

PM2.5 exacerbates asthma and TRPA1 and TRPV1 expression

Exacerbating effects of PM2.5 in OVA-sensitized and challenged mice and the expression of TRPA1 and TRPV1 protein in lung

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Abstract

Objective: To investigate the effects of PM2.5 on asthma-related phenotypes and on the expression of TRPA1, V1 protein in lung in a mouse model of asthma. *Methods:* Female Balb/c mice were utilized to establish 28 and 42 day asthma models. Mice were sensitized with OVA and challenged with OVA, OVA plus normal saline (NS), or OVA plus PM2.5 at two doses, 1.6mg·kg⁻¹ or 8.0mg·kg⁻¹. PM2.5 was administrated by intratracheal instillation without anesthesia. 24 hours after the final OVA challenge changes in airway resistance (RI) and lung

compliance (Cdyn) in response to acetylcholine chloride (ACH) were evaluated, and blood, bronchoalveolar lavage fluid (BALF) and lung tissue samples were taken. Number of eosinophils in blood and various leukocytes in BALF were determined. Lung protein was extracted and probed by immunoblot for TRPA1, V1 expression. Levels of IL-13, SP, PGD₂ and NGF in BALF were measured by ELISA. *Results:* PM2.5 treated mice showed significantly greater changes in number of inflammatory cells in blood and BALF, in RI and Cdyn in response to ACH, and in lung injury, as indicated by inflammatory cell infiltration, increased tracheal secretions and thickened bronchial smooth muscles, compared to controls. In addition, higher expression of TRPA1, V1 in lung and IL-13, SP, PGD₂ and NGF in BALF were seen in mice exposed to PM2.5. All effects were most pronounced in mice in the 42 day model exposed to 8.0mg·kg⁻¹ PM2.5. *Conclusions:* PM2.5 exacerbates effects of asthma in this animal model, possibly by regulating TRPA1, V1 and the relevant neurokines.

Keywords

PM2.5, ovalbumin, asthma, TRPA1, TRPV1, neurokines, Immunopathogenesis, Mechanisms, Epidemiology, Pathophysiology

Introduction

The incidence and mortality of bronchial asthma, a chronic airway inflammatory disease, has increased over recent decades in many countries. Among 300 million asthma patients, approximately 250,000 die each year, with a higher risk in urban areas than in rural areas [1-3].

Both genetic predisposition and environmental exposures are associated with the complicated pathogenesis of asthma. However, variations in asthma prevalence among individuals with common racial background but different environmental exposures suggest that air pollutants might be a dominant factor in the etiology of asthma [4-5]. Epidemiological studies indicate that air pollution, especially elevated concentrations of particulate matter 2.5 microns or less in size (PM_{2.5}), is one of the major factors responsible for the increased incidence of asthma [6-8]. Acute toxicity experiments confirm that ambient PM_{2.5} exposure can stimulate macrophages, bronchial epithelial cells and alveolar epithelial cells to release large amounts of proinflammatory factors, chemotactic factors, and oxygen free radicals (ROS), attracting inflammatory factors and neutrophils, which in turn mediate the pathological damage process in the lung [8-9]. Asthma exacerbation by PM_{2.5} is thought to be related to airway inflammation, oxidative damage, and imbalance of Th1/Th2 or Th17/Treg [10-14]. Polycyclic aromatic hydrocarbons found in PM_{2.5} have been shown to induce bronchial mucosa damage, alveolar epithelial hyperplasia and chronic inflammation [15-16]. Most countries in the world have become aware of the dangers of PM_{2.5} and have formulated testing standards to closely monitor

PM_{2.5} concentration. For example, criterion value of PM_{2.5} in *PM_{2.5} air quality guidelines* set by the World Health Organization (WHO) is 10µg/m³ (annual average concentration of PM_{2.5}) and 25µg/m³ (daily average concentration of PM_{2.5}).

In recent years, transient receptor potential ion channel proteins (TRPs) including TRPA1 and TRPV1 have been found to be involved in asthma pathophysiology [17-20]. TRPA1-activating stimuli such as cigarette smoke, chlorine, aldehydes, and scents are among the most prevalent triggers of asthma. A TRPA1 antagonist inhibited eosinophil (EOS) infiltration and prevented the development of airway hyper-reactivity when administered during airway allergen challenge [18]. TRPV1 has been demonstrated to regulate the activation and inflammatory properties of CD4(+) cells. Targeting TRPV1 using either genetically modified mice or a pharmacological inhibitor tended to decrease IgE levels, airway inflammation and lung function changes [21]. TRPA1 and TRPV1 are widely distributed in the body, which can be found both on sensory neurons and non-neuronal cells (such as mast cells and macrophages) [22-23]. Several relevant effectors of TRPA1 and / or TRPV1 have been identified, including Th2 cytokine IL-13 [31], neurogenic inflammation mediator substance P (SP) [38], mast cell activation marker prostaglandin D₂ (PGD₂) [40] and nerve growth factor (NGF) [43], have been identified. These same factors have been shown to play a role in asthma airway inflammation [31-43]. Cardiac oxidative stress and electrophysiological changes in rats exposed to concentrated ambient particles have been shown to be mediated by TRP-dependent pulmonary reflexes. Thus TRPs

may play a role in the exacerbating effects of PM_{2.5} on asthma [24].

In this study, we use the OVA mouse model of asthma to investigate effects of PM_{2.5} on asthma severity, as determined by lung function assays, pulmonary histopathology, presence of inflammatory cells in blood and bronchoalveolar lavage fluid (BALF) and levels of cytokines and neurokinines in BALF. In addition, to begin to elucidate the mechanism by which PM_{2.5} affects airway function, we compared TRPA1 and TRPV1 protein expression and expression of relevant effectors IL-13, SP, PGD₂ and NGF in control, OVA challenged and OVA plus PM_{2.5} treated mice.

Methods

Animals and ethics statement

Female Balb/c mice (8-12 weeks old, 20±2g) were obtained from the Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu, China), with a certification (No. SCXK (Su) 2012-0004). The mice were acclimated to laboratory conditions for one week before experiments commenced. All animal procedures were approved by the Laboratory Animal Center of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China) and conform to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, 2011).

PM_{2.5} sampling and processing

Atmospheric PM_{2.5} samples were gathered using a Thermo Scientific TEOM1405-D particle monitor from March 14 to December 14, 2015 by Nanjing Environmental Monitoring

Center. The sampling site was on the rooftop of Caochang Men, one of the state control points in Nanjing. PM_{2.5} was collected on filters, and the total mass was measured with a Tapered Element Oscillating Microbalance. The filter membranes containing the PM_{2.5} samples were immersed in deionized distilled water and particulate matter was eluted by ultrasonic vibration 40min×3 times. The eluate was filtered through six pieces of sterile gauze, which filters out larger impurities but allows PM_{2.5} to pass through. Extracted PM_{2.5} was formulated with saline in two concentrations (0.8mg·mL⁻¹ and 4.0mg·mL⁻¹), then stored at 4°C for subsequent experiments.

Mouse model of asthma

Two variants of the OVA sensitization/challenge mouse model of asthma were used, one with 12 OVA challenges over a total of 28 days and one with 21 OVA challenges over a total of 42 days (Fig. 1). For each model, 100 mice were randomly divided into five equal groups: (1) control group (n=20); (2) OVA-sensitized and challenged group (OVA, n=20); (3) OVA plus normal saline group (OVA+NS, n=20); (4) OVA plus low dose PM_{2.5}(1.6mg·kg⁻¹) group (OVA+PM_{2.5}-L, n=20); (5) OVA plus high dose PM_{2.5}(8.0mg·kg⁻¹) group (OVA+PM_{2.5}-H, n=20). Mice in all groups except the control group were given two injections, one subcutaneous and one intraperitoneal, of 0.5mL sensitizing solution containing 25μg OVA and 2mg aluminium hydroxide gel on day 1 and day 8.

28 day model: Mice in the OVA+NS, OVA+PM_{2.5}-L and OVA+PM_{2.5}-H groups were

challenged with inhalation of 5% OVA solution for 20 min from day 15 through day 23, and on days 25, 26 and 28. On days 24 and 27 mice received 40 μ L normal saline, low dose PM2.5 solution, or high dose PM2.5 solution, depending on their group, via intratracheal instillation using a pulmonary drug delivery system (Penn-century, Inc., USA) without anesthesia. Mice in the OVA group were OVA-challenged daily from day 15 through day 28 [25].

42 day model: Mice in the OVA+NS, OVA+PM2.5-L and OVA+PM2.5-H groups were OVA-challenged for 20 min daily from day 15 through day 28 and every other day from day 30 to day 42. Mice received 40 μ L normal saline, low dose PM2.5 solution, or high dose PM2.5 solution, depending on their group, on day 29, 33, 37 and 41. Mice in the OVA group were OVA-challenged daily from day 15 through day 28 and every other day from day 30 to day 42 [26].

All mice were anaesthetized with 4% chloral hydrate 24h after the last OVA challenge for collection of blood, BALF and lung tissues.

Eosinophil (EOS) count in peripheral blood

20 μ L of peripheral blood was mixed with 0.38 mL EOS count solution (Nanjing Jiancheng Bioengineering Institute, China). The amount of EOS was observed and counted in a hemocytometer under 10X and 100X magnification.

BALF collection and inflammatory cell counts

BALF was collected from a subset of 10 mice from each experimental group. These mice

were anaesthetized and cannulas were inserted into their tracheas. Bronchoalveolar lavage was performed with 3 aliquots of 0.5 mL PBS (pH 7.2) withdrawn three times each. The fluid recovery rate was 65%-75%. BALF samples were centrifuged ($1000 \text{ r}\cdot\text{min}^{-1}$) for 10 min. The pelleted cells were resuspended in 0.5 mL PBS, smeared and fixed. The percentage of different leukocytes in BALF was counted double-blindly using an oil immersion microscope at magnification of 10X and 100X (Wright and Giemsa's staining; two counts per slide, 200 cells per count).

Airway responsiveness determination

Airway responsiveness was determined for a separate subset of 6 mice from each experimental group. Airway responsiveness was analyzed 24h after the last OVA challenge with acetylcholine chloride (ACH) stimulation of restrained mice using a Buxco FinePointeTM RC system (Buxco Inc., USA). Briefly, mice were anesthetized and their tracheas were isolated by blunt dissection. A small caliber "Y" figure tube was inserted and secured in the airway. Airway resistance (RI) and dynamic lung compliance (C_{dyn}) in response to increasing concentrations of ACH (0, 6.25, 12.5, 25, 50mg·mL⁻¹) were recorded. RI is defined as the pressure driving respiration divided by flow and C_{dyn} is change in volume divided by change in pressure.

Histological assessment

The right lung tissues were fixed in 10% formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin and inspected under a light microscope. Lung

injury scores were determined using a semi-quantitative scoring method [27] in which lung injury was graded from 0 (normal) to 4 (severe) in three categories: inflammatory cell infiltration, thickened bronchial smooth muscle and increased tracheal secretions. The total lung injury score (TLIS) was calculated by adding the individual scores for each category.

Western blot analyses

Mouse lungs were collected 24h after the final OVA challenge and stored at -80°C . One week later total protein was extracted from the lung tissue, quantified using the Bradford method, and diluted in denaturing Lysis Buffer. The samples were incubated at $95-100^{\circ}\text{C}$ for 5 min. Then, samples containing 50 μg proteins each were subjected to SDS-PAGE (Bio-Rad Laboratories Inc., USA). After electrophoresis, proteins were transferred to PVDF membrane, blocked for 2h in 5% skim milk powder blocking buffer and washed with PBST 4 times. Blots were incubated with mouse anti-TRPA1 or anti-TRPV1 antibodies (Abcam Inc., England) and β -Tubulin antibodies (CST Inc., USA) overnight at 4°C . After washing with PBST 4 times, blots were incubated with secondary antibodies for 2h at room temperature. The membranes were washed with PBST 4 times, incubated with developing solution for 5 min at room temperature, and exposed with G: BOX chemiXR5 (Bio-Rad Laboratories Inc., USA). The results were analyzed with Gel-Pro32 software (Media Cybernetics Inc., USA).

Measurement of cytokine and neurokinine levels in BALF

Levels of IL-13, SP, PGD_2 and NGF in BALF were measured using enzyme-linked

immunosorbent assay (ELISA) kits (Shanghai Yunhan Biological Technology Co., Ltd, China) according to manufacturer's instructions.

Statistical analyses

Data were evaluated using one-way analysis of variance (ANOVA) followed by LSD or Dunnett's T3 post hoc analysis. $P < 0.05$ was considered as significant. Data were presented as $\bar{x} \pm sd$ (mean $\pm$ standard deviation). Statistical analysis was performed with SPSS22.0 software.

Results

Impact of PM2.5 on inflammatory cell counts

Number of EOS in peripheral blood and percentages of EOS, neutrophils, lymphocytes and macrophages in BALF were determined in control, OVA, OVA+NS, OVA+PM2.5-L and OVA+PM2.5-H mice in the two asthma models as an indication of inflammatory state. There was a significant increase in blood EOS in the four asthma groups compared with the control group ($P < 0.01$) and in the OVA+PM2.5-L and OVA+PM2.5-H groups compared to the OVA+NS group ($P < 0.05$) in both models. There was also a significant increase in blood EOS in OVA+PM2.5-H compared to OVA group in the 42 day model ($P < 0.05$) (Fig. 2A).

Similarly, in BALF there was a significant increase in EOS percentage in the four asthma groups compared with control group ($P < 0.01$) and of the OVA+PM2.5-L and OVA+PM2.5-H groups compared with OVA and OVA+NS groups ($P < 0.05$, $P < 0.01$) in both models. For BALF neutrophils, the only significant difference was an increase in the OVA+PM2.5-H group

compared to control in the 42 day model ($P<0.05$). Percentage of BALF lymphocytes was also similar among the various groups, with the only significant difference being an increase in the OVA+NS group compared to control in the 42 day model ($P<0.05$). The percentage of macrophages in BALF was significantly lower in the OVA+PM2.5-L and OVA+PM2.5-H groups compared with the control group ($P<0.01$) the OVA group ($P<0.05$) and the OVA+NS group ($P<0.05$, $P<0.01$) in both models (Fig. 2B). Taken together, these results suggest that PM2.5 exacerbates inflammation in OVA sensitized and challenged mice.

Effect of PM2.5 on airway responsiveness and lung histopathology

The effect of PM2.5 on airway responsiveness after exposure to increasing concentrations of ACH was determined by measuring changes in RI and Cdyn. RI values increased and Cdyn values decreased with exposure to increasing concentrations of ACH in all mice tested, but the extent of change was more pronounced in OVA groups and was most pronounced in OVA+PM2.5 groups (Fig. 3). At $50\text{mg}\cdot\text{ml}^{-1}$ ACH, mean RI values for the four asthmatic groups were significantly higher than control group values in both asthma models ($P<0.05$, $P<0.01$) and mean RI value for the OVA+PM2.5-H group was significantly higher than OVA and OVA+NS groups in the 42 day model ($P<0.05$) (Fig. 3A and 3C). Mean Cdyn values for OVA+PM2.5-L and OVA+PM2.5-H groups were significantly lower than control values at $50\text{mg}\cdot\text{ml}^{-1}$ ACH in both asthma models ($P<0.05$, $P<0.01$). Mean Cdyn values for the OVA+PM2.5-H group was also significantly lower than OVA and OVA+NS groups in the 42 day asthma model ($P<0.05$) (Fig.3B

and 3D).

To evaluate the effect of PM_{2.5} on pulmonary histology in the OVA asthma model, lungs were harvested 24h after the last OVA challenge and sections were prepared, subjected to hematoxylin and eosin staining and observed. Representative images are shown in Figure 4. A semi quantitative system in which inflammatory cell infiltration, thickened bronchial smooth muscle and increased tracheal secretions were each scored on a 0 to 4 scale, and the three scores were added together, was used to measure lung injury. The mean total lung injury scores (TLIS) for mice in the four asthma groups were significantly higher than scores for control mice. Mean TLIS was significantly higher for the OVA+PM_{2.5}-H group compared to the OVA+NS group in both asthma models ($P<0.05$). For the OVA+PM_{2.5}-L group mean TLIS was significantly higher than the OVA+NS score only in the 42 day model ($P<0.05$) (Fig. 5).

Effect of PM_{2.5} on lung expression of TRPA1 and TRPV1 in the mouse asthma models

Total protein was extracted from mouse lungs and TRPA1 and TRPV1 expression were determined by Western blotting. In both the 28 and 42 day models, TRPA1 was more highly expressed in OVA, OVA+PM_{2.5}-L and OVA+PM_{2.5}-H groups compared with the control group ($P<0.05$), and OVA+PM_{2.5}-L and OVA+PM_{2.5}-H groups with a larger difference in the 42 day model ($P<0.01$). TRPA1 expression was also significantly higher in OVA+PM_{2.5}-L and OVA+PM_{2.5}-H groups compared with OVA and OVA+NS groups in the 42 day model ($P<0.05$) (Fig 6).

Compared with the control group, TRPV1 was more highly expressed in OVA and OVA+PM2.5-H groups in both the 28 and 42 day models, and OVA+PM2.5-L group in the 28 day model ($P<0.01$). Compared with OVA+NS group, TRPV1 expression was significantly higher in OVA+PM2.5-L and OVA+PM2.5-H groups in the 28 day model and OVA+PM2.5-H group in the 42 day model as well ($P<0.05$). Compared with OVA group, TRPV1 was more highly expressed in OVA+PM2.5-L group in 42 day model ($P<0.05$) (Fig 7).

Cytokines and neurokines in BALF

BALF was collected from mice 24h after the final OVA challenge and levels of IL-13, SP, PGD₂ and NGF were determined by ELISA. IL-13 and SP levels were significantly higher in the four asthmatic groups compared to control in both the 28 and 42 day models ($P<0.05$, $P<0.01$) (Fig. 8A and 8B). IL-13 and SP levels were also significantly higher in the OVA+PM2.5-L and OVA+PM2.5-H groups compared to the OVA+NS group in both models. In the 42 day model, IL-13 was significantly higher in the OVA+PM2.5-H group and SP was significantly higher in the OVA+PM2.5-L group compared to the OVA group ($P<0.05$). For PGD₂ and NGF levels were significantly higher in the four asthma groups compared to control in both the 28 and 42 day models, except for OVA+NS group in the 28 day model (Fig. 8C and 8D). PGD₂ levels were also significantly higher in the OVA+PM2.5-L and OVA+PM2.5-H groups compared to the OVA+NS group in both models and compared to OVA in the 42 day model ($P<0.05$). NGF was significantly higher in the OVA+PM2.5-L group compared to OVA+NS in the 42 day model

($P < 0.05$), and in the OVA+PM2.5-H group compared to OVA+NS in both models and compared to OVA in the 42 day model ($P < 0.05$).

Discussion

In this study we investigated the effects of PM2.5 on asthma using 28 day and 42 day OVA sensitization/challenge mouse models of asthma and administering PM2.5 intratracheally, without anesthesia, rather than the more common approaches of oropharyngeal aspiration or intratracheal instillation under anesthesia [28-29]. We found that PM2.5 exacerbates the changes in inflammatory cells in blood and BALF, the degree of change in RI and Cdyn in response to ACH, and lung injury, as indicated by inflammatory cell infiltration, increased tracheal secretions and thickened bronchial smooth muscles, in the OVA model of asthma. In addition, higher expression of TRPA1, TRPV1, IL-13, SP, PDG₂ and NGF were observed in mice exposed to PM2.5 along with OVA. All effects were most pronounced in PM2.5-H mice in the 42 day asthma model.

Several studies have shown an exacerbating effect of PM2.5 in experimental asthma models. He et al found that in the presence of OVA, PM2.5-rich dust caused severe eosinophil infiltration and goblet cell proliferation in airways, along with a marked induction of the Th2 cytokines interleukin (IL)-4 and IL-13, and the eosinophil-related cytokine IL-5 in BALF [15]. Zhang et al suggested that acute exposure of PM2.5 (intratracheally administered) could synergize with allergens in the subsequent challenge to aggravate the severity of asthma in sensitized mice, possibly by promoting a Th2-biased immune response [30]. Wagner et al

confirmed that asthmatic rats (induced by OVA) exposed to concentrated air particles had 200% increase in airway mucus and had more BALF neutrophils (250% increase), eosinophils (90%), and total protein (300%) compared to controls [28]. Our findings support these previous findings, adding to the evidence that PM_{2.5} does exacerbate asthma in animal models.

The disease mechanism of asthma is complicated, but many aspects have been revealed by studies focusing on immune, smooth muscle and airway epithelial function. Several asthma therapies have been developed to target the immune system, but their limited efficacy suggests that other mechanisms, perhaps including neurological mechanisms, are involved in asthma etiology. TRPA1 has been shown to be a key integrator of interactions between the immune and nervous systems in the airway, driving asthmatic airway inflammation following inhaled allergen challenge [17]. TRPV1 inhibition attenuates IL-13 mediated asthma features in mice by reducing airway epithelial injury [31]. In this study we found increases in TRPA1 and TRPV1 expression in OVA+PM_{2.5} mice, suggesting that TRPA1 and TRPV1 inhibition could be beneficial in attenuating lung injury and asthma symptoms. Therefore, the potential use of TRPV1 and TRPA1 antagonists may be a novel therapy for asthma.

Some cytokines and neurokinins including IL-13, SP, PGD₂ and NGF are relevant to TRPA1 and / or TRPV1 channels. IL-13 is a pleiotropic cytokine secreted by CD4⁺ TH₂-cells which participates in the inflammatory response of asthma [31-33]. SP is an important mediator of neurogenic inflammation in airways and is known to be a sensory neuropeptide. Because of its

rapid spasmogenic effect on smooth muscle of airways, SP is also considered to be a tachykinin [34-35]. When TRP channels are open Ca^{2+} enters cells and causes release of tachykinins [36-37]. TRPV1 nerve fibers were found to contain CGRP, SP and nNOS [38]. PGD_2 can inhibit platelet aggregation, blood vessel expansion and bronchial smooth muscle contraction. It functions as a mast cell-derived mediator to trigger asthmatic responses [39]. Several studies have shown that PGD_2 metabolite 15-delta PGJ_2 and other prostaglandins are involved in inflammatory pain, a symptom of asthma, probably through activation of TRPA1 [40-41]. NGF, an essential neurotrophic factor, may lead to airway inflammation, airway hyperresponsiveness and bronchial remodeling by regulating immune cells and releasing inflammatory cells [42]. NGF probably induces neurogenic inflammation in response to asthma triggers, and promotes cough and respiratory tract obstruction through TrkA receptor, TRPA1 and TRPV1 channels [43]. In this study we have shown that IL-13, SP, PGD_2 and NGF all increase with $\text{PM}_{2.5}$ exposure in the OVA asthma model, particularly with high $\text{PM}_{2.5}$ in the 42 day model.

According to our results, the exacerbating effects of $\text{PM}_{2.5}$ on asthma were more obvious in the 42 day model than in the 28 day model. At the same time there was no dose-response effect of $\text{PM}_{2.5}$ on total lung injury score and BALF eosinophilia. This suggests that the exacerbating effects of $\text{PM}_{2.5}$ depend mainly on the length $\text{PM}_{2.5}$ exposure rather than the dose. It has been reported that long-term repeated $\text{PM}_{2.5}$ exposure is associated with increased risk of asthma mortality in broad-based subjects [44-45]. Further investigation of the effect of length of

PM2.5 exposure is warranted.

Many other questions remain to be answered about the effects of PM2.5 on asthma and the possible role of TRPA1 and TRPV1. Future studies will focus on what happens when mice are sensitized by OVA and challenged with PM2.5, or are exposed to PM2.5 without sensitization and challenge with OVA. Components of PM2.5, including water soluble, organic carbon and elemental carbon factors, should be tested to determine which ones contribute to asthma exacerbation. The role of TRPA1 and TRPV1 in PM2.5 induced asthma exacerbation should be investigated by determining the effect of TRPA1 and TRPV1 inhibition on PM2.5 induced asthma exacerbation, the localization and distribution of the overexpressed proteins and their functions in bronchial epithelial cells and bronchial smooth muscle cells. Additionally, the relationship of TRPA1, TRPV1 proteins and the four relevant factors (IL-13, SP, PGD₂ and NGF) should be studied further to help determine the neuroimmunologic mechanism of asthma exacerbation by PM2.5 exposure. In our study, SP, PGD₂ and NGF levels were evaluated 24 hours after the final challenge. Expression of these factors may have peaked before that time point, particularly SP and PGD₂ which have short half-lives. Future experiments will follow expression of these factors over time following the final challenge to more accurately gauge the effect of PM2.5 on their expression.

In conclusion, PM2.5 exposure in combination with OVA sensitization and challenge in 28 day or 42 day asthma models was utilized to investigate whether PM2.5 exacerbates the severity

of asthma. Our findings demonstrated that PM_{2.5} could aggravate the changes in inflammatory cells in blood and BALF, the degree of change in RI and C_{dyn} in response to ACH and lung injury, especially in the 42 day asthma model, possibly by regulating TRPA1 and TRPV1 channels and the relevant neurokines.

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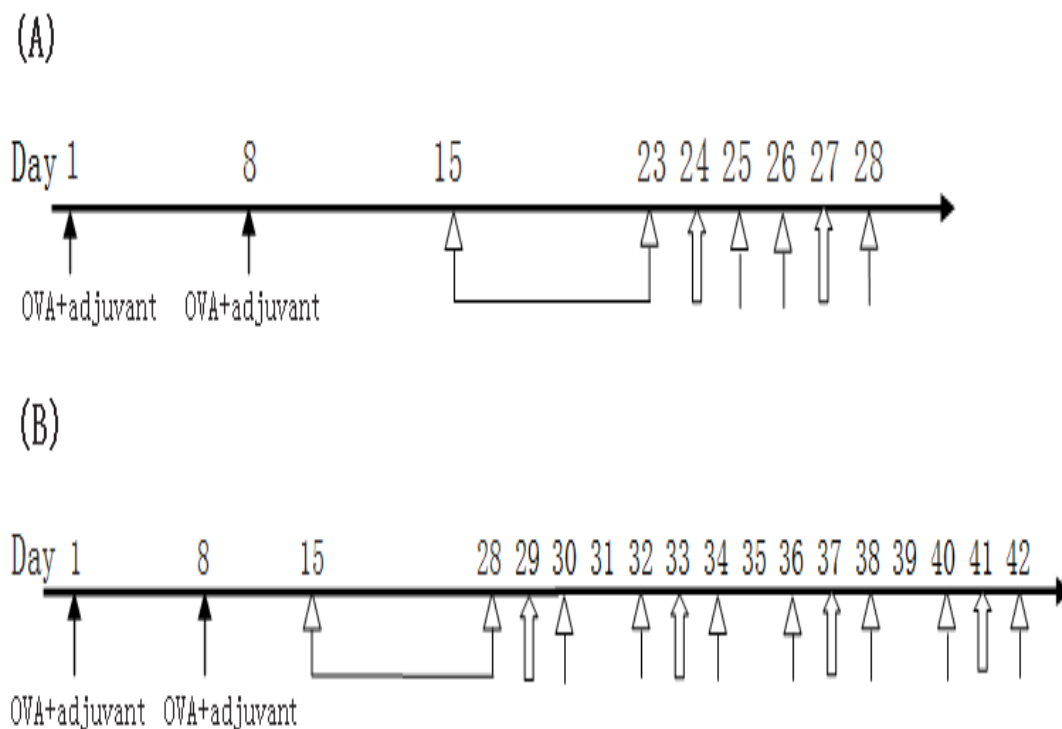


Figure 1. Timeline for asthma models. (A) 28 day asthma model (B) 42 day asthma model. Thin arrow: inhalation of 5% OVA solution; Thick arrow: pulmonary administration of 40 μ L PM2.5 solution or normal saline

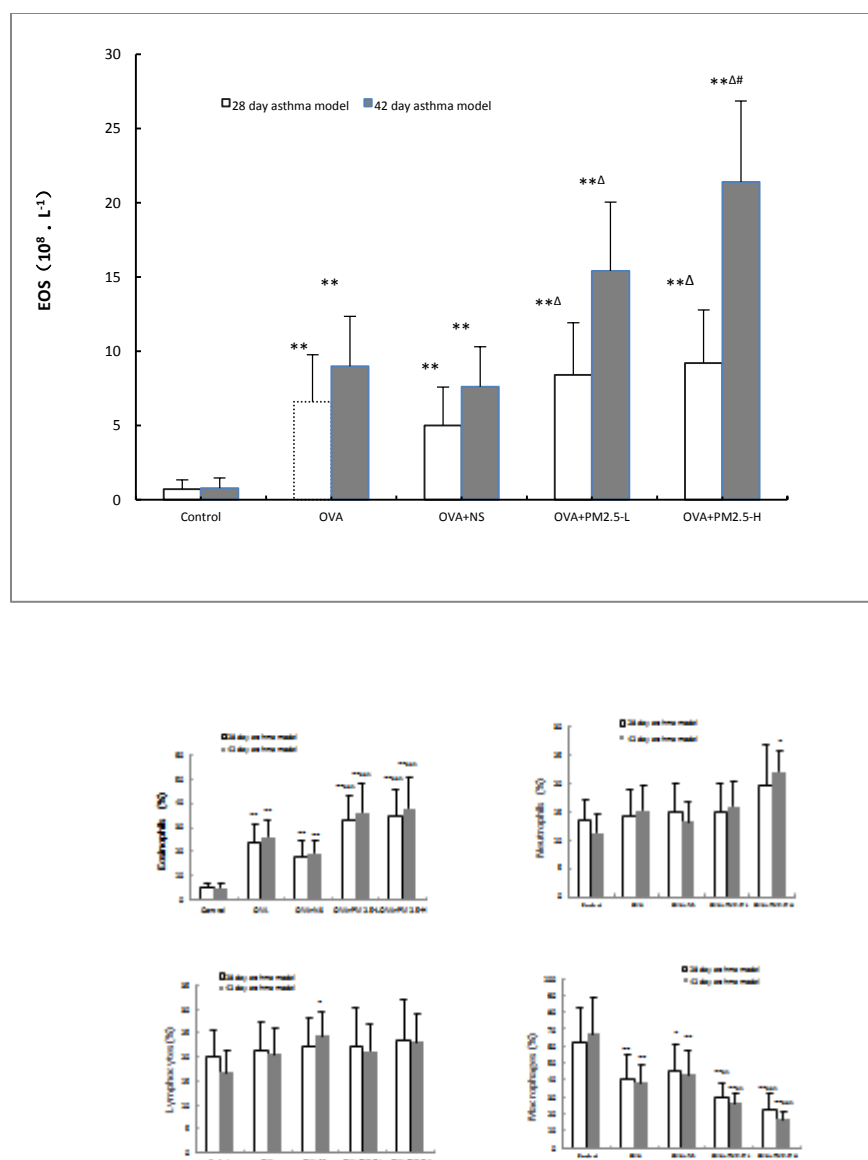
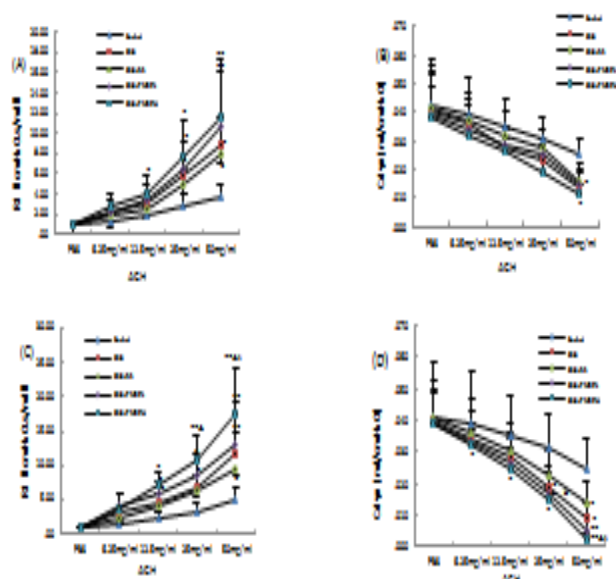


Figure 2. Inflammatory cells in blood and BALF. (A) Eosinophil counts in peripheral blood ($n=10$, $\bar{x} \pm sd$). (B) Percentage of inflammatory cells in BALF of 28 and 42 day asthma models ($n=10$, $\bar{x} \pm sd$). * $P < 0.05$ vs control group, ** $P < 0.01$ vs control group; Δ $P < 0.05$ vs OVA+NS group; $\Delta\Delta$ $P < 0.01$ vs OVA+NS group; $\#$ $P < 0.05$ vs OVA group



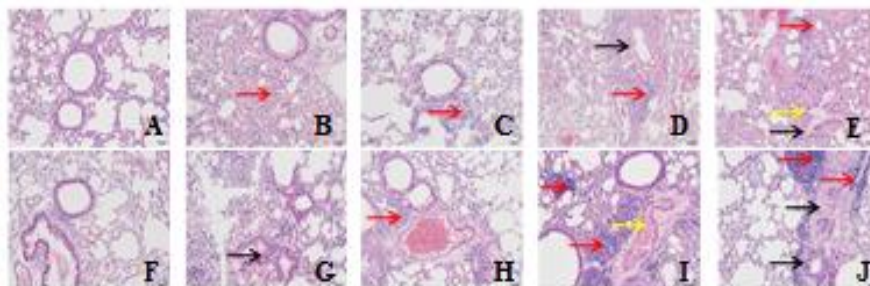


Figure 4. Pulmonary histopathological changes (HE $\times 100$). (A)-(E) lung sections of mice in the 28 day asthma model. (F)-(J) lung sections of mice in the 42 day model. (A) and (F): control group; (B) and (G): OVA group; (C) and (H): OVA+NS group; (D) and (I): OVA+PM2.5-L group; (E) and (J): OVA+PM2.5-H group. Red arrow: inflammatory cell infiltration; black arrow: thickened bronchial smooth muscle; yellow arrow: increased tracheal secretions.

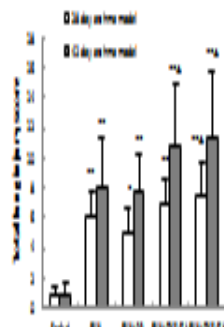


Figure 5. Quantification of lung injury. Lung injury was graded from 0 (normal) to 4 (severe) in three categories: inflammatory cell infiltration, thickened bronchial smooth muscle and increased tracheal secretions. The three scores were added to get total lung injury score (n=8, $\bar{x} \pm \text{sd}$). ** P<0.01 vs control group; [^] P<0.05 vs OVA+NS group

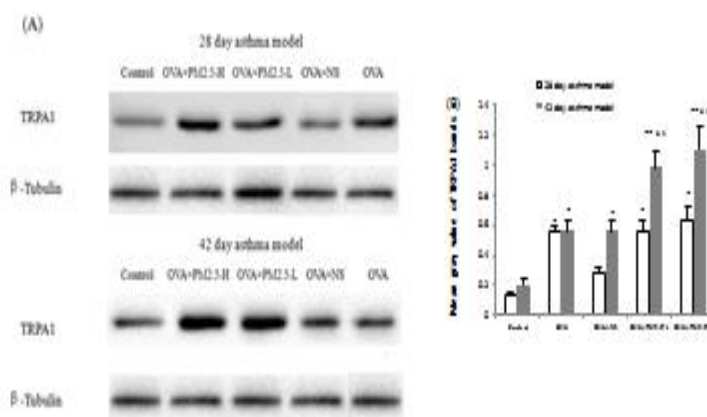


Figure 6. Expression of TRPA1 in lung tissues. (A) TRPA1 protein expression in individual representatives of each group. β tubulin was used as a loading control. (B) Quantification of TRPA1 protein expression. (n=6, $\bar{x} \pm sd$) * P<0.05, ** P<0.01 vs control group; # P<0.05 vs OVA group. Mean gray value: a measure of intensity calculated as sum of the gray value of all pixels divided by the number of pixels.

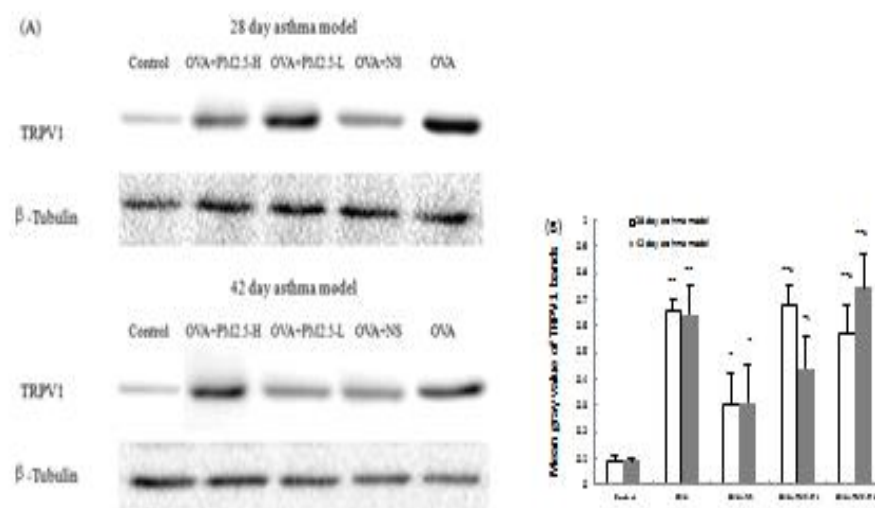


Figure 7. Expression of TRPV1 in lung tissues. (A) TRPV1 protein expression in individual representatives of each group. β tubulin was used as a loading control. (B) Quantification of TRPV1 protein expression. (n=6, $\bar{x} \pm sd$) * $P < 0.05$, ** $P < 0.01$ vs control group; # $P < 0.05$ vs OVA group. Mean gray value: a measure of intensity calculated as sum of the gray value of all pixels divided by the number of pixels.

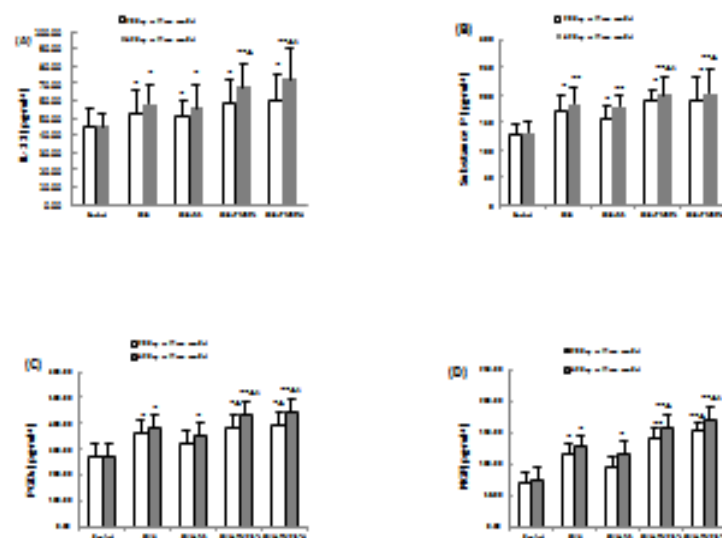


Figure 8. Cytokines and neurokinins in BALF (n=8, $\bar{x} \pm \text{sd}$). (A) IL-13; (B) SP; (C) NGF; (D) PGD₂ * P<0.05, ** P<0.01 vs control group; Δ P<0.05, $\Delta\Delta$ P<0.01 vs OVA +NS group; # P<0.05 vs OVA group