuclei of DEPs after extraction, predominantly aggravate allergen-induced

airway inflammation by enhancing Th2 responses in mice (Yanagisawa *et al.*, 2006). In addition, 9, 10-phenanthraquinone and 1, 2-naphthoquinone, quinone compounds in DEPs, partially contribute to the exacerbation of allergic airway inflammation in mice (Hiyoshi *et al.*, 2005; Inoue *et al.*, 2007a, 2007b). However, the components in DEPs that are mainly associated with aggravation of allergic airway inflammation remain unclear.

Benzo[a]pyrene (BaP) (Fig. 1) is a ubiquitous environmental pollutant primarily derived from cigarette smoke, DEPs, industrial wastes and food products (Birnbaum, 1994; Rubin, 2001) and is recognized as an environmental mutagenic and carcinogenic PAH. BaP also plays a role as an adjuvant immunoglobulin E (IgE) producer in allergen immunized mice. Mizutani *et al.* (2007) suggested that BaP exposure aggravates allergic rhinitis in a guinea pig model. Intranasal (Kadkhoda *et al.*, 2005) and oral (Kadkhoda *et al.*, 2004) administration with BaP and allergen enhances allergen-specific IgE production and systemic Th1/Th2 responses.

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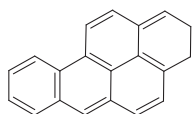


Figure 1. Structural formula of benzo[a]pyrene (BaP)

BaP exerts proallergic effects on sensitized basophils by enhancing mediator release from basophils in allergic individuals to birch pollen (Lubitz *et al.*, 2010). However, whether BaP exposure affects respiratory diseases, including allergic airway inflammation, remains unclear.

The aim of the present study was to determine the effects of intratracheal BaP exposure on allergic airway inflammation and mediastinal lymph node (MLN) cell activation/proliferation in mice.

Materials and Methods

Animals

Five-week-old male C3H/HeJcl mice were purchased from Japan Clea Co. (Tokyo, Japan) and used for the experiments. The animals were provided a commercial diet (CE-2; Japan Clea) and water *ad libitum* and housed in an animal facility maintained at 22–26 °C and 40%–69% humidity under a 12-h light/dark cycle. All procedures were approved by the Animal Care and Use Committee of National Institute for Environmental Studies, which was conducted in accordance with the guideline for the Care and Use of Laboratory Animals of National Institute for Environmental Studies. Animals were humanely treated and were alleviated of suffering.

Study protocol

Mice were divided into eight experimental groups: (1) vehicle, (2) 0.05 pmol BaP per animal per week, (3) 1 pmol BaP per animal per week [BaP; B1760; Sigma-Aldrich Co., St. Louis, MO, USA, purity $\geq 96\%$ (HPLC)], (4) 20 pmol BaP per animal per week, (5) OVA, (6) OVA + 0.05 pmol BaP per animal per week, (7) OVA + 1 pmol BaP per animal per week and (8) OVA + 20 pmol BaP per animal per week (Fig. 2). The vehicle group received phosphate-buffered saline (PBS; pH, 7.4; Invitrogen Corp., Carlsbad, CA, USA) containing 0.01% dimethyl sulfoxide [DMSO; Nacalai Tesque, Kyoto, Japan, purity 99.5% (GC)] once per week for 6 weeks. The OVA group received 1 μg of OVA (10 $\mu\text{g mL}^{-1}$; Sigma Chemical Co, St Louis, MO, USA) dissolved in PBS containing 0.01% DMSO every 2 weeks for 6 weeks. BaP was dissolved in 100% DMSO and subsequently diluted with PBS. BaP solutions were administered at doses of 0.05, 1 and 20 pmol BaP per animal per week once per week for 6 weeks. The OVA + BaP groups received combined administration of BaP

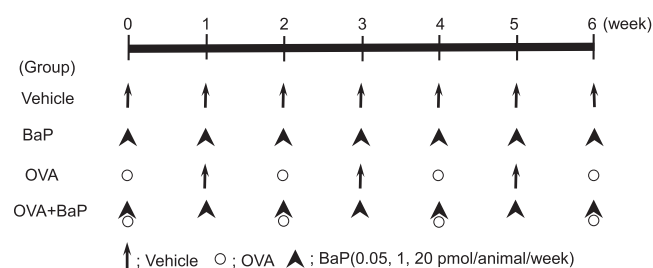


Figure 2. Experimental design for intratracheal instillation of benzo[a]pyrene (BaP) and ovalbumin (OVA).

together with OVA in the same vehicle. Mice were anesthetized with 4% halothane (Takeda Chemical Industries, Ltd, Osaka, Japan), and intratracheally instilled with 100- μL aliquots of an aqueous suspension.

Blood retrieval and bronchoalveolar lavage (BAL)

All mice were euthanized under anesthesia 24 h after the final intratracheal instillation (4–5 animals per group). The chest and abdominal walls were opened, and blood was retrieved by cardiac puncture. The lungs were lavaged with 1 ml of sterile saline at 37 °C three times by syringe. The lavaged fluid was harvested by gentle aspiration. The average volume was 90% of the amount instilled. The lavage fluid was centrifuged at 300 g for 10 min at 4 °C to recover alveolar-free cells. The total cell count was determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were prepared using an Autosmear (Sakura Seiki Co., Tokyo, Japan) and stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 500 cells were counted under a microscope (AX80; Olympus, Tokyo, Japan). The lungs were retrieved immediately after BAL. The serum and lungs were stored at -80°C until further use.

Histopathological examination

The lungs were fixed in 10% phosphate-buffered formalin (pH 7.4) 24 h after the final intratracheal instillation. Tissue sections were embedded in paraffin and cut into 4- μm thick slices (5–6 animals per group). The histological specimens were stained with hematoxylin and eosin (H&E) to evaluate eosinophil and lymphocyte infiltration in the airway. The sections were also subjected to periodic acid–Schiff (PAS) staining to evaluate goblet cell proliferation in the bronchial epithelium. Histological changes were assessed using an Olympus AX80 microscope. The degree of eosinophil and lymphocyte infiltration in the airways or goblet cell proliferation in the bronchial epithelium was graded in a blind fashion as follows: 0 = not present, 0.5 = slight, 1 = mild, 1.5 = mild to moderate, 2 = moderate, 2.5 = moderate to marked and 3 = marked. 0.5 was defined as an inflammatory reaction affecting < 10% of the airways or goblet cells stained with PAS 1 = 10–20%, 1.5 = 20–30%, 2 = 30–40%, 2.5 = 40–50% and 3 = >50% of the airways or goblet cells affected.

Enzyme-linked immunosorbent assay (ELISA)

The lungs (4–5 animals per group) were removed after BAL and homogenized with 10 mM potassium phosphate buffer (pH 7.4), containing 0.1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich), 0.1 mM phenylmethanesulfonyl fluoride (Nacalai Tesque), 1 μM pepstatin (Peptide Institute, Inc., Osaka, Japan) and 2 μM leupeptin (Peptide Institute). The homogenates were subsequently centrifuged at 105 000 g for 1 h at 4 °C. The supernatants were dispensed and stored at -80°C . ELISAs for interleukin (IL)-4 (Thermo Fisher Scientific Inc., IL, USA), IL-5 (Thermo Fisher Scientific), IL-13 (R&D Systems, Minneapolis, MN, USA), IL-33 (R&D Systems), interferon gamma (IFN- γ ; Thermo Fisher Scientific), IL-12 (Thermo Fisher Scientific), monocyte chemoattractant protein-1 (MCP-1; R&D Systems), chemokine (C-C motif) ligand 5/regulated and normal T-cell expressed and secreted (CCL5/RANTES; R&D Systems), CCL11/eotaxin (R&D Systems) in lung tissue supernatants and IL-4 (e-Bioscience, San Diego, CA, USA), IL-5 (e-Bioscience), interferon gamma (IFN- γ ; e-Bioscience) in culture supernatants of the

OVA-restimulated MLN cells were conducted. OVA-specific IgE was measured using a mouse anti-OVA IgE ELISA KIT (Shibayagi Co., Gunma, Japan). Serum OVA-specific IgG₁ was measured as previously reported (Yanagisawa *et al.*, 2006).

Preparation of MLN cells in mice

MLN cells were isolated (five animals per group) 24 h after the last intratracheal administration. The lymph nodes were pushed through a sterile stainless wire mesh. Subsequently, the lymph node cells were centrifuged at 400 *g* for 5 min at 20 °C. After washing with PBS, the cells were resuspended in culture medium R10, consisting of Gibco RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals Inc., Eschwege, Germany), 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (Sigma) and 50 µM 2-mercaptoethanol (Invitrogen). Cell numbers and viability were determined by the trypan blue (Invitrogen) exclusion method.

Fluorescence-activated cell sorting (FACS) analysis

The following monoclonal antibodies were used for FACS analysis. MHC class II, I-A/I-E (2G9, Rat IgG_{2a} κ, FITC-conjugated; BD Biosciences, San Diego, CA, USA); CD86 (GL1, Rat IgG_{2a} κ PE-conjugated, BD Biosciences); TCR β-chain (H57-597, FITC-conjugated, BD Biosciences); CD4 (RM4-5, PE-conjugated, BD Biosciences); CD8 (53-6.7, PE-conjugated, Miltenyi Biotec GmbH, Germany). LNCs were resuspended in 100 µL of PBS with 0.3% bovine serum albumin and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) and were incubated with each antibody for 30 min on ice. After incubation, the cells were washed and fluorescence was measured by an FACSCalibur (Becton, Dickinson and Co., Parsippany, NJ, USA). Fluorescence data from 10 000 cells in each sample were collected, and positive cells were expressed as % total events.

Cell proliferation assay

MLN cells (2×10^5) were cultured with 20 µg of OVA in 200 µL of R10 medium in 96-well flat-bottom plates. These cultures were

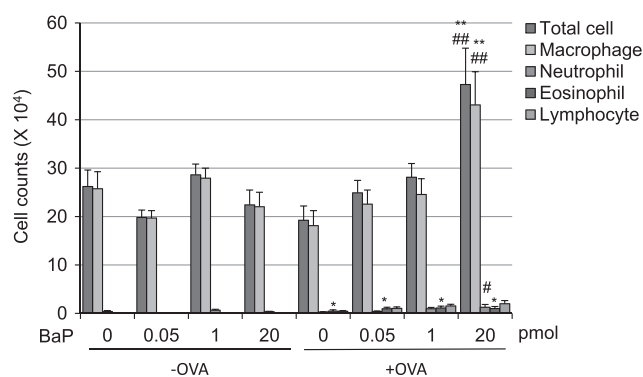


Figure 3. The number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid after exposure to benzo[a]pyrene (BaP) in allergic airway inflammation in mice 24 h after the final intratracheal administration. Data were expressed as the means $\pm$ SE for 4–5 animals per group. * $P < 0.05$ versus vehicle group, ** $P < 0.01$ versus vehicle group, # $P < 0.05$ versus the ovalbumin (OVA) group.

performed in triplicate at 37 °C in a 5% CO₂/95% air atmosphere. After 65 h, we examined cell proliferation by adding 5-bromo-2'-deoxyuridine to each well 20 h before the measurement. Cell proliferation was measured with a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions.

Statistical analysis

Data were expressed as the means $\pm$ standard errors. Differences among the groups were determined by one-way analysis of variance or the Kruskal–Wallis test using Ekuseru-Toukei 2010 statistical software (Social Survey Research Information Co., Tokyo, Japan). Differences between the experimental and control groups were determined by Dunnett's multiple comparison test or Steel's multiple comparison test. P -values of < 0.05 were considered statistically significant.

Table 1. Protein levels of Th1/Th2 cytokines in the lungs after exposure to benzo[a]pyrene (BaP) on allergic airway inflammation in mice

Group	Pg per total lung					
	IL-5	IL-13	IL-33	IL-6	TNF- α	IL-12
Vehicle	11.2 $\pm$ 0.23	8.89 $\pm$ 0.78	5672 $\pm$ 673	84.5 $\pm$ 6.37	289 $\pm$ 24.1	967 $\pm$ 52.4
0.05 pmol BaP	11.8 $\pm$ 0.43	6.48 $\pm$ 1.58	4297 $\pm$ 208	84.8 $\pm$ 7.68	352 $\pm$ 33.1	937 $\pm$ 45.0
1 pmol BaP	11.2 $\pm$ 0.21	8.48 $\pm$ 2.15	5566 $\pm$ 439	87.5 $\pm$ 5.47	342 $\pm$ 41.7*	1021 $\pm$ 28.7
20 pmol BaP	10.3 $\pm$ 0.22	3.81 $\pm$ 1.07	4641 $\pm$ 187	102 $\pm$ 1.39	412 $\pm$ 23.6	1057 $\pm$ 51.8
OVA	94.2 $\pm$ 25.0**	104 $\pm$ 33.1	7952 $\pm$ 1078	91.2 $\pm$ 6.97	333 $\pm$ 17.6	1092 $\pm$ 30.4
OVA + 0.05 pmol BaP	225 $\pm$ 41.5*	265 $\pm$ 63.5*	12963 $\pm$ 2441*	97.3 $\pm$ 4.73	345 $\pm$ 35.0	1076 $\pm$ 92.7
OVA + 1 pmol BaP	205 $\pm$ 55.0*	248 $\pm$ 66.7*	12782 $\pm$ 2691	121 $\pm$ 10.4**	437 $\pm$ 35.1**	1074 $\pm$ 51.4
OVA + 20 pmol BaP	152 $\pm$ 53.1*	176 $\pm$ 58.3*	8700 $\pm$ 402*	110 $\pm$ 9.96	394 $\pm$ 11.8	1173 $\pm$ 88.57*

^aThe supernatants of homogenized lungs were measured by enzyme-linked immunosorbent assay (ELISA). Data were expressed as the means $\pm$ SE for 4–5 animals per group.

* $P < 0.05$ versus the Vehicle group,

** $P < 0.01$ versus the Vehicle group,

$P < 0.05$ versus the OVA group.

Table 2. Protein levels of chemokines in the lungs after exposure to benzo[a]pyrene (BaP) on allergic airway inflammation in mice

Group	Pg per total lung		
	MCP-1	RANTES	eotaxin
Vehicle	5.64 ± 2.44	55.5 ± 6.98	40.2 ± 3.51
0.05 pmol BaP	1.22 ± 1.13	39.7 ± 3.87	40.8 ± 2.55
1 pmol BaP	5.36 ± 3.42	60.0 ± 4.57	43.4 ± 5.07
20 pmol BaP	4.11 ± 2.61	51.1 ± 5.20	43.1 ± 1.92
OVA	43.1 ± 11.9	101 ± 13.0*	194 ± 50.6*
OVA + 0.05 pmol BaP	78.5 ± 24.4*	121 ± 17.3*	399 ± 77.4*
OVA + 1 pmol BaP	90.0 ± 22.7*	109 ± 6.61*	484 ± 125*
OVA + 20 pmol BaP	57.1 ± 13.6*	104 ± 7.82**	343 ± 89.2*

^aThe supernatants of homogenized lungs were measured by enzyme-linked immunosorbent assay (ELISA). Data were expressed as the means ± SE for 4–5 animals per group.

* $P < 0.05$ versus the Vehicle group,

** $P < 0.01$ versus the Vehicle group.

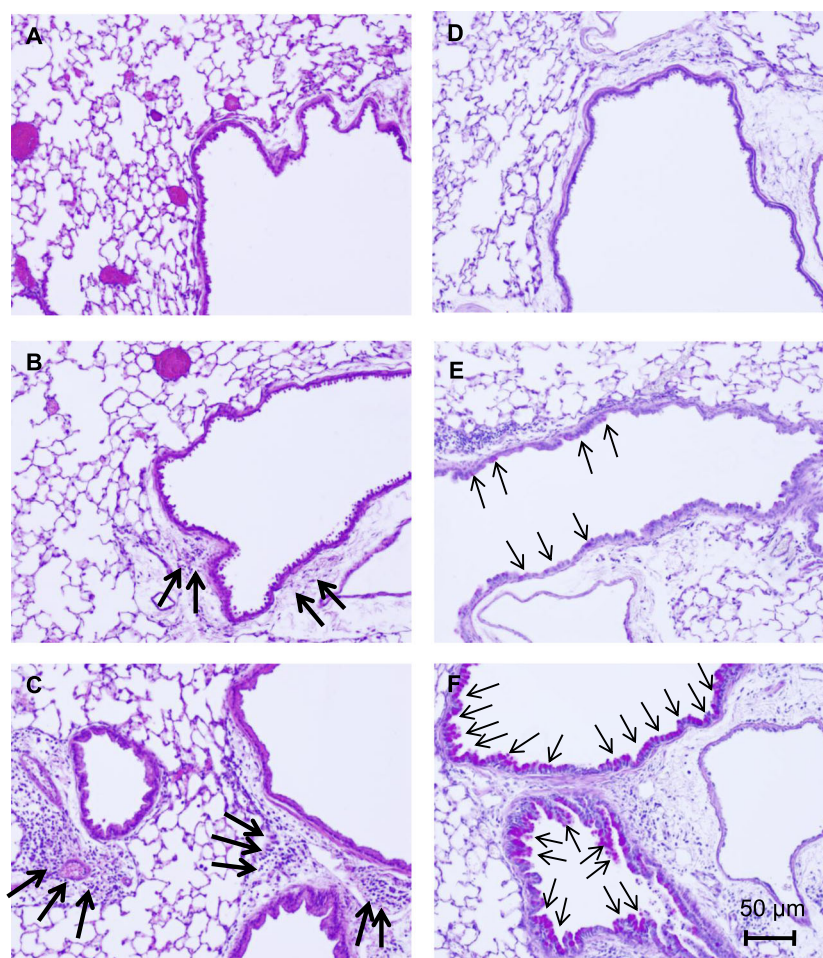


Figure 4. Histological findings in the lungs after exposure to benzo[a]pyrene (BaP) in allergic airway inflammation in mice. The infiltration of inflammatory cells into the lungs using hematoxylin and eosin (H&E) staining and goblet cell hyperplasia of the airway epithelium using periodic acid–Schiff (PAS) staining were evaluated 24 h after the last intratracheal instillation (5–6 animals per group). H&E staining; (A–C), PAS staining (D–F), (A, D) the vehicle group, (B, E) the ovalbumin (OVA) group, (C, F) the OVA + 20 pmol BaP group. Arrows show accumulation of eosinophils and lymphocytes (thick) and proliferation of goblet cells (thin), respectively.

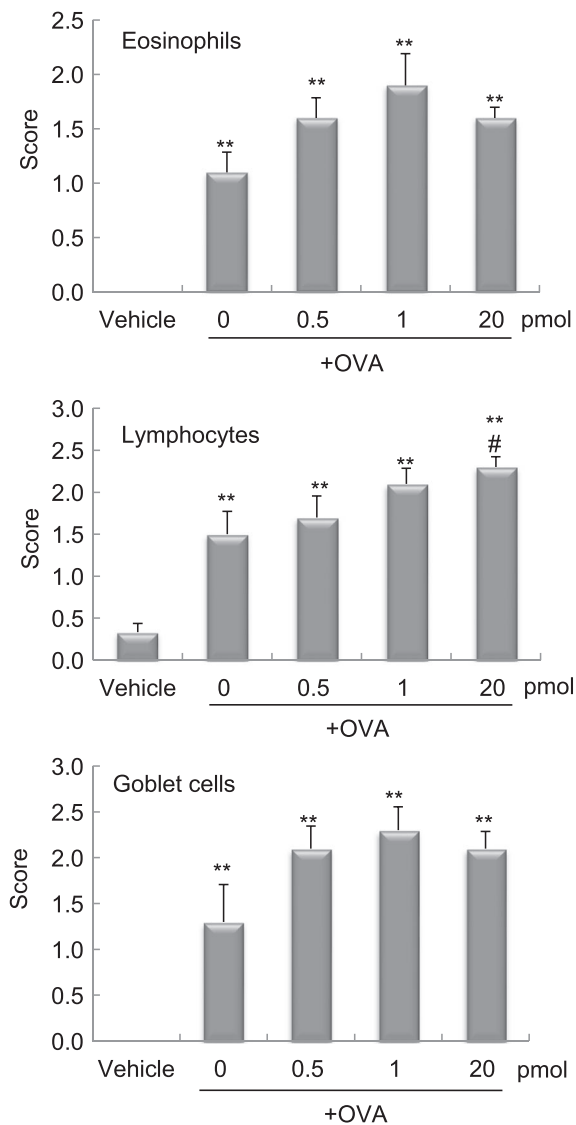


Figure 5. Evaluation of histological findings in the lungs after exposure to benzo[a]pyrene (BaP) in allergic airway inflammation in mice. The degree of eosinophil and lymphocyte infiltration in the airways or goblet cell proliferation in the bronchial epithelium was graded as: 0 = not present; 0.5 = slight; 1 = mild; 1.5 = mild to moderate; 2 = moderate; 2.5 = moderate to marked; 3 = marked. Data were expressed as the means $\pm$ SE for 5–6 animals per group. ** $P < 0.01$ versus vehicle group, # $P < 0.05$ versus the ovalbumin (OVA) group.

Results

Effects of BaP on cellular profiles in BAL fluid

We confirmed cellular infiltration into BAL fluid 24 h after the final intratracheal instillation to estimate the effects of BaP on allergen-induced airway inflammation. OVA administration increased the number of eosinophils compared with vehicle administration (Fig. 3). Increases in the number of macrophages and neutrophils were more prominent in the OVA + 20 pmol BaP group than those in the OVA group (macrophages, $P < 0.01$; neutrophils, $P < 0.05$). The administration of 0.05 or 1 pmol BaP with OVA also increased the number of neutrophils, eosinophils and lymphocytes compared with OVA administration, but the result was not statistically significant.

Effects of BaP on cytokine and chemokine expression in the lungs

We investigated the effect of BaP on the protein levels of proinflammatory molecules in the lungs 24 h after the final instillation. OVA administration caused a significant increase in IL-5 and RANTES protein expression in the lungs compared with those in the vehicle administration (IL-5, $P < 0.01$; RANTES, $P < 0.05$; Tables 1, 2). Co-exposure to OVA and BaP increased IL-5, IL-13, IL-33, MCP-1 and eotaxin levels compared with OVA exposure alone, but no significant changes were observed. IL-6 and TNF- α levels were higher in the OVA + 1 pmol BaP group compared with the OVA group ($P < 0.05$). IL-12 levels were higher in the OVA + 20 pmol BaP group than in the vehicle group ($P < 0.05$). IL-1 β , IL-4 and IL-10 levels had no significant changes in either group (data not shown). IFN- γ production was undetectable in all the groups (data not shown).

Effects of BaP on lung histopathological findings

Next, a histopathological analysis of H&E- and PAS-stained lung sections was performed 24 h after the final intratracheal instillation. We noted no remarkable differences in pathological alterations in the vehicle-exposed lungs (Fig. 4A, D). OVA administration caused an increase in eosinophil and lymphocyte accumulation in the peribronchial and perivascular regions, as well as goblet cell proliferation in the airway epithelium (Figs 4B, E and 5). Co-exposure to OVA and BaP elevated eosinophilic inflammation and goblet cell hyperplasia compared with exposure to OVA alone, even although the difference was not statistically significant (Fig. 4C, F). In addition, lymphocyte accumulation was higher in the OVA + 20 pmol BaP group than in the vehicle or OVA group.

Effects of BaP on serum OVA-specific immunoglobulin

To evaluate the effects of BaP on allergen-induced Ig production, OVA-specific IgE and IgG₁ in serum 24 h after the last intratracheal instillation were measured. As shown in Fig. 6, OVA administration significantly increased production of OVA-specific IgE and IgG₁ compared with the vehicle alone ($P < 0.01$). Co-exposure to OVA and 1 pmol BaP significantly increased OVA-specific IgG₁ production compared with OVA exposure alone ($P < 0.05$).

Effects of BaP on MLN cell proliferation/activation

We evaluated cell proliferation and cell surface molecule expression 24 h after the last intratracheal instillation to evaluate the effects of BaP on MLN cell activation/proliferation. The number of MLN cells increased after OVA exposure, but it was not affected by BaP exposure (Fig. 7A). Exposure to 1 or 20 pmol BaP with OVA significantly increased the percentage of MHC class II⁺CD86⁺ cells (Fig. 7B). The CD4⁺/CD8⁺ T-cell ratio was slightly elevated after co-exposure to OVA and BaP (Fig. 7C). Exposure to BaP with OVA enhanced MLN cell proliferation by OVA-re-stimulation for 65 h, even although the increase was not statistically significant (Fig. 7D). Overall, IL-4, IL-5 and IFN- γ levels in culture supernatants of the OVA-re-stimulated MLN cells were higher in the OVA + BaP groups than in the OVA group and this effect was more prominent in the OVA + 0.05 pmol BaP group (Fig. 7E, F, G).

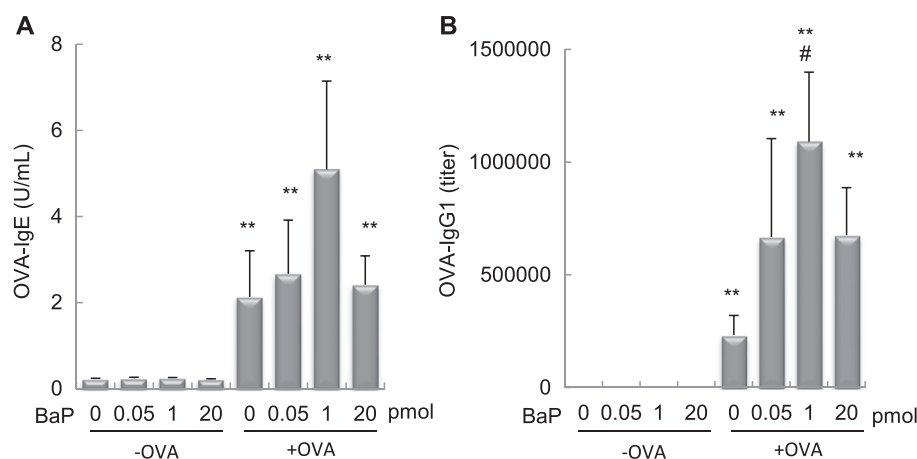


Figure 6. Ovalbumin (OVA)-specific Ig antibody levels in serum after exposure to benzo[a]pyrene (BaP) in allergic airway inflammation in mice. OVA-specific IgE and IgG₁ in serum were measured 24 h after the last intratracheal instillation using enzyme-linked immunosorbent assay (ELISA). Data were expressed as the means $\pm$ SE for 4–5 animals per group. ** $P < 0.01$ versus the Vehicle group, # $P < 0.05$ versus the OVA group.

Discussion

The present study demonstrated that intratracheal exposure to low-dose BaP enhanced pulmonary inflammation in OVA-immunized mice; however, the result was not statistically significant. These results were paralleled by the expression of proinflammatory molecules in the lungs and the production of OVA-specific IgG₁ in serum. *Ex vivo* studies showed that co-exposure to OVA and BaP slightly enhanced cell proliferation and activated antigen presenting cells (APCs) and CD4 positive T cells in the MLN cells.

Experimental studies have shown that DEP exposure can exacerbate allergic airway responses, including allergen-specific IgE, pulmonary eosinophilia and airway hyperresponsiveness (Maes *et al.*, 2010). In particular, PAHs in DEP contribute to the development of allergic responses. We reported that extracted organic chemicals from DEPs exacerbate OVA-induced airway inflammation such as eosinophilic inflammation, OVA-specific IgG₁ production and Th2 cytokine secretion in mice (Yanagisawa, *et al.*, 2006). 9, 10-Phenanthraquinone and 1, 2-naphthoquinone partially contribute to aggravating allergic airway inflammation (Hiyoshi *et al.*, 2005; Inoue, *et al.*, 2007a, 2007b). Takenaka *et al.* (1995) reported that phenanthrene, a major PAH in DEP, increases *in vitro* IgE production in human B cells. Intraperitoneal administration of pyrene, another major PAH in DEP, with OVA increases OVA-specific IgE in mice compared with that of OVA administration alone (Suzuki *et al.*, 1993).

BaP is a PAH consisting of DEPs that induce immune responses, including an adjuvant effect. Kadkhoda *et al.* (2004, 2005) reported that intranasal or oral administration with BaP enhances allergen-specific IgE production in mite allergen-immunized mice. Intranasal exposure to BaP aggravates Japanese cedar pollen-induced allergic rhinitis in guinea pigs (Mizutani *et al.*, 2007). In the present study, co-exposure to OVA and a medium dose of BaP elevated OVA-specific IgG₁ in serum compared with OVA exposure alone. Our previous and present studies suggest that BaP can, at least in part, contribute to the adjuvant activity of DEP, resulting in an enhanced allergic response.

This study showed that the administration of a high dose of BaP with OVA resulted in an increase in the infiltration of macrophages and neutrophils in the lungs. Eosinophils in BAL fluid and IL-5, IL-13, IL-33, MCP-1 and eotaxin levels in the lungs increased

after co-exposure to OVA and BaP compared with exposure to OVA alone, even although the increase was not statistically significant. The administration of 1 pmol BaP with OVA significantly elevated the IL-6 and TNF- α levels compared with administration of OVA alone. Th2 cytokines such as IL-5, IL-13 and IL-33 play an important role in the pathogenesis of allergic asthma (Galli *et al.*, 2008). IL-5 is essential for eosinophil migration (Faccioli *et al.*, 1996) and specifically supports eosinophil differentiation, maturation and activation (Clutterbuck and Sanderson, 1988). IL-13 is released from Th2 lymphocytes and promotes mucous secretion and mucous cell hyperplasia in bronchial epithelium (Tesfagzi, 2008). IL-33 is an IL-1 family cytokine that contributes to the development of type 2 immune responses by inducing IL-5, IL-13 and IgE production (Liew *et al.*, 2010; Oboki *et al.*, 2010b). IL-33 overexpression in transgenic mice leads to increased airway inflammation, goblet cell hyperplasia and eosinophilia (Oboki *et al.*, 2010a; Xiang *et al.*, 2010). MCP-1 is a possible modulator of eosinophilic inflammation and predominantly activates mononuclear macrophage. Eotaxin, a CC chemokine, is chemotactic for eosinophils, basophils and Th2 cells. Administration with eotaxin antibodies suppresses airway hyperresponsiveness and inflammation in a murine model of allergic airway inflammation (Scheerens *et al.*, 2002). Proinflammatory cytokines, IL-6 and TNF- α , play a critical role in aggravating the inflammatory conditions during allergic asthma. IL-6 promotes Th2 differentiation of CD4⁺ T cells while suppressing Th1 differentiation (Dienz and Rincon, 2009). TNF induces the development of airway hyperresponsiveness and pulmonary infiltration of lymphocytes, eosinophils and neutrophils in a murine model of OVA-induced allergic asthma (Nakae *et al.*, 2007). These findings suggest that BaP exposure in an allergic airway inflammation model may, at least in part, enhance pulmonary inflammation owing to Th2 cytokine and chemokine expression. In this study, 20 pmol BaP with OVA increased IL-12 levels in the lungs and macrophages and neutrophils in BAL fluid. IL-12 is a key inducer of the Th1 response such as Th1-type CD4 differentiation. Alveolar macrophage depletion in OVA-sensitized mice markedly enhances airway inflammation and airway hyperresponsiveness (Tang *et al.*, 2001). Ren *et al.* (2014) reported that intratracheal instillation of tar (PAH-rich fraction in DEPs) results in an increased accumulation of macrophages and neutrophils in the lungs on OVA-induced allergic airway inflammation, although eosinophilic

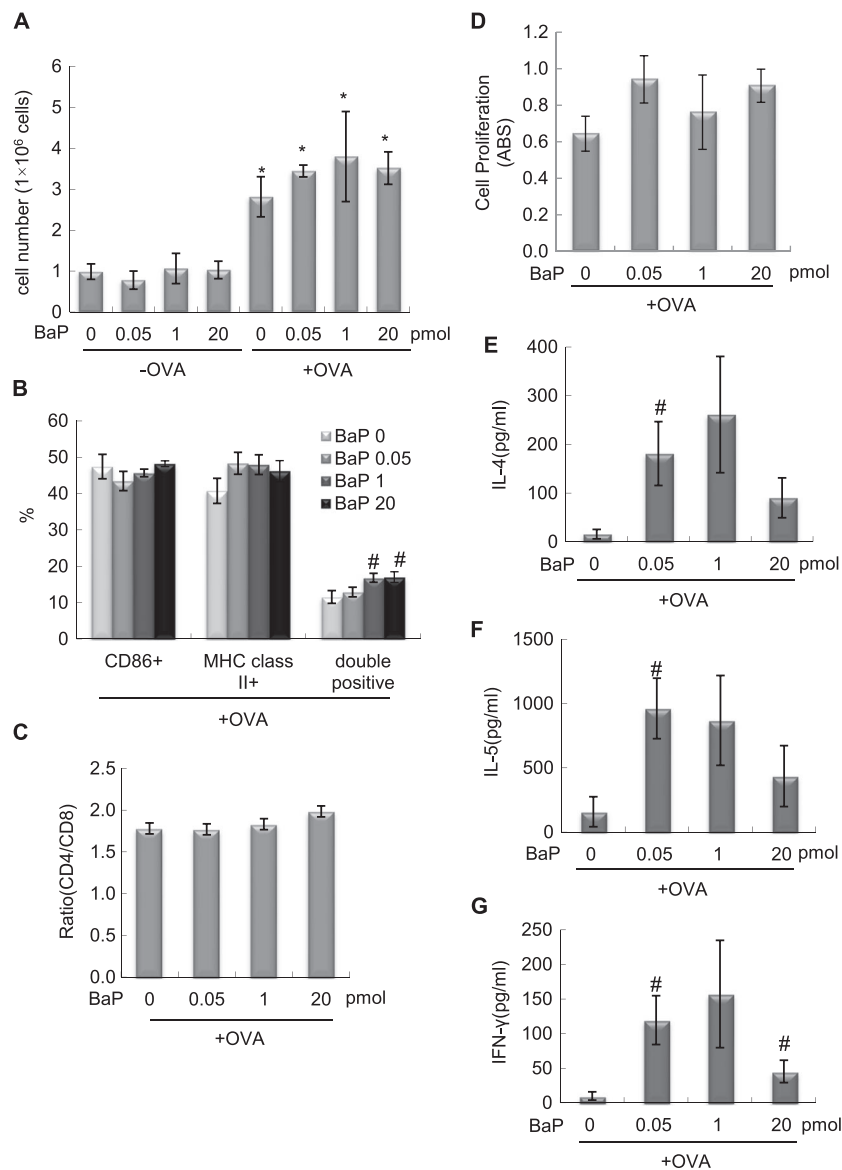


Figure 7. The proliferation/activation of mediastinal lymph node (MLN) cells after exposure to benzo[a]pyrene (BaP) in allergic airway inflammation in mice. Cell number and cell surface molecule expression in the MLN cells were performed by fluorescence-activated cell sorting (FACS) 24 h after the last intratracheal instillation. Cell proliferation and cytokine expression in culture supernatants were examined after 65 h of culture in the presence of ovalbumin (OVA). (A) Cell number. (B) The percentage of MHC class II and CD86 expression. (C) The ratio of TCR⁺CD4⁺/TCR⁺CD8⁺ T cells. (D) Cell proliferation with OVA-restimulation (Abs). (E) Interleukin (IL)-4. (F) IL-5. (G) IFN- γ . Data were expressed as the means $\pm$ SE for five animals per group. * $P < 0.05$ versus the Vehicle group, # $P < 0.05$ versus the OVA group.

inflammation was mild. Intranasal exposure to BaP with mite allergen induces Th1 (IL-12p70 and IFN- γ) and Th2 (IL-4 and IL-10) cytokine production in splenocyte culture supernatants (Kadkhoda *et al.*, 2005). These results suggest that the administration of a high dose of BaP with OVA induces both Th1 and Th2 responses and may, therefore, reduce the Th2-polarized response by activating alveolar macrophages and producing IL-12. Although the molecular signaling mechanism for the induction of cytokines/chemokines by BaP with OVA is yet to be determined, it has been recently recognized that these effects are mainly mediated through the binding of environmental contaminants to the aryl hydrocarbon receptor (AhR). All major human cell types express AhR, including bronchial epithelial cells, suggesting that the respiratory system is sensitive to AhR ligands (Alexandrov *et al.*, 2010). BaP exerts its biological effects via

the AhR signaling pathway. A recent report suggested that PM exposure enhances allergic airway inflammation including the elevation of Th2 cytokines (IL-4, IL-5 and IL-13) through an AhR-notch signaling cascade (Xia *et al.*, 2015). These results suggest that BaP exposure with allergen can, at least in part, enhance Th2-polarized pulmonary inflammation through the activation of AhR.

We observed that exposure to medium- and high-dose BaP with OVA increases MHC class II and CD86 expression in the MLN cells, whereas TCR β expression does not change (data not shown). MHC class II (Niederhuber and Shreffler, 1977) and the costimulatory molecule CD86 (Freeman *et al.*, 1993) are essential for antigen presentation. In addition, co-exposure to BaP with OVA slightly increased the CD4⁺/CD8⁺ T-cell ratio and OVA-re-stimulated proliferation and cytokine secretion in the MLN cells. The

activation of APCs and cytokine expression by BaP with allergens may contribute to aggravating allergic responses.

The predicted maximum exposure of BaP to humans via the ambient air is estimated to be $0.0009 \mu\text{g kg}^{-1} \text{ day}^{-1}$ (Ministry of Environment Government of Japan Website), which is equivalent to a dose of $0.062 \text{ pmol } 25 \text{ g}^{-1} \text{ BW week}^{-1}$. In the present study, we showed that the administration of $0.05\text{--}20 \text{ pmol BaP}$ with OVA showed a tendency to increase allergic airway inflammation. BaP exposure may facilitate the allergy epidemic in humans.

In conclusion, real-world exposure levels of BaP may contribute, at least in part, to exacerbation of allergic airway inflammation induced by DEP through Th2 cytokine and chemokine expression in the lungs and the proliferation/activation in the MLN cells. In addition, a high dose of BaP with allergen may be associated with activation of both Th1 and Th2 responses.

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Conflict of interest

The authors did not report any conflict of interest.

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