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Biological Response-Enhancing Activity with Antigens in A549 Cells Exposed to Representative Polycyclic Aromatic Hydrocarbons

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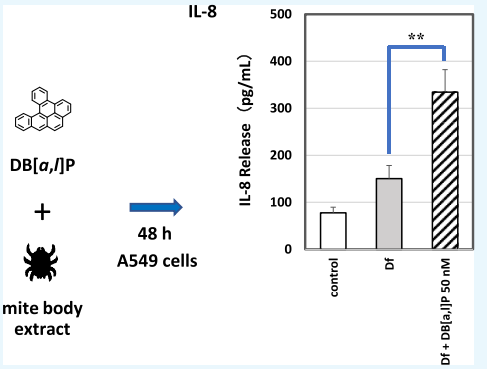
ABSTRACT: The question of what kinds of airborne particles, including diesel exhaust particles and their adherent chemical constituents, exacerbate the activity of allergic and inflammatory respiratory diseases has not been elucidated in detail. Therefore, chemicals that have amplifying actions on *Dermatophagoides farinae* (Df) body extract-induced IL-8, the inflammatory cytokines of the innate immune system, were comprehensively examined using commonly used human alveolar epithelial cells, A549, as simple screening for 17 polycyclic aromatic hydrocarbons (PAHs), which are representative organic constituents in atmospheric samples. The significant amplifying actions of two PAHs, dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) at 50 nM and dibenzo[*a*,*i*]pyrene (DB[*a*,*i*]P) at 2 μM for 48 h, for IL-8 protein release induced by mite antigens in epithelial cells were observed for the first time. In contrast, the enhancement of IL-8 was not observed in protein levels for these PAHs without the antigens. Meanwhile, the significant synergistic amplifying effect of DB[*a*,*l*]P at 50 nM on proinflammatory actions was measured in gene expression (i.e., IL-8, IL-6, ICAM-1, and TNF-α) levels in the experimental setting; for the results, the induction of TNF-α may have been the essential factor that enhanced the amplifying activity of DB[*a*,*l*]P for IL-8 gene expression and protein release. Examining the exacerbating effect on allergic pathophysiological states for DB[*a*,*l*]P is planned for further study.



INTRODUCTION

Diesel exhaust particles (DEPs) account for most of the airborne particulate matter (PM) 2.5, which are much finer particles in suspended PM (SPM) in large cities. Previous epidemiological studies[1−3] and animal experiments[4−6] have indicated that the influence of SPM is positively related to the prevalence of allergic pulmonary disorders, including bronchial asthma. Although the amount of DEPs released into the atmosphere has been decreasing in recent years because of strict emission regulations in Japan, the measurement bureau with the unattained emission standard exists and SPM originating from the inclusion of Chinese yellow and internal combustion engines has become a concern in recent years.[7,8] DEP comprises carbon black as a core material and various adherent contaminants such as polycyclic aromatic compounds, aliphatic hydrocarbons, quinone compounds, heavy metals, sulfates, nitrates, and ammonium salts. The extent to which particle and organic components contribute to pulmonary disease is contentious;[4−10] however, organic extracts were found to have a high enhancement contribution to inflammatory pulmonary disorders, cytokine production with lipopolysaccharides (LPSs),[9] and induction of allergic cytokines in mice.[5,6]

Polycyclic aromatic compounds, including polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (B[*a*]P) together with particular compounds, are leading candidates for the enhancement contributions. In our in vivo study, B[*a*]P[11] and two polycyclic aromatic quinones (1,2-naphthoquinone[12,13] and phenanthrenequinone[14]) in the oxygenated PAHs (oxy-PAHs) were reported to exacerbate allergic response and cytokine release with ovalbumin in respiratory organs. For comparison, the effect of PM, namely, carbon nanoparticles and nanomaterials, with different sizes on LPS-inducing pulmonary disorders[15,16] and allergic airway inflammation[17] has also been elucidated. Nevertheless, the question what kinds of airborne particles, including DEPs and their adherent chemical constituents, exacerbate allergic and inflammatory respiratory diseases has not yet been answered in detail.[6−10,18−20] Moreover, the mechanism for the activity of these constituents in PM has not been well revealed.

IL-8 (CXCL8) is the most potent neutrophile-recruiting chemokine and participates in several pathophysiological states in the lungs, including broncho construction, edema, and neutrophilia, and has been linked to airway diseases such as

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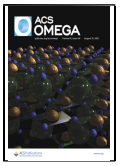

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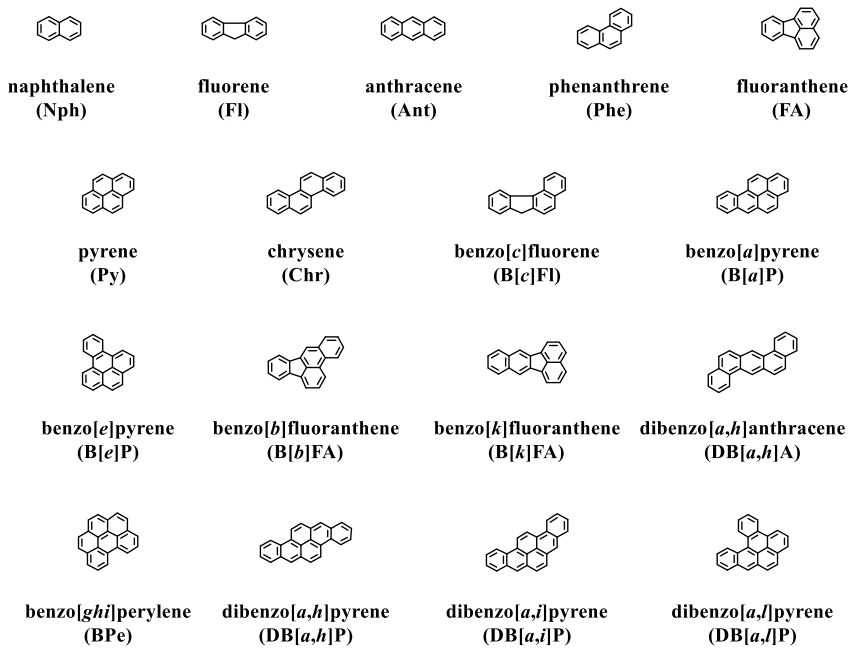

Figure 1. PAHs examined in the current study.

chronic obstructive pulmonary disease, cystic fibrosis, and severe asthma.[21−25] In human bronchial epithelial (BEAS-2B) cells, the expression of the inflammatory chemokine IL-8 protein was induced for pyrene (Py), one of the PAHs,[26] several nitrated derivatives of PAHs (nitro-PAHs) such as 1-nitropyrene (1-NPy) and 3-nitrofluoranthene,[24−29] and other PM constituents (Zn^{2+}, Fe^{2+}, carbon black, SiO_2, etc.)[24,25,30] in the absence of an antigen; however, the gene expression and protein production of IL-8 were not induced by the representative PAH, B[*a*]P.[24,25] Meanwhile, exposure to aeroallergens poses a major risk for allergic sensitization and the development of perennial rhinitis and allergic asthma.[31] It has been reported that mite antigens strongly induce inflammatory chemokine proteins such as IL-8 in human bronchial and alveolar epithelial cells.[31,32]

The chronic aggravating effects of B[*a*]P on mite-induced-asthma mice have been reported in recent in vivo studies.[33−35] It is possible that many unknown compounds contribute to the aggravating activity of antigen-induced asthma and a proinflammatory response. Therefore, in the current study, amplifying actions in mite antigen-induced IL-8, the inflammatory chemokine of the innate immune system, were comprehensively investigated using commonly used human alveolar epithelial adenocarcinoma (A549) cells[36−39] as simple screening for a series of PAHs, representative organic constituents found in atmospheric samples. The gene expressions of several representative cytokines and chemokines in epithelial cells were also examined for the remarkable amplification-inducing chemicals.

RESULTS

IL-8 Release after Exposure to Antigens and PAHs. To determine the chemical possessing an amplification action in chemokine release under coexposure to antigens, A549 cells were exposed to 17 representative PAHs (Figure 1) in the absence and presence of the *Dermatophagoides farinae* (Df) body extract antigen (10 μg/L) for 24 and 48 h. The IL-8 release was detected for the vehicle control, and a pronounced increase was confirmed only when the antigen level was approximately two times (1.6−2.2 times) higher than that of the control at 24 and 48 h, respectively, in an enzyme-linked immunosorbent assay (ELISA) (Figures 2A and 3). IL-6, ICAM-1, and TNF-α releases could not be detected under the vehicle control or any exposure sample (data not shown). The release of the IL-8 protein was not enhanced by 5 μM of most PAHs in the presence of mite antigens, so only the results for nine PAHs are shown in Figure 2A. Conversely, a significant amplifying action against antigen exposure was confirmed with dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) at 50 nM for 48 h (1.4−2.2 times higher than with antigen only) and with dibenzo[*a*,*i*]-pyrene (DB[*a*,*i*]P) at 2 μM for 48 h (1.3 times higher than with antigen only) (Figures 2A and 3). For 5 μM and 500 nM DB[*a*,*l*]P for 48 h, cytotoxicity was confirmed (cell viability was less than 70%) (Figure 2B). For 5 μM DB[*a*,*i*]P at 48 h, cytotoxicity was not observed; however, the amplifying activity for the IL-8 release was also not observed (data not shown). DB[*a*,*l*]P did not exhibit any amplifying activity compared with antigen alone at either 50 nM for 24 h or 5 nM for 48 h (Figure 3).

Dose- and Time-Dependent Induction of the mRNA of Several Cytokines and Chemokines after Exposure to B[*a*]P and DB[*a*,*l*]P. The significant amplifying action of the IL-8 protein release against antigen exposure was confirmed by DB[*a*,*l*]P at 50 nM, which was being more prominent than DB[*a*,*i*]P at 2 μM for 48 h (Figures 2A and 3). Therefore, the amounts of mRNA for representative inflammation-related genes in A549 cells induced by DB[*a*,*l*]P as high amplifying chemicals and B[*a*]P for comparison were measured after 12 and 24 h (Figures 4 and 5). IL-8 mRNA was significantly induced by a mite antigen after 24 h, and synergistically significant enhancement of the IL-8 gene expression (3.9 times that found for antigen only) was observed with 50 nM DB[*a*,*l*]P after 24 h (Figure 4A). Significant enhancement by DB[*a*,*l*]P was not measured at 50 nM after 12 h or at 5 nM after 24 h. For 5 μM B[*a*]P, significant enhancement against antigen exposure was not induced after either 12 or 24 h. The

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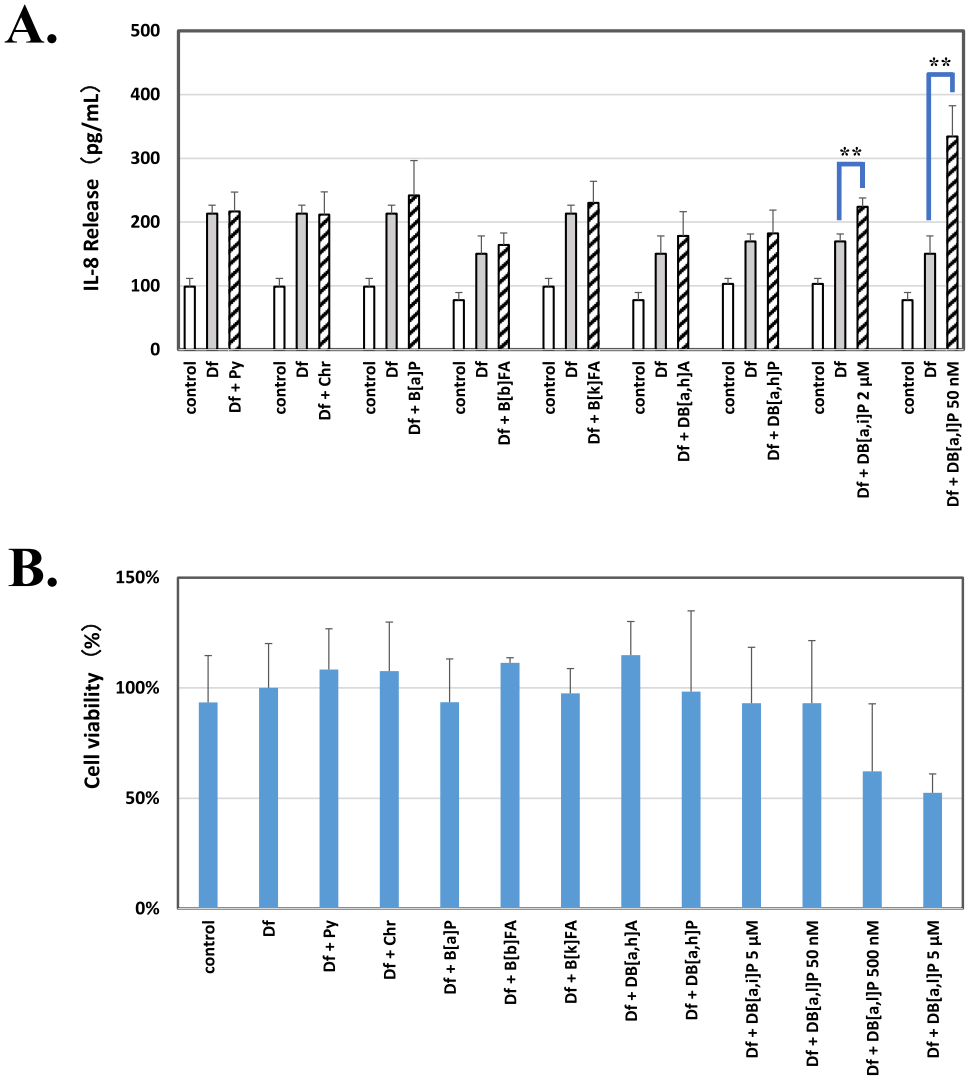

Figure 2. Release of IL-8 from and cell viability in A549 cells exposed to PAHs for 48 h. (A) IL-8 release as measured by ELISA. (B) Cell viability as measured by the MTS assay. Exposure to PAHs was performed at 5 μM unless otherwise specified. ** indicates a highly significant difference from only antigen-treated cells ($p < 0.01$) as determined by Student's *t*-test ($n = 3$). Release of IL-8 under only antigen exposure was significantly increased (almost $p < 0.01$ and at most $p < 0.05$) compared with the vehicle control.

significant increase against the vehicle control was observed for 5 μM B[*a*]P and for 5 and 50 nM DB[*a,l*]P after 24 h (1.5, 2, and 2.8 times the control, respectively).

The synergistic increase of IL-6 mRNA compared with the case with only the antigen (3.3 times) was confirmed only at 50 nM DB[*a,l*]P after 24 h (Figure 4B). The significant increase against the vehicle control was obtained for 5 μM B[*a*]P and for 50 nM DB[*a,l*]P after 12 h (3.0 and 3.6 times the control, respectively). The synergistically significant increase of ICAM-1 mRNA against the antigen (2.7 times) was observed for 50 nM DB[*a,l*]P after 12 h and maintained at the same dose after 24 h (Figure 4C).

Detection was difficult for the TNF-α mRNA at the vehicle control so that analysis of TNF-α mRNA was performed by assuming a value of 0 in the case of the detection limit. The mRNA of TNF-α could be measured for antigen exposure such that the expression levels for TNF-α were calculated as fold induction against the case with antigen only at 12 h (Figure 4D). The synergistically significant increase of TNF-α mRNA was confirmed at 50 nM DB[*a,l*]P after both 12 and 24 h (6.5 and 8.4 times higher than the antigen control at the respective exposure times).

Gene expression was also measured for IL-1α, IL-1β, CCL5 (RANTES), and CCL17 (TARC); however, the amplifying expression was not found for these genes although a significant increase was confirmed for IL-1β and CCL5 with antigen only and for IL-1β with 5 μM B[*a*]P alone, in comparison with the vehicle control at 24 h (Figure 5). A typical aryl hydrocarbon receptor (AhR) response metabolic gene (CYP1A1) and an antioxidant-responsive gene (HO-1) were measured for B[*a*]P and DB[*a,l*]P (Figure 6A,B). The remarkable CYP1A1 expression was measured at 12 and 24 h for 5 μM B[*a*]P; however, the enhancement was not found for 5 μM B[*a*]P in the presence of the antigen. The increase of CYP1A1 was not induced by antigen alone. No significant variation was observed for HO-1 during the coexposure of PAH and antigen although a decrease in significance was found by Student's *t*-test at 50 nM DB[*a,l*]P against the case with antigen only after 24 h. A significant increase in HO-1 mRNA against the vehicle control was confirmed only under 12 h of antigen exposure.

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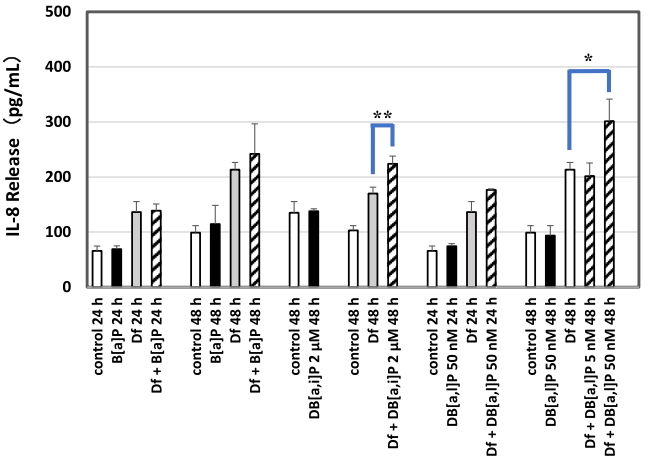

Figure 3. Release of IL-8 from A549 cells exposed to B[*a*]P, DB[*a,i*]P, and DB[*a,l*]P under several conditions. The IL-8 release was measured by ELISA. Exposure to PAHs was performed at 5 μM unless otherwise specified. ** indicates a highly significant difference from only antigen-treated cells ($p < 0.01$), and * indicates a significant difference from only antigen-treated cells ($p < 0.05$, $n = 3$). Release of IL-8 under only antigen exposure was significantly increased ($p < 0.01$) compared with the vehicle control.

DISCUSSION

IL-8 (CXCL8) is the most potent neutrophile-recruiting chemokine and an important target for the treatment of severe allergic asthma patients.[21−25] IL-8 protein levels were examined after 24 and 48 h in human alveolar epithelial (A549) cells, whereas the measurement of mRNA for several cytokines and chemokines was conducted after 12 and 24 h because the increase in mRNA expression for various genes proceeds for quite a while before protein production and release, as shown in other reports.[18,24−26,28,29,40] As a result of comprehensive screening evaluation of a series of PAHs, the enhancement of IL-8 protein release against antigen exposure was confirmed for 50 nM DB[*a,l*]P and 2 μM DB[*a,i*]P after 48 h in the presence of mite antigens (Figures 2 and 3). By contrast, the enhancement of IL-8 was not observed in protein levels for these PAHs without the antigen although the enhancing expression of IL-8 mRNA of DB[*a,l*]P was confirmed without the antigen (Figure 4A). This is thought to be derived from the expression of mRNA being more sensitive than protein formation. The remarkable increase of the IL-8 protein release was measured in antigen-only exposure compared with the vehicle control (Figures 2 and 3). Df mite body extracts probably include both proteolytic (Df 1) and nonproteolytic (Df 2) allergens, with the former affecting the IL-8 increase in A549 cells because, reportedly, nonproteolytic mite allergens do not increase IL-8 in A549 cells (unlike bronchial epithelial cells such as BEAS-2 cells).[31,32] The amount of IL-8 protein release in the vehicle control at 24 and 48 h was less than that in several previous studies with A549 cells;[29,32,40,41] this is probably because the cell response may be more sensitive in subconfluent growth than in the confluent conditions of our study. However, in another study, the amount of IL-8 protein similar to the current study was reported.[41]

The absolute mRNA amount of IL-6 and ICAM-1 was approximately 1∼2 orders of magnitude lower than that of IL-8 at the control level, and the absolute mRNA amount of TNF-α mRNA was approximately 1 order of magnitude lower than

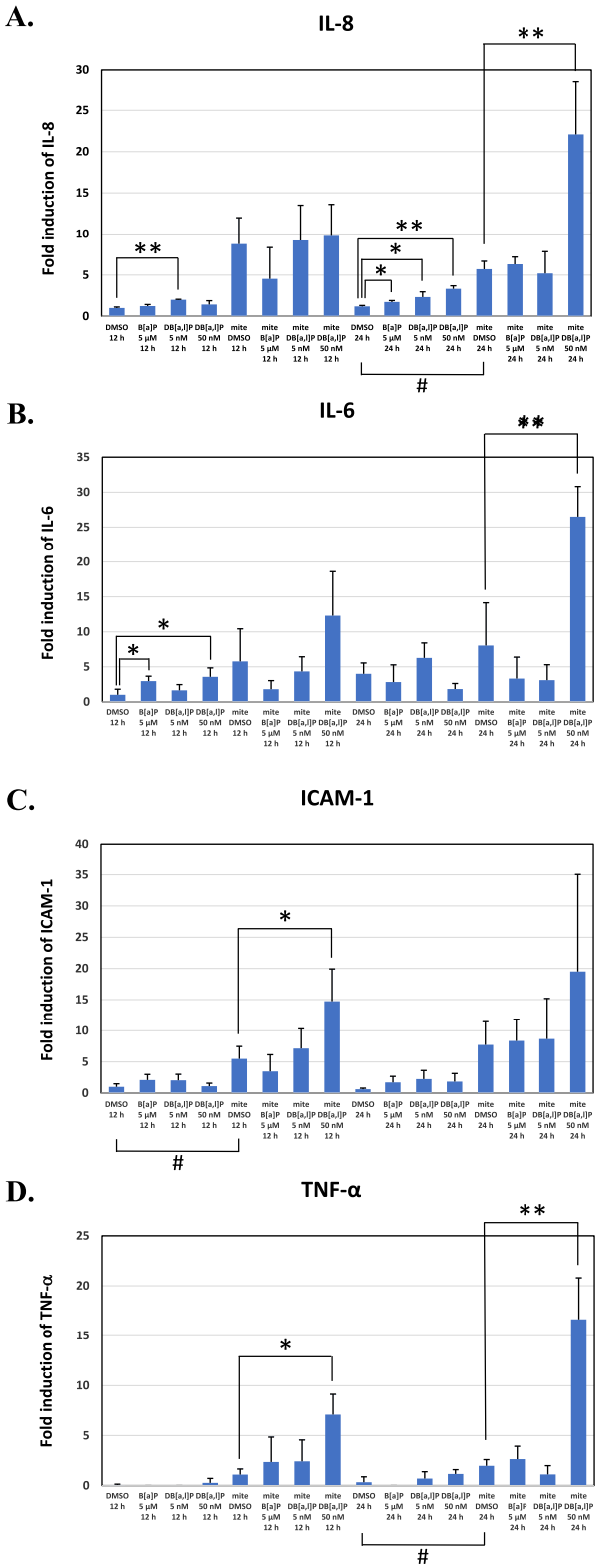

Figure 4. Dose- and time-dependent induction for mRNA of inflammatory cytokines and chemokines measured by RT-PCR in A549 cells exposed to B[*a*]P and DB[*a,l*]P for 12 and 24 h. (A) IL-8 mRNA, (B) IL-6 mRNA, (C) ICAM-1 mRNA, and (D) TNF-α mRNA. ** indicates a highly significant difference from cells treated only with antigen ($p < 0.01$) and * indicates a significant difference from cells treated only with antigen ($p < 0.05$, $n = 3$). # indicates a significant increase with only antigen exposure compared with the vehicle control ($p < 0.05$).

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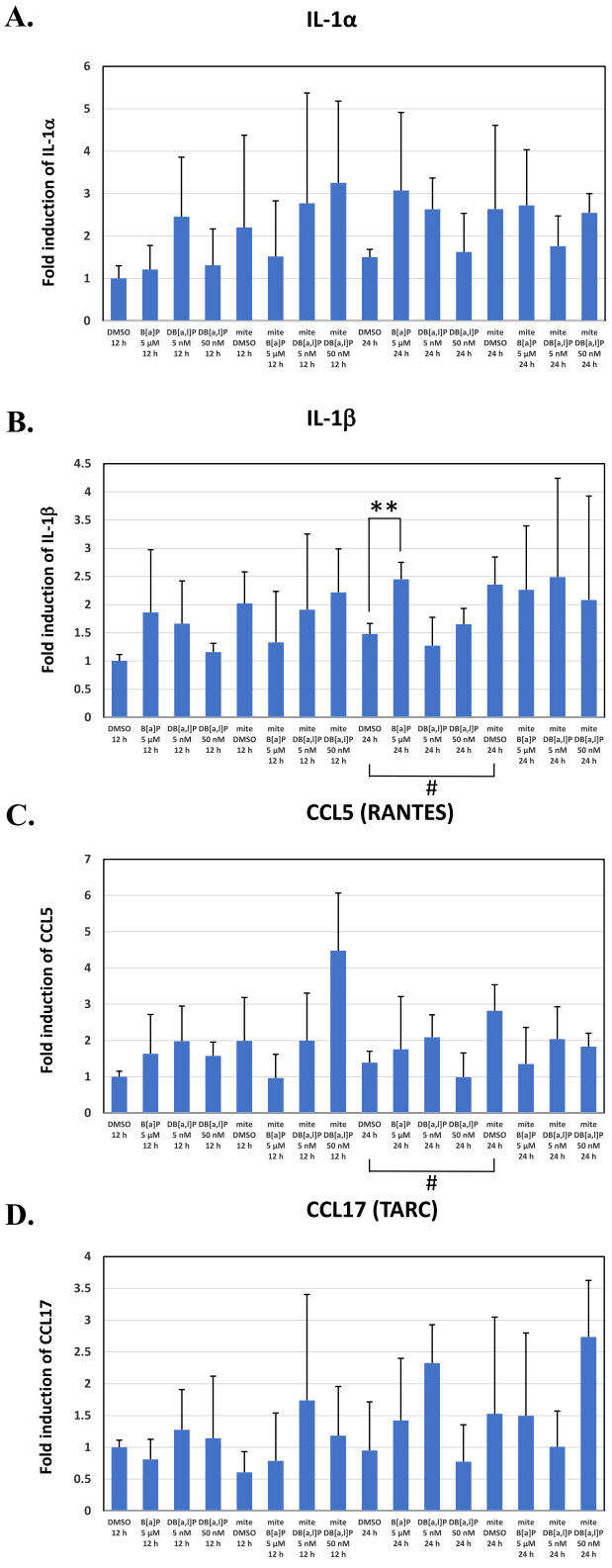

Figure 5. Dose- and time-dependent induction for mRNA of inflammatory cytokines and chemokines measured by RT-PCR in A549 cells exposed to B[*a*]P and DB[*a,l*]P for 12 and 24 h. (A) IL-1α mRNA, (B) IL-1β mRNA, (C) CCL5 (RANTES) mRNA, and (D) CCL17 (TARC) mRNA. ** indicates a highly significant difference from only antigen-treated cells ($p < 0.01$, $n = 3$). # indicates a significant increase with only antigen exposure compared with the vehicle control ($p < 0.05$).

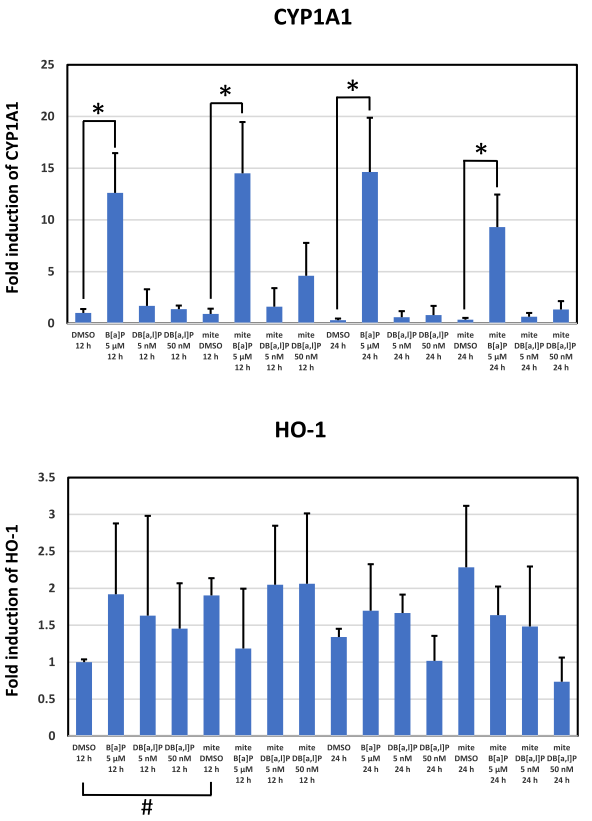

Figure 6. Time-dependent induction for mRNA of metabolic enzymes measured using RT-PCR in A549 cells exposed to B[*a*]P and DB[*a,l*]P for 12 and 24 h. (A) CYP1A1 mRNA and (B) HO-1 mRNA. * indicates a significant difference from only antigen-treated cells ($p < 0.01$, $n = 3$). # indicates a significant increase with only antigen exposure compared with the vehicle control ($p < 0.05$).

that of IL-8 at the exposure of antigen only (data not shown). The undetectable IL-6, ICAM-1, and TNF-α protein releases (data not shown) are thought be derived from the low expression levels of these genes. The enhancement expression of the IL-6 gene was confirmed at 50 nM DB[*a,l*]P at 48 h in A549 cells in the current study, with IL-6 being a pleiotropic cytokine that plays a key role in the acute-phase response.[4,24] ICAM-1 (intercellular adhesion molecule-1, CD45) is expressed in both hematopoietic and nonhematopoietic cells and functions through cell−cell and cell−matrix adhesive interactions. Cell adhesion mediated by ICAM-1 is related to transendothelial migration of leukocytes and the activation of T cells.[42] TNF-α, IL-1α, and IL-1β are believed to be pivotal in the onset of inflammatory processes and may regulate the expression of other cytokines and chemokines such as IL-8, IL-6, ICAM-1, and CCL5 (RANTES) through the activation of transcription factors such as NF-κB and AP-1.[4,24,25,28,43,44] The remarkable expression of TNF-α mRNA was measured at 50 nM DB[*a,l*]P for 48 h, whereas the gene expressions of IL-1α and IL-1β were not affected by DB[*a,l*]P in A549 cells (Figures 4D and 5A,B) though the expression of IL-1α mRNA was reported for benzo[*e*]pyrene (B[*e*]P) and benzo[*b*]-fluoranthene (B[*b*]FA) in human primary bronchial epithelial cells.[45] The gene expression of TNF-α may be related to the IL-8 expression-amplifying activity of DB[*a,l*]P in A549 cells.[45] The epidermal hyperplasia caused by DB[*a,l*]P in mouse skin and its relation to gene expression of cytokines such as TNF-α and IL-1β have been reported.[46] The aggravation of

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inflammation in the skin is predicted in combination of DB[*a*,*l*]P with mite antigen on the basis of the results of the current study. It was reported that DEP and $PM_{2.5}$ induced chemokine and cytokine proteins such as IL-8, IL-6, and TNF-α in human alveolar epithelial (A549) cells.[19,20,40] Several organic constituents and particulate components included in DEP and $PM_{2.5}$ also produced chemokines and cytokines in these cells.[24,29,30] In the current study, the significant amplifying effect of DB[*a*,*l*]P for mite antigen exposure was observed in gene expressions (IL-8, IL-6, ICAM-1, and TNF-α) and the importance of organic constituents was confirmed in inflammatory responses of atmospheric pollutants.

CCL17 (TARC; thymus- and activation-regulated chemokine) is known as the chemotactic factor of Th2 cells.[47] The lack of an observed significant variation of CCL17 mRNA in A549 cells in the current study may result from the sensitivity of cells and less activators such as IL-4 (Figure 5D).[47] Meanwhile, CCL5 (RANTES) is an essential chemoattractant for eosinophils and is often linked to the development of allergic asthma.[24,26−28] In this study, the significant mRNA increase was confirmed with antigen only against the vehicle control at 24 h (Figure 5C). This result might have occurred because of the sensitivity of the detection of mRNA though the release of the CCL5 protein was not induced by mite allergens but by a virus in epithelial cells[48] and chronic ovalbumin exposure.[11]

An extremely remarkable CYP1A1 mRNA expression was confirmed for B[*a*]P although the effect of coexposure to an antigen was not observed (Figure 6A). CYP1A1 gene expression by DB[*a*,*l*]P was not significant but was higher than the vehicle control level both at 12 and 24 h. A representative metabolic enzyme, CYP1A1, is induced via AhR by B[*a*]P; AhR is present in the cytoplasm as a complex with a dimeric heat shock protein 90 before ligand interactions. Ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and B[*a*]P activate AhR, which then transfers into the nucleus and mediates the regulation of gene expression including specific CYPs, GSTs, NQO1, and several growth factors and cytokines.[4,18,28,49,50] The high AhR activity of B[*a*]P and the slight AhR activity of DB[*a*,*l*]P in liver carcinoma cells are known and the present result reflects such properties.[50,51] The expression of several inflammatory genes (IL-8, IL-1β, etc.) by B[*a*]P is believed to also be mediated by TNF-α/NF-κB activation, whereas the AhR/RelB complex may be involved in the regulation of these genes.[18,28,49] The significant variation of mRNA expression for the antioxidant enzyme HO-1 was not confirmed at 5 μM B[*a*]P (Figure 6B); it is believed that the decreasing tendency of HO-1 mRNA during the coexposure of 50 nM DB[*a*,*l*]P with mite antigens at 24 h was caused by depletion of the representative antioxidant enzyme HO-1 under strong oxidative stress.[4,18,50]

Recently, it has been reported that the IL-8 protein release in A549 cells exposed to Df (5 μg/mL) for 24 h was significantly upregulated by pretreatment with 10 nM B[*a*]P for 24 h.[41] The pretreatment with our targeted PAHs may lead to an increase of PAHs enhancing the IL-8 release compared with the case with the antigen alone. The increase in the expression of cytokine genes such as IL-8 and TNF-α using promoter-transfected A549 cells was measured for several PAHs such as B[*a*]P, chrysene (Chr), and phenanthrene (Phe) with high concentrations in the absence of antigen.[52] The improvement of the exposure condition of PAHs may also help us in determining other PAHs possessing amplifying activation upon coexposure to the antigen only for untransfected cells.

In conclusion, the amplifying action of 2 PAHs, DB[*a*,*l*]P and DB[*a*,*i*]P, upon IL-8 protein release induced by mite antigens in human epithelial cells was identified in the current study for the first time. The significant synergistic amplifying effect of DB[*a*,*l*]P on the proinflammatory action was confirmed in the gene expressions (IL-8, IL-6, ICAM-1, and TNF-α). The approach of the current study was limited to the screening of amplifying PAHs in IL-8 generation of the innate immune system and did not evaluate the effects of chronic exposure to these PAHs on allergic symptoms in vivo.[33−35] However, it is important to examine the exacerbating effect on allergic pathophysiological states for DB[*a*,*l*]P, whose prominent amplifying action was found in IL-8 generation. Future studies are warranted to advance the search for PAHs and PAH derivatives, such as oxy-PAHs[12−14] and nitro-PAHs,[24−29] that possess amplifying activities for various inflammatory cytokine and chemokine releases. Studies are also needed to elucidate the variation in the operating points and upstream mechanisms for these activities using inhibitors for several receptors and signal pathways.[18,24,27,28,30]

MATERIALS AND METHODS

Chemicals. Chr, B[*a*]P, and dibenzo[*a*,*h*]anthracene (DB[*a*,*h*]A) were supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan); fluoranthene (FA) was supplied by Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan); anthracene (Ant) and Py were supplied by Nacalai Tesque, Inc. (Kyoto, Japan); benzo[*c*]fluorene (B[*c*]Fl) was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany); dibenzo[*a*,*h*]pyrene (DB[*a*,*h*]P), DB[*a*,*i*]P, and DB[*a*,*l*]P were supplied by AccuStandard, Inc. (New Haven, CT, USA); other test chemicals were supplied by Sigma-Aldrich Co. LLC. (St. Louis, MS, USA). The purities of many test chemicals were 99−100%, those of FA, B[*c*]Fl, B[*b*]FA, and benzo[*ghi*]-perylene (BPe) were ≥98%, and that of DB[*a*,*h*]A was 96%. The mite antigen, Df body extract, was supplied by ITEA Inc. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were Gibco products purchased from Thermo Fisher Scientific (Waltham, MA, USA). The RNA extraction reagent was supplied by Qiagen N. V. (Venlo, the Netherlands). Dimethyl sulfoxide (DMSO) was supplied by Wako Pure Chemical Industries, Ltd.

Cell Culture. The human adenocarcinoma-derived alveolar basal epithelial cell line A549 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Sennan, Osaka, Japan). The cells were cultured in DMEM with 1% penicillin and 1% streptomycin and supplemented with 10% (v/v) FBS at 37 °C in a humidified atmosphere containing 5% CO_2 upon confluence. The cells were plated in the serum-free DMEM with 1% penicillin and 1% streptomycin in a collagen-coated microplate (1.5×10^5 cells/well) over 1.5 days. Then, the medium was changed to a fresh medium including either mite antigen (Df body extract) (10 μg/mL), each chemical, or both. All chemicals were dissolved in DMSO, and the final concentration of the solvent in the culture medium was 0.1% v/v. Control cells were treated with 0.1% v/v DMSO. The mite antigen was dissolved in pure water (10 mg/mL), the concentrated solution was filtrated using an Ultrafree column with a 0.22 μm hydrophilic polyvinylidene difluoride membrane (Merck Millipore Corp., Darmstadt, Germany) at 12,000 rpm for 5 min at 4 °C, and the final concentration of

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the filtrate in the culture medium was 0.1% v/v. The exposure conditions for each assay are described below.

ELISA. The cells were exposed to 1 mL of medium with either mite antigen, each of the individual compounds, or both at a concentration below that with cytotoxicity (cell viability below 70%) ($n = 3$) in a 1 mL 24-well microplate. After 24 and 48 h, the supernatant aliquots were collected in microtubes. Centrifugation was performed for the tubes at 1000 rpm for 5 min at 4 °C; then, the supernatants were preserved in a freezer at −80 °C before the assay. The protein levels of IL-8, IL-6, ICAM-1, and TNF-α were measured in triplicate using commercially available ELISA kits [Quantikine (R&D Systems Inc., Minneapolis, MN, USA), high-sensitivity ELISA (Invitrogen product, Thermo Fisher Scientific), OmniKine (Assay Biotech. Inc., Fremont, CA, USA), and Quantikine HS (R&D Systems Inc.), respectively]. ELISA analysis was conducted basically according to the kit instructions. The assay samples were used in the assay after being diluted twice with a diluent solution. The optical density at 450 nm was measured using a plate reader (Model 680, Bio-Rad Laboratories, Inc., Hercules, CA, USA). A significant increase ($p < 0.01$ or $p < 0.05$) in fold induction only against mite antigen or vehicle control was evaluated using Student's t-test for a simple two-comparison test and the Dunnett test for dose−response multiple comparisons among three groups using KaleidaGraph version 4.0 (Hulinks Inc., Tokyo, Japan).

Cell Viability Assay. Cytotoxicity was confirmed along with cell viability through the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt (MTS) assay (Promega Corp., Madison, WI, USA).[53] Cell viability in response to each chemical was measured in triplicate using the MTS assay, basically in accordance with the manufacturer's protocol. Cells were cultured in each well of a 24-well microplate. After 24 and 48 h of exposure to either the antigen, each chemical, or both, 200 μL of MTS solution was added to the medium; after 1 h, the optical density at 490 nm was measured using a plate reader (Model 680).

RNA Isolation, Reverse Transcription (RT), and Real-Time Polymerase Chain Reaction (PCR). Cells were cultured in each well of a 12-well microplate. After 12 and 24 h of exposure to either the antigen, each chemical, or both, the total RNA was isolated from the cells using the RNeasy mini kit (Qiagen N. V.) and the DNA-free kit (Applied Biosystems products purchased from Thermo Fisher Scientific) in accordance with the manufacturers' protocols. The total RNA was dissolved in RNase-free water, and the concentration was determined spectrometrically. Cell cultures were prepared in triplicate for each exposure condition, and the total RNA (100 ng) was added to a reaction mixture containing 100 pmol of random hexamers, Prime Script RT enzyme mix, and deoxyribonucleoside triphosphates in a final volume of 20 μL and reverse-transcribed using the Prime Script RT reagent kit (Perfect Real Time) (Takara Bio, Inc., Otsu, Japan). The reaction mixture was incubated at 37 °C for 15 min and heated at 85 °C for 5 s to inactivate the enzyme in accordance with the manufacturer's protocol. The oligonucleotides used for PCR were commercially synthesized by Fasmac Co., Ltd. (Atsugi, Japan) as shown in Table 1.

Quantification of cDNA was conducted using the ECO 48 Real-Time PCR system (As One Corp., Osaka, Japan), and staining was performed with SYBR Green I. A 2 μL aliquot of the RT mixture was added to a PCR mixture containing 0.2 μM of each primer and TB Green Premix Ex Taq DNA polymerase in a final volume of 25 μL; it was then amplified using the TB Green Premix Ex Taq II kit (Takara Bio, Inc.) in accordance with the manufacturer's protocol. The PCR reactions were performed with 1 cycle at 50 °C for 2 min and at 95 °C for 30 s and then with 40−60 cycles at 95 °C for 5 s and at 60 °C for 30 s (the cycle number was selected for each gene). The specificity of the PCR products was determined by melting curve analysis; the expression levels for each gene were obtained from the fluorescence of SYBR Green I adjusted by ROX, and the levels were normalized against those of 18S rRNA. A significant increase ($p < 0.01$ or $p < 0.05$) in fold induction against the vehicle control at 12 h (compared with the exposure of mite antigen at 12 h for only TNF-α) was evaluated using Student's t-test for a simple two-comparison test and the Dunnett test for dose−response multiple comparisons among three groups using KaleidaGraph version 4.0.

Table 1. Oligonucleotides Used for PCR

18S rRNA	5′-CAACACGGGAAACCTCACC-3′ (117 bp)
	5′-CAGACAAATCGCTCCACCAA-3′
IL-8	5′-GAATCAGTGAAGATGCCAGTGAA-3′ (80 bp)
	5′-CAACCCTACAACAGACCCACAC-3′
IL-6	5′-CACACAGACAGCCACTCACCT-3′ (128 bp)
	5′-TGCCTCTTTGCTGCTTTCAC-3′
ICAM-1	5′-CTAAAACACTAGGCCACGCATCT-3′ (120 bp)
	5′-CCACCACTTCCCCTCTCATC-3′
IL-1α	5′-CTGGGACCTCAGTTTTATCATTTTC-3′ (144 bp)
	5′-TTCGTGCTTTGCCTTCATCTT-3′
IL-1β	5′-CGACCACCACTACAGCAAGG-3′ (137 bp)
	5′-CAAAGATGAAGGGAAAGAAGGTG-3′
CCL5	5′-TGGAGATGAGCTAGGATGGAGAG-3′ (108 bp)
	5′-GGGGTAGGATAGTGAGGGGAAG-3′
CCL17	5′-CCCCAACAACAAGAGAGTGAAG-3′ (88 bp)
	5′-CAGTCAGGAGTCTGGGGTGA-3′
CYP1A1	5′-TGCCAAGAGTGAAGGGAAGAG-3′ (133 bp)
	5′-GAAGGGCAGAGGAATGTGATG-3′
HO-1	5′-TCTTGGCTGGCTTCCTTACC-3′ (92 bp)
	5′-GGCTCCTTCCTCCTTTCCA-3′

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Notes

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NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP August 19, 2021, a correction was made to the caption of Figure 3. The corrected version was reposted August 31, 2021.

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