

Ym1 and Ym2 Expression in a Mouse Model Exposed to Diesel Exhaust Particles

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ABSTRACT: Background: Chitinase may play a role in regulating allergic diseases. **Objective:** We studied the role of chitinase in a mouse model exposed to diesel exhaust particles (DEP). Mice were exposed to intranasal DEP (0.6 mg/mL) for 5 days and challenged with aerosolized DEP (6 mg/m³) on days 6–8. Enhanced pause (Penh), as an airway obstruction marker, was measured on day 9, and bronchoalveolar lavage (BAL) fluid and lung tissues were collected on day 10. The expression of Ym1 and Ym2 mRNA was assessed in lung tissue extracts by reverse transcription-polymerase chain reaction. **Results:** DEP induced significant increases in methacholine-induced Penh and IL-4 levels in BAL fluid relative to the control group. Peribronchial and perivascular inflammatory cell infiltrates were prominent in the DEP group. DEP induced Ym1 and Ym2 mRNA expression in lung tissue extracts relative to the control group. **Conclusion:** These results demonstrate that DEP induced airway hyperresponsiveness and Ym mRNA expression via a Th2 cell-biased response, suggesting that chitinase may play an important role in airway inflammation and responsiveness upon exposure to DEP in a mouse model, and may therefore be involved in regulating allergic diseases. © 2008 Wiley Periodicals, Inc. *Environ Toxicol* 23: 110–116, 2008.

Keywords: chitinase; diesel exhaust particles; asthma

INTRODUCTION

Increased exposure to air pollutants such as diesel exhaust particles (DEP) has been proposed as one mechanism to explain the rise in allergic disorders. A growing body of research supports that the inhalation of DEP may have a role in development or exacerbation of asthma and allergic diseases through altering airway immune responses (van Zijverden et al., 2000; Bleck et al., 2006; Chang et al.,

2006; Cha et al., 2007). However, the immunologic mechanisms by which DEP enhance allergic sensitization and asthma remain unclear.

Alveolar macrophages play an important role in particle-induced lung inflammation via direct induction of IL-13 production, suggesting that alveolar macrophages may act as major effectors of innate immunity by modulating immune and inflammatory responses towards a Th2-like condition via the production of IL-13, as seen in the adaptive immune response (Kang et al., 2005).

Chitin is a common element in organisms including parasites, fungi, and bacteria, but does not occur in mammalian tissues (Guo et al., 2000), allowing for selective antimicrobial activity of chitinase. Ym1 and Ym2 synthesized by activated macrophages are homologous to chitinase and have chitinase activity (Jin et al., 1998; Sun et al., 2001).

Ym1 is involved in allergic peritonitis through the IL-4/STAT 6 signal transduction pathway (Welch et al., 2002).

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Zhu et al. (2004) reported that acid mammalian chitinase may be an important mediator of IL13-induced responses in Th2-dominated disorders such as asthma. An association between acid mammalian chitinase polymorphisms and asthma further supports the involvement of acid mammalian chitinase in asthma development (Bierbaum et al., 2005).

Nitric oxide (NO) is a short-lived molecule that causes vasodilation and bronchodilation (Moncada et al., 1989, 1991). We demonstrated that concentrations of NO metabolites increased with asthma severity in the tracheo-bronchial secretions of asthmatic subjects, and that NO metabolites in sputum are a more valuable indicator for monitoring asthmatic airway inflammation than those in serum (Jang and Choi, 1999; Jang et al., 1999). The nitric oxide synthase (NOS) isoforms play different roles in airway inflammation after ozone exposure (Jang et al., 2004).

The regulation and function of chitinase has not been well explored in air pollution asthma models. Therefore, we developed a mouse model exposed to DEP, and the effect of DEP exposure, as an air pollutant, on Ym1 and Ym2 mRNA expression was examined.

MATERIALS AND METHODS

DEP Sources and Preparation

The DEP used in this study were obtained from Hajime Takizawa and were collected using the following method. A 4JB1-type, light-duty (2740 cc), four-cylinder diesel engine (Isuzu Automatic Co., Tokyo, Japan) was connected to an EDGY dynamometer (Meiden-Sya, Tokyo, Japan), and was operated using a standard diesel fuel at speeds of 1500 rpm under a load of 10 torque (kg/m). The exhaust was introduced into a stainless-steel dilution tunnel (300 mm ϕ $\times$ 8400 mm) in a constant-volume sampler system equipped to the end of the dilution tunnel. The temperature at the sampling point was below 50°C. The diameter of the particles was measured using a low-pressure-type Anderson Air Sampler (Anderson, 1966), and the mean diameter was 0.4 μ m. Most of the shapes analyzed by scanning electron microscope were globular. The details of the DEP used were described previously (Sagai et al., 1996; Ohtoshi et al., 1998).

DEP Exposure

DEP were sterilized by autoclaving and suspended in serum-free media after being coated with bovine serum albumin (BSA) to minimize particle aggregation and hydrophobicity (Sahoo et al., 2002). After sonication, the endotoxin concentration of the DEP suspension was less than 0.064 ng/mL (0.32 EU/mL), based on a Limulus Amebocyte Lysate assay (QCL-1000; BioWhittaker, Walkersville, MD;

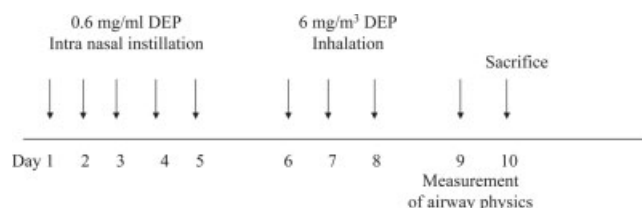


Fig. 1. Schematic of the experimental protocol. Mice were sensitized intranasally on days 1–5. On days 6 and 8, after the initial sensitization, the mice were exposed to 6 mg DEP/m³ saline or to a straight saline control for 1 h using an ultrasonic nebulizer.

Grunig et al., 1998). Female Balb/c mice, 5- to 6-weeks-old and free of mice-specific pathogens, were obtained from Orient Co. (Charles River Laboratories, Seoul, Korea). The mice were housed throughout the experiments in a laminar flow cabinet and were maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were treated according to guidelines approved by the Institutional Animal Care and Use Committee of Soon-chunhyang University Medical School. DEP were resuspended in saline solution for 30 min before being administered. DEP (0.6 mg/mL DEP in 50 μ L of saline) were administered intranasally for the first 5 days; on days 6–8, the mice were exposed to 6 mg/m³ DEP for 1 h (Fig. 1) in a closed system chamber attached to an ultrasonic nebulizer (NE-UO7; Omron Corporation, Tokyo, Japan) with an output of 1 mL/min and a particle size of 1–5 μ m.

The control mice were only administered and exposed to saline solution. Mice were killed with an overdose of pentobarbital sodium (65 mg/kg body weight, administered intraperitoneally). The chest cavity was exposed, and the catheter was carefully inserted into the trachea and secured with ligatures. Bronchoalveolar lavage (BAL) was performed four times by instillation of 1-mL normal saline and gentle retrieval. Cell numbers were counted using a haemocytometer and differential cell counts were performed on slides prepared by cyto-centrifugation and Diff-Quik staining (Scientific Products, Gibbstowne, NJ). Supernatants were separated by centrifugation (500 $\times$ g, 5 min) and kept at –70°C until use. After ligation of the right main bronchus, the left lung was fixed with 4% paraformaldehyde in phosphate buffered saline and paraffin-embedded. The right lung was excised and immersed in TRI reagent (guanidium thiocyanate-phenol mixture, Molecular Research Center, Cincinnati, OH) and immediately frozen in liquid nitrogen.

Determination of Airway Responsiveness to Methacholine

Airway responsiveness was measured in unrestrained, conscious mice 1 day after the last challenge, as previously described (Hamelmann et al., 1997). Mice were placed in a

barometric plethysmographic chamber (All Medicus Co., Seoul, Korea), and baseline readings were taken and averaged for 3 min. Increasing concentrations of aerosolized methacholine, from 2.5 to 50 mg/mL, were nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization and enhanced pause (Penh) was determined. Penh, calculated as $(\text{expiratory time/relaxation time} - 1) \times (\text{peak expiratory flow/peak inspiratory flow})$, according to the manufacturer's protocol, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh is used as a measure of airway responsiveness to methacholine. Results are expressed as the percentage increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) is expressed as 100%. Penh values averaged for 3 min after each nebulization were evaluated.

Macrophage Cell Culture in the Presence of DEP

Alveolar macrophages (RAW 264.7 macrophages, ATCC[®] Number: TIB-71TM) were grown in 10% fetal bovine serum (FBS) and RPMI 1640 (WelGENE, Daegu, Korea), and 5×10^5 cells were seeded on a 24-well plate and cultured in 1-mL RPMI 1640 (WelGENE, Daegu, Korea) supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) at 37°C, and 5% CO₂ in the presence or absence of various concentrations of BSA-treated DEP. Supernatants were removed and stored at -70°C until analysis and RNA was extracted from the cell pellets.

Histology of the Lung Tissue

Sections of fixed embedded tissues 4 µm thick were cut using a Leica model 2165 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and placed on glass slides. The tissues were processed, embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

Measurement of IL-4 and IFN-γ Protein Levels and Nitrite and Nitrate in BAL Fluids

The levels of IL-4, IFN-γ, nitrite, and nitrate in BAL fluid were quantified using a sandwich enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (Biosource International, Camarillo, CA). Each sample was determined in duplicate. The lower detection limits for IL-4, IFN-γ, nitrite, and nitrate were 7.8 pg/mL, 4.7 pg/mL, 1.56 µM/L, and 0.54 µM/L, respectively. Values below these limits were considered to be 0 for statistical analysis.

Inter- and intra-assay coefficients of variance were less than 10%.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was subsequently extracted using TRI Reagent (guanidium thiocyanate-phenol mixture, Molecular Research Center) and chloroform according to the manufacturer's instructions. The following primer pairs were used for PCR: YM1, CTGGAATTGGTGCCCTACA and CAAGCATGGTGGTTTACAGGA; YM2, CAGAACCGTCAGACATTCATTA and ATGGTCCTTCCAGTAGGTAATA and mice GAPDH (5'-GGCATTGCTCTCAATGACAA-3' and 5'-AGGGCCTCTCTCTTGCTCTC-3'). The RNA was reverse-transcribed by incubation with 10 mM dNTP, 0.1 M DTT, 1 µL of Oligo (dT) (500 µg/mL), and 1 µL of SuperScript[®] II (200 units/µL; Life Technologies, Grand Island, NY) at 42°C for 50 min, and then heat-inactivated at 70°C for 15 min. After reverse transcription, aliquots of the cDNA were transferred to tubes containing specific primer pairs for mouse Ym1, mouse Ym2, and mice GAPDH genes for PCR. Ym1 and Ym2 amplification was performed in a thermocycler for 28 cycles (one cycle: 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, respectively) with the initial denaturation at 94°C for 5 min and a final extension at 72°C for 10 min. Amplified PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide stain alongside a 100-bp DNA ladder marker (Bioneer Co., Daejeon, Korea).

Statistical Analysis

Differences between independent samples were compared using the nonparametric Kruskal-Wallis *H* test for continuous data. The Mann-Whitney *U* test was applied to compare significant differences between two samples. Differences were considered significant for *p*-values < 0.05. Results are expressed as means ± standard error of the mean (SEM), unless otherwise stated.

RESULTS

To investigate the role of DEP in induction of airway hyperresponsiveness (AHR), in a modification of the classical OVA sensitization and challenge model, mice were exposed to intranasal DEP and challenged with aerosolized DEP on days 6–8. Penh was measured on day 9 and sample collection occurred on day 10. The negative controls included animals receiving intranasal saline followed by aerosolized saline on days 6–8. Delivery of aerosolized DEP following exposure with intranasal DEP induced a

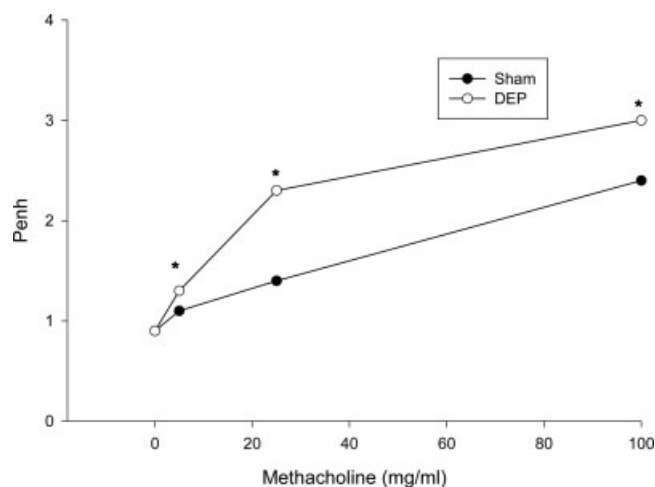


Fig. 2. The effect of DEP on airway responsiveness in DEP-sensitized and challenged mice. Airway responsiveness was measured 24 h after the last challenge in mice. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed in the main chamber of a barometric plethysmograph and treated, first with saline, and then with increasing doses, from 2.5 to 50 mg/mL, of nebulized methacholine for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization, during which time Penh values were determined. Data represent mean $\pm$ SEM from six independent experiments. * $p < 0.05$ versus the control group.

significant increase in methacholine-induced Penh, indicating AHR (Fig. 2).

No significant increase in the levels of IFN- γ were seen in the BAL fluid in the DEP treatment group, although DEP treatment did cause a statistically significant increase in IL-4 (Fig. 3), indicating that DEP modulated Th2 cytokines in the mouse model exposed to DEP. The level of IFN- γ was not statistically significant. However, the absolute increase shown is larger than for IL-4, and the total levels of IFN-gamma are always much higher than for IL-4.

Examination of the BAL fluid showed an increase in total and differential cell counts with DEP treatment. There was an increase in neutrophils and lymphocytes in the DEP group relative to the saline group [$(0.2 \pm 0.05) \times 10^4$ and $(0.6 \pm 0.01) \times 10^4$ neutrophils/mL; $(0.6 \pm 0.01) \times 10^4$ and $(0.9 \pm 0.02) \times 10^4$ lymphocytes/mL, respectively].

Histological assessment of lung tissue samples from each group revealed that peribronchial and perivascular inflammatory cell infiltrates were prominent in the DEP treatment group compared with the saline group. Taken together, these data indicate that DEP increased AHR and airway inflammation, and appears to be able to induce AHR.

The nitrite concentration in BAL fluids, which indicates the *in vivo* generation of NO in the airways, was significantly greater in the DEP exposure group than in the con-

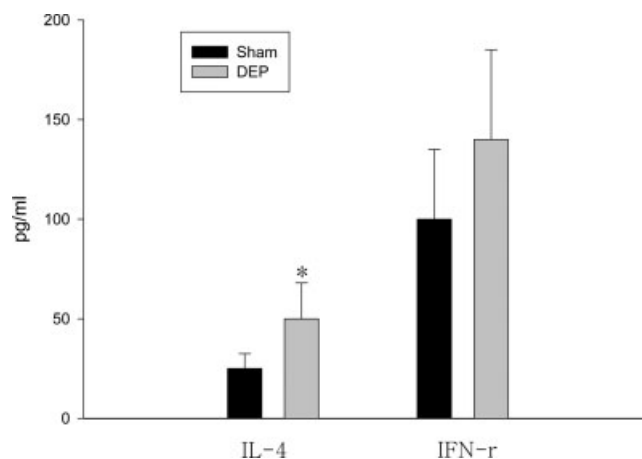


Fig. 3. The effect of DEP on IL-4 and IFN- γ levels in mice BAL fluids. Sampling was performed 24 h after the last challenge in mice treated as described in Figure 1. Bars represent mean $\pm$ SEM from six independent experiments. * $p < 0.05$ versus the control group.

trol group (52.3 ± 15.1 and 29.5 ± 5.7 μ mol/L, respectively; $p < 0.05$, Fig. 4). The alveolar macrophages produced nitrite during *in vitro* stimulation with DEP particles (50 μ g/mL), with maximal induction at 4 h after stimulation (Fig. 5).

Ym1 was one of the most highly induced IL-4 target genes, exhibiting a 70-fold or greater increase in induction in multiple macrophage populations. Ym1 was most expressed in the spleen and lungs, with lower expression in the thymus, intestine, and kidney, whereas Ym2 was most expressed in the stomach, with lower levels of expression in the thymus and kidney (Finkelman and Urban, 2001;

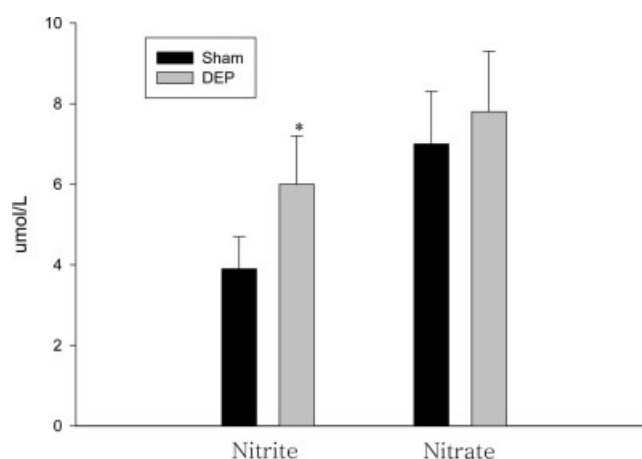


Fig. 4. The effect of DEP on nitrite and nitrate levels in mice BAL fluids. Sampling was performed 24 h after the last challenge in mice treated as described in Figure 1. Bars represent mean $\pm$ SEM from six independent experiments. * $p < 0.05$ versus the control group.

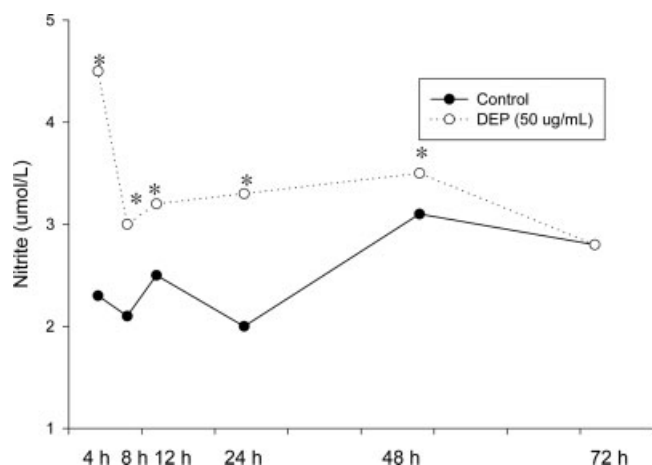


Fig. 5. Nitrite production by alveolar macrophages (Raw 264.7 cells) exposed to 50 μ g DEP/mL. * $p < 0.05$ versus the control group.

Ward et al., 2001). The conservation of STAT6 sites probably accounts for the similar, striking induction of Ym1 and Ym2 expression in Th2-type environments. We examined the possibility that Ym1 and Ym2 may be expressed in a murine model when exposed to DEP and found that TH2-biased BALB/c mice exposed intranasally with DEP, followed by a DEP challenge, upregulated lung expression of Ym1 and Ym2 transcripts in comparison with mice neither exposed nor similarly challenged (Fig. 6).

DISCUSSION

In this study, we found that DEP induce chitinase expression in the mouse lung, possibly via inducing IL-4 or IL-13 in the absence of antigen. It has been demonstrated previously, by extensive epidemiological studies, that particulate air pollutants, such as DEP, are related to allergic diseases including asthma and allergic rhinitis (Diaz-Sanchez et al., 2003). Although a number of animal and human studies have demonstrated that DEP act as an adjuvant during allergen exposure, and affect acute asthma exacerbations (Sagai et al., 1996; Takano et al., 1997, 1998; Lim et al., 1998;

Miyabara et al., 1998; Nel et al., 1998; Ohta et al., 1999; Walters et al., 2001; Ichinose et al., 2002), questions remain concerning the ability of DEP to induce asthma in the absence of an allergen.

We hypothesized that DEP play a role in increasing asthma prevalence, although a causal relationship has yet to be established. To investigate the effects of DEP exposure on airway inflammation and AHR, we exposed and challenged DEP in mice. DEP exposure increased airway inflammation and AHR, suggesting that DEP can exacerbate asthma independently of allergens.

Many studies (Sagai et al., 1996; Takano et al., 1997, 1998; Lim et al., 1998; Miyabara et al., 1998; Nel et al., 1998; Ohta et al., 1999; Walters et al., 2001; Ichinose et al., 2002) have demonstrated that long-term exposure to DEP affects AHR and airway inflammation, depending on dose, frequency, and time of DEP treatment. Such chronic exposure protocols require diesel engines, which are costly and not widely available, to investigate the effect of DEP on asthma phenotypes such as airway inflammation and AHR. Therefore, we developed a mouse model of short-term exposure to DEP and demonstrated that DEP increased AHR and airway inflammation.

Macrophages are responsible for antigen presentation and destruction of microbes and neoplastic cells. They may be subjected to modulation by selective cytokines for enhanced competence in response to air pollutants such as DEP. The phagocytosis of DEP by macrophages may induce an adaptive immune response for the development of asthma. In this study, macrophages exposed to DEP produced nitrite, indicating that they have a role in the immune response in the air pollutant model.

Chitin is a common element in some organisms including parasites, fungi, and bacteria, but does not occur in mammalian tissue (Guo et al., 2000), allowing for selective antimicrobial activity of a chitinase. Alternatively, Ym1 homologous to chitinase may have a role in the removal of chitin-containing antigens following invasions by such microorganisms (Harbord et al., 2002). A significant sequence homology (42–57%) exists between Ym1 and several other chitinase-like proteins without known functions, such as HC-gp39 (Nyirkos and Golds, 1990; Kirkpatrick et al., 1997), chitotriosidase (Renkema et al., 1999),

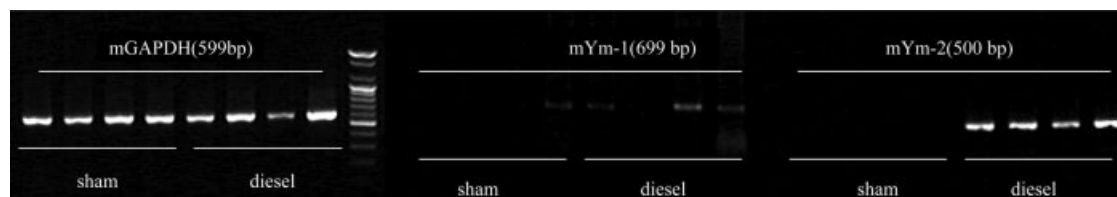


Fig. 6. The expression of Ym1 and Ym2 mRNA in the lungs of DEP-treated and control mice. Lung tissues from DEP-treated and control mice were stained with monoclonal antibodies directed against Ym1 and Ym2.

chitinase A1 (Watanabe et al., 1993), gp38k (Shackelton et al., 1995), oviductin (Malette et al., 1995), and DS-47 (Kirkpatrick, 1995). Ym1, a novel mammalian lectin with a binding specificity to GlcN (Guo et al., 2000), has been purified and characterized as a single-chain polypeptide with an estimated molecular mass of 45 kDa and a pI of 5.7. The induced expression of Ym1 by activated macrophages and the profound cellular changes paralleling its appearance suggest that Ym1 may be functionally significant to the development of either host defense against or tolerance of nematodes (Guo et al., 2000). Ym1 and Ym2 proteins were expressed in the allergic lung model in a manner that was critically dependent on CD4⁺ T cells and on IL-4-mediated or IL-13-mediated signaling via IL-4R α subunits (Jin et al., 1998). Upregulation of Ym1 and Ym2 expression occurred in the allergic asthma model by proteomic analysis (Jeong et al., 2005). Zhu et al. (Zhu et al., 2004) suggested that AMCase might be an important mediator of IL13-induced responses in Th2-dominated disorders such as asthma.

To identify the molecular mediator in the DEP model, a comparison was made of the lung tissue, which demonstrated that the Ym1 and Ym2 mRNA was upregulated in the lung during DEP exposure in the mouse model and that the level of Ym2 was more higher than that of Ym1, suggesting that Ym1 and Ym2 proteins are important in AHR and inflammation related to air pollutants such as DEP. In parallel with previous studies (Welch et al., 2002; Zhu et al., 2004), levels of Th2 cytokines such as IL-4 increased in the BAL fluids of mice exposed to DEP, suggesting that Ym1 and Ym2 are involved in the mouse model exposed to DEP via the Th2 cell biased response in the lung.

NOS is an enzyme active in airway epithelial cells, macrophages, neutrophils, mast cells, autonomic neurons, smooth muscle cells, fibroblasts, and endothelial cells. The chemical products of NOS in the lung vary with disease state and are involved in pulmonary neurotransmission, host defence, and airway and vascular smooth muscle relaxation (Moncada et al., 1989, 1991). In our study, the levels of NO metabolites increased in alveolar macrophages after DEP exposure, suggesting that NO contributes to airway inflammation in this mouse model exposed to DEP.

In conclusion we demonstrated that DEP increase AHR, and that DEP induce chitinase expression in the mouse lung, possibly via inducing IL-4 or IL-13 in the absence of antigen.

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