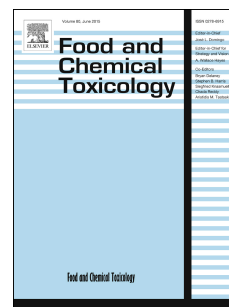


Accepted Manuscript

Exposure to diisodecyl phthalate exacerbated Th2 and Th17-mediated asthma through aggravating oxidative stress and the activation of p38 MAPK

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PII: S0278-6915(18)30093-0

DOI: [10.1016/j.fct.2018.02.028](https://doi.org/10.1016/j.fct.2018.02.028)

Reference: FCT 9599

To appear in: *Food and Chemical Toxicology*

Received Date: 7 August 2017

Revised Date: 7 February 2018

Accepted Date: 10 February 2018

Please cite this article as: Qin, W., Deng, T., Cui, H., Zhang, Q., Liu, X., Yang, X., Chen, M., Exposure to diisodecyl phthalate exacerbated Th2 and Th17-mediated asthma through aggravating oxidative stress and the activation of p38 MAPK, *Food and Chemical Toxicology* (2018), doi: 10.1016/j.fct.2018.02.028.

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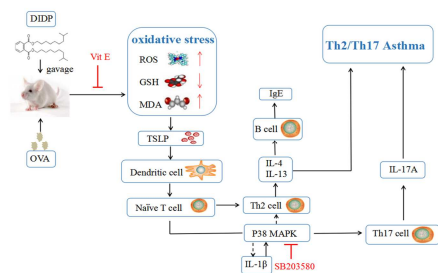
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Running title: DIDP exacerbated asthma



1. Introduction

Allergic asthma is a chronic disease (Newcomb and Peebles, 2013) characterized by airway inflammation, airway remodeling, mucous cell metaplasia, and increased airway reactivity (AR). It is estimated that asthma kills 346000 people worldwide each year (Lozano et al., 2013). Studies have found that asthma is associated with long-term exposure to pollutants (Eder et al., 2006). Phthalates are often used as a plasticizer, and can leach, migrate or emit into the air, food or other materials. Diisodecyl phthalate (DIDP) is considered to be a less toxic phthalate and is widely used as a plasticizer in the manufacture of polymers and consumer products. It has been reported that Di-(2-ethylhexyl) phthalate (DEHP) and Diisononyl phthalate (DINP) have an adjuvant effect on asthmatic mice (Kimber and Dearman, 2010; Larsen et al., 2001; Larsen et al., 2002). An epidemiological study found that Diisodecyl phthalate (DIDP) oxide metabolites are associated with the occurrence of asthma by looking at urine markers (Bertelsen et al., 2013). However, the effect of DIDP exposure and the underlying mechanisms are not clear.

Increasing evidence suggests that asthma is a heterogeneous disease. An imbalance between Th1/Th2 plays a prominent role in asthma (Mak et al., 2013). The Th2 immune response is activated in a large subset of subjects with allergic asthma, and Th2-type cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 are over-produced (Lloyd and Hessel, 2010). IL-4 is crucial for allergic sensitization, and considered to be an important cytokine for the development of Th2 cells and the production of immunoglobulin E (IgE). IL-5 is vital to eosinophil migration and existence. IL-13 can trigger airway hyperresponsiveness and tissue remodeling, as well as IgE production (Finkelman et al., 2010; Lloyd and Hessel, 2010). Thymic stromal lymphopoietin (TSLP), a pro-inflammatory cytokine expressed mainly in epithelial cells, triggers allergic inflammation through the epithelial-dendritic cell interface (Tsicopoulos et al., 2013). TSLP can differentiate naive CD4⁺ T lymphocytes into Th2 cells and produce IL-4, IL-5, IL-13, while reducing the expression of Th1 related interferon- γ (IFN- γ) (West et al., 2012).

There is a large body of evidence that oxidative stress is an important mechanism resulting in allergic asthma (Murta et al., 2016). Asthma is associated with the production of high levels of reactive oxygen species (ROS), which leads to severe airway hyperresponsiveness (AHR), airway inflammation, depletion of glutathione (GSH), and increased lipid peroxidation (MDA) in vivo (Shang et al., 2017). Vitamin E, an antioxidant, is important for antioxidant defense against oxidative stress. Studies have found that vitamin E contributes to a lower incidence of asthma (Pearson et al., 2004). Vitamin E (30 mg/kg) can antagonize oxidative stress caused by DEHP in a mouse asthma model (You et al., 2016).

Studies have shown that IL-17A is associated with the more severe phenotypes of allergic asthma (Al-Ramli et al., 2009). IL-17A is a member of the IL-17 family and is secreted by the Th17 cells which are a subset of the CD4⁺ T cells (Iwakura et al., 2011). IL-1 β , an effective pro-inflammatory factor in allergic asthma, can trigger IL-17 cytokine expression (Krause et al., 2012; Wang et al., 2010b). Studies have proved that IL-1 β induced the activation of p38 mitogen-activated protein kinase (p38 MAPK) pathway that is thought to play a central role in the inflammatory response and tissue remodeling (Kim et al., 2012; Wuyts et al., 2003). p38 MAPK is reported to be essential in the production of IL-17 (Tang et al., 2016). SB203580, a specific inhibitor for p38 MAPK, has been proven to effectively reduce the expression of IL-17A (Coulthard et al., 2009).

In this study, we investigate the effect of DIDP exposure on allergic asthma and explore whether the asthma induced by DIDP is implicated in the Th2 and Th17 responses and in oxidative stress.

2. Materials and methods

2.1 Ethics statement

Animals used in this study were approved by the Office of Scientific Research Management of Central China Normal University, with the certification on the Application for the Use of Animals dated Oct.17, 2016 (approval ID: CCNU-IACUC-2016-003). Animals were reared in the

experimental animal center of Central China Normal University School of life sciences. Food and drinking water were available ad libitum.

2.2 Main reagents and kits

DIDP (>99%, Sigma, St. Louis, MO, USA) was dissolved in Tween 80 (analytical reagent, Shanghai, China) (DIDP:Tween-80 is 1:1 in w/w) together with a saline solution. Vit E ($\geq$ 99%, St. Louis, MO, USA) and OVA (>99%, St. Louis, MO, USA) were purchased from Sigma. SB203580 (>98%, Houston, TX, USA) and $\text{Al}(\text{OH})_3$ (>99%, Newburyport, MA, USA) were purchased from Abmole Bioscience and Strem Chemicals respectively. The ELISA kits for total-IgE, IL-4, IL-13, IFN- γ , IL-1 β and IL-17A were purchased from eBioscience (San Diego, CA, USA). The ELISA kit for TSLP was obtained from Bluegene (Shanghai, China). The GSH test kit was purchased from Nanjing Jiancheng (Nanjing, Jiangsu, China).

2.3 Animals

SPF (specific pathogen free) male BALB/c mice (5-6 weeks; 18-20g) were purchased from the Hubei experimental animal center (Wuhan, China). We tried to keep these mice away from allergens and maintained the temperature between 20-25 °C and humidity between 50-70%. The mice were kept in these conditions for 7 days prior to being used in the experiments.

2.4 Exposure and immunization

The mice were randomly divided into 11 groups of eight each: (1) saline group (saline); (2) 15 mg/kg/day DIDP group (DIDP 15); (3) ovalbumin sensitization group (OVA); (4) 0.15 mg/kg/day DIDP combined with OVA group (DIDP 0.15+OVA); (5) 1.5 mg/kg/day DIDP combined with OVA group (DIDP 1.5+OVA); (6) 15 mg/kg/day DIDP combined with OVA group (DIDP 15+OVA); (7) 150 mg/kg/day DIDP combined with OVA group (DIDP 150+OVA); (8) 15 mg/kg/day DIDP combined with OVA along with Vit E (30 mg/kg/day) group (DIDP 15+OVA+VE); (9) 15 mg/kg/day DIDP combined with OVA along with SB203580 (5 mg/kg) group (DIDP 15+OVA+SB203580); (10) OVA combined with Vit E group (OVA+VE); (11) OVA combined with SB203580 group (OVA+SB203580). The particular grouping and protocol for exposure and

sensitization is shown in Fig.1.

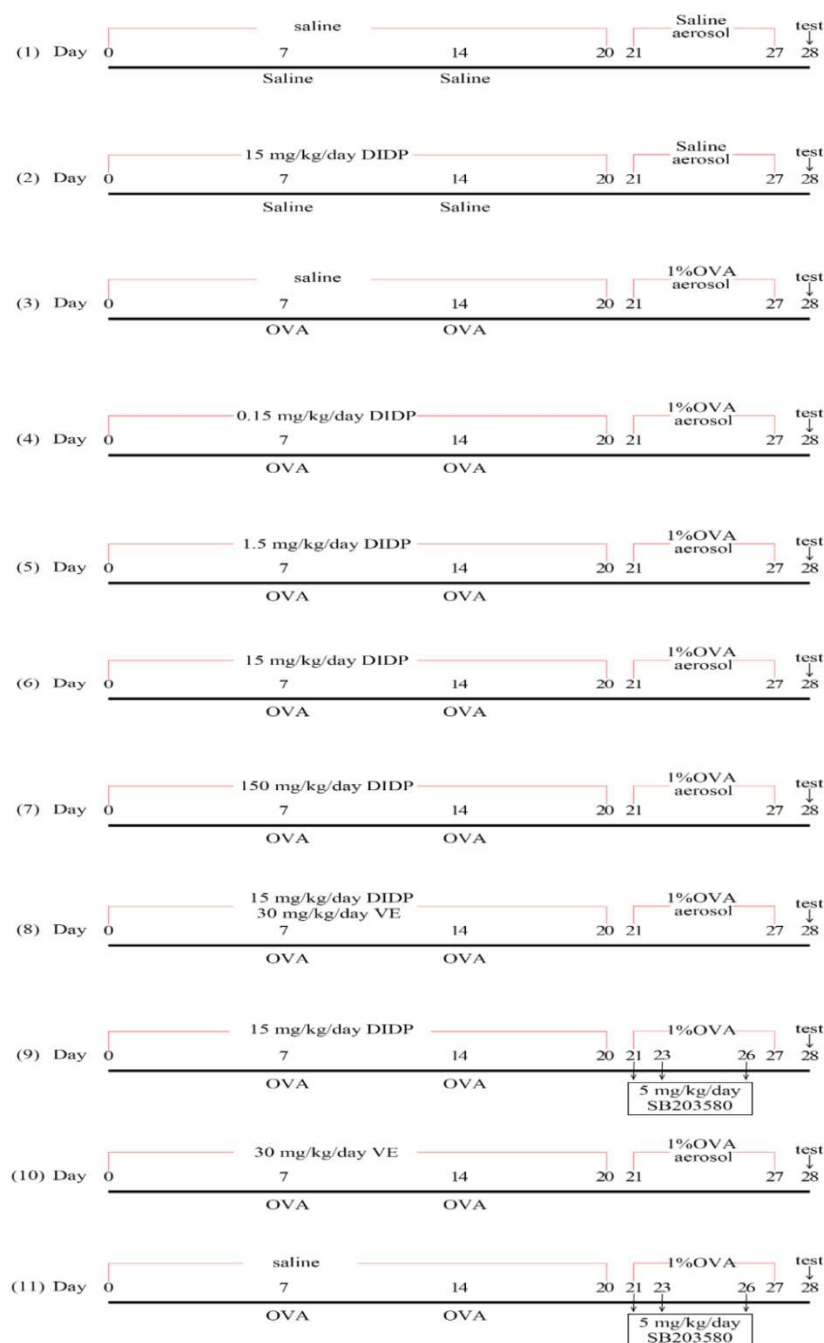


Fig.1. Experimental protocol.

The allergic asthma model was established by sensitization with OVA + Al(OH)₃ (50mg OVA and 1.75 mg Al(OH)₃ in 300ml 0.9% saline) on days 7 and 14 via subcutaneous injection. From day 21 to 27, the mice were subjected to an aerosol of 1 % OVA or saline (30 min/day) using an ultrasonic nebulizer (Yuyue 402A type I, China). DIDP and Vit E were given by intragastric gavage

from day 0 to 20. Blocker SB203580 was injected intraperitoneally 1 hour before OVA sensitization on days 21, 23 and 26.

2.5 Lung function measurement

An AniRes 2005 system (Bestlab, version 2.0, China) was used to measure airway hyperresponsiveness (AhR) 24 h after the final aerosol delivery. Lung function was assessed by increasing the concentration of methacholine (0.025, 0.05, 0.1 and 0.2 mg/kg), at intervals of at least 5 minutes for each concentration. The inspiratory resistance (Ri), expiratory resistance (Re) and lung dynamic compliance (Cldyn) were recorded to evaluate AhR. The Rarea of Ri and Re represent the area between the peak value and baseline during a 250s period, and the lowest value of Cldyn for latter analysis. To avoid the effect of methacholine (MHC) on the detection of biological indicators of the mice, we conducted the AhR tests in a separate set of experiments under the same conditions.

2.6 Collection of blood and bronchoalveolar lavage fluid (BALF)

Blood was collected by removing the eyeball. After centrifugation (845g, 25 °C, 10 min), the serum was obtained and stored at -80 °C for use in the total-IgE test. The BALF was obtained by cannulating the trachea and lavaging with 0.9 ml ice-cold saline twice using a 1 ml polyethylene injector. The final volume of qualified BALF samples was between 1.3 and 1.5 ml. After centrifugation (845g, 4 °C, 10 min), the BALF supernatant was collected and stored at -80 °C for analysis.

2.7 Histopathological analysis of lung tissue and preparing lung homogenate

The lung tissues were collected and washed with PBS buffer. The tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut into slices. These slices were then stained with hematoxylin and eosin (H&E). The other lung tissues were weighed and homogenized by 10 ml/g ice-cold PBS to obtain a concentration of 10%. After centrifugation (9,400 g, 4° C, 10 min), the supernatant of homogenate was collected and stored at -80 °C for later analysis.

2.8 Immunohistochemistry

After the paraffin section was dewaxed, rehydrated and antigens retrieved, an endogenous peroxidase inhibitor was added to block endogenous peroxidase activity, and serum was added to block nonspecific background activity. After incubation in primary antibodies, Anti-p38 (phospho Y182) (Abcam, MA, USA), the sections were incubated in both appropriate biotinylated immunoglobulins, as well as in an avidin-biotin peroxidase complex. After color development, the images were recorded using a microscope. The average optical density (AOD) of phospho-p38 positive expression was determined using an Image Pro Plus 6.0 (Media Cybernetics, USA). Three views (200×) of each slice were randomly selected, and the intensity of positive expression was calculated as the average optical density of the slice.

2.9 Measurement of the levels of serum total-IgE, TSLP and cytokines

Serum total-IgE, TSLP, IL-4, IL-13, IFN- γ , IL-1 β and IL-17A were measured using ELISA kits according to the manufacturer's instructions. Sensitivities of ELISA kits were 4 ng/ml for total-IgE, 1 pg/ml for TSLP, 4 pg/ml for IL-4, 4 pg/ml for IL-13, 15 pg/ml for IFN- γ , 8 pg/ml for IL-1 β , 4 pg/ml for IL-17A.

2.10 Determining reactive oxygen species (ROS), glutathione (GSH), Malondialdehyde (MDA)

ROS was detected using DCFH-DA. The lung tissue homogenate of the mice was diluted 100-fold in PBS (pH =7.5). 100 μ L of this diluted solution was added to 96-well plates, and 100 μ L of 0.2 μ mol/L DCFH-DA was added to the well plates. The reaction mixture was protected from light for 10 min at 37 °C. Under an excitation wavelength of 480 nm and an emission wavelength of 520 nm, the fluorescence intensity was measured by the fluorescence reader (FLx 800; BioTek Instruments, Winooski, VT, USA).

GSH content was detected by assay kit (Nanjing Jiancheng, China) according to the manufacturer's instructions.

To assay MDA, 100 μ L of tissue homogenate and 0.4 ml mass fraction of 0.6% TBA (0.6 g solid TBA dissolved in 100 ml 10 % TCA) were collected in a 1.5 ml EP tube, kept in a bath of

boiling water for 15 min, cooled using cold water, and centrifuged at 9,391g for 10 min. The content of MDA was calculated by detecting the absorbance at 450 nm, 532 nm and 600 nm.

2.11 Statistical analysis

All data are presented as mean $\pm$ SD. All analyses were carried out using SPSS 17.0, while statistical graphs were generated using GraphPad Prism 5.0 software. Differences between groups were assessed by one-way ANOVA analysis. A $p < 0.05$ or $p < 0.01$ was considered a significant or extremely significant difference respectively between groups.

3. Results

3.1 Exposure to DIDP aggravated airway remodeling and AhR induced by OVA

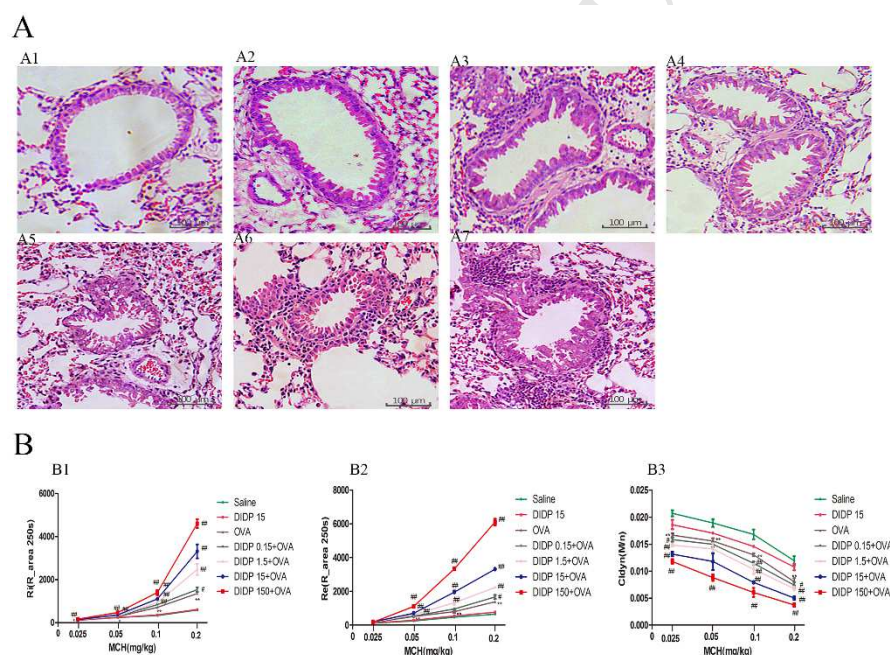


Fig.2.Exposure to DIDP aggravated airway remodeling and AhR induced by OVA. (A) Histological analysis of lung tissue by hematoxylin & eosin staining. Pictures taken at 20 x .H&E staining: showing the changes in the bronchial lumen. (A1) saline group. (A2) DIDP 15. (A3) OVA. (A4) DIDP 0.15+OVA. (A5) DIDP 1.5+OVA. (A6) DIDP 15+OVA. (A7) DIDP 150+OVA. (B) Airway hyperresponsiveness. (B1) inspiratory resistance (Ri). (B2) expiratory resistance (Re). (B3) lung dynamic compliance (Cldyn). Group data were expressed as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$, significant difference compared with the saline group; # $p < 0.05$ and ## $p < 0.01$, significant difference compared with the OVA group. (n=3)

To investigate the histopathological changes resulting from exposure to DIDP, the lung tissues were stained with H&E. As shown in Fig.2, for the saline group (Fig.2A1), the bronchial wall is normal, the alveolar structure has integrity, and there were no inflammatory cells infiltrating the bronchial wall. In contrast, the bronchial wall of the DIDP 15 group (Fig.2A2) was slightly thicker, while the bronchial wall of the OVA group (Fig.2A3) was significantly thickened, with increased mucosal wrinkling, bronchial stenosis, and infiltration of inflammatory cells into the alveolar wall and surrounding areas. Importantly, smooth muscle thickening and lumen stenosis were more obvious in the DIDP groups (0.15, 1.5, 15, 150) +OVA (Fig.2A4, 5, 6, 7), as compared with the OVA group. With increasing DIDP exposure concentrations, airway remodeling was aggravated and the alveolar wall became thinner or ruptured causing emphysema and blisters.

To determine the impact of exposure to DIDP on AhR, we measured Ri (Fig.2B1), Re (Fig.2B2) and Cldyn (Fig.2B3) in each group for the different concentrations of methacholine. As the methacholine concentration increased, Ri and Re (Fig.2B1 and B2) increased gradually, while the Cldyn (Fig.2B3) decreased gradually. Compared with the saline group, the OVA- treated groups demonstrated a significant increase in Ri, Re and a decrease in Cldyn under the same concentration of methacholine. Notably, in the presence of OVA sensitization, Ri and Re (Fig.2B1 and B2) in groups exposed to DIDP showed a marked increase, compared with OVA group, and the Cldyn (Fig.2B3) demonstrated a significant decrease. As the DIDP concentration increased, an upward trend in Ri, Re and a decrease in Cldyn were very obvious. When the concentration of methacholine was 0.2 mg/kg, Ri and Re (Fig.2B1 and B2) in the DIDP (0.15, 1.5, 15, 150) +OVA groups increased markedly ($p < 0.01$), while the Cldyn (Fig.2B3) for these groups decreased significantly ($p < 0.01$).

These results suggest that exposure to DIDP aggravated airway remodeling and AhR in the presence of OVA sensitization.

3.2 Exposure to DIDP exacerbating Th2 and Th17 immune response

To further explore the role of exposure to DIDP on allergic asthma, the levels of total-IgE, TSLP, and cytokines were determined. The results of ELISA tests (Fig.3) showed that the expression of total-IgE and TSLP (Fig.3A and B) in the DIDP 15 group had no significant difference from the saline group. Comparing the OVA group with the saline group, sensitization with OVA induced a significant increase in the total-IgE and TSLP ($p < 0.01$). Interestingly, in the presence of OVA, exposure to DIDP elevated the levels of total-IgE and TSLP. And with the increase of DIDP concentration, the levels of total-IgE and TSLP showed an obvious upward trend. Compared with the OVA group, the DIDP (1.5, 15, 150)+OVA groups showed a significant increase in the total-IgE and TSLP expression ($P < 0.01$).

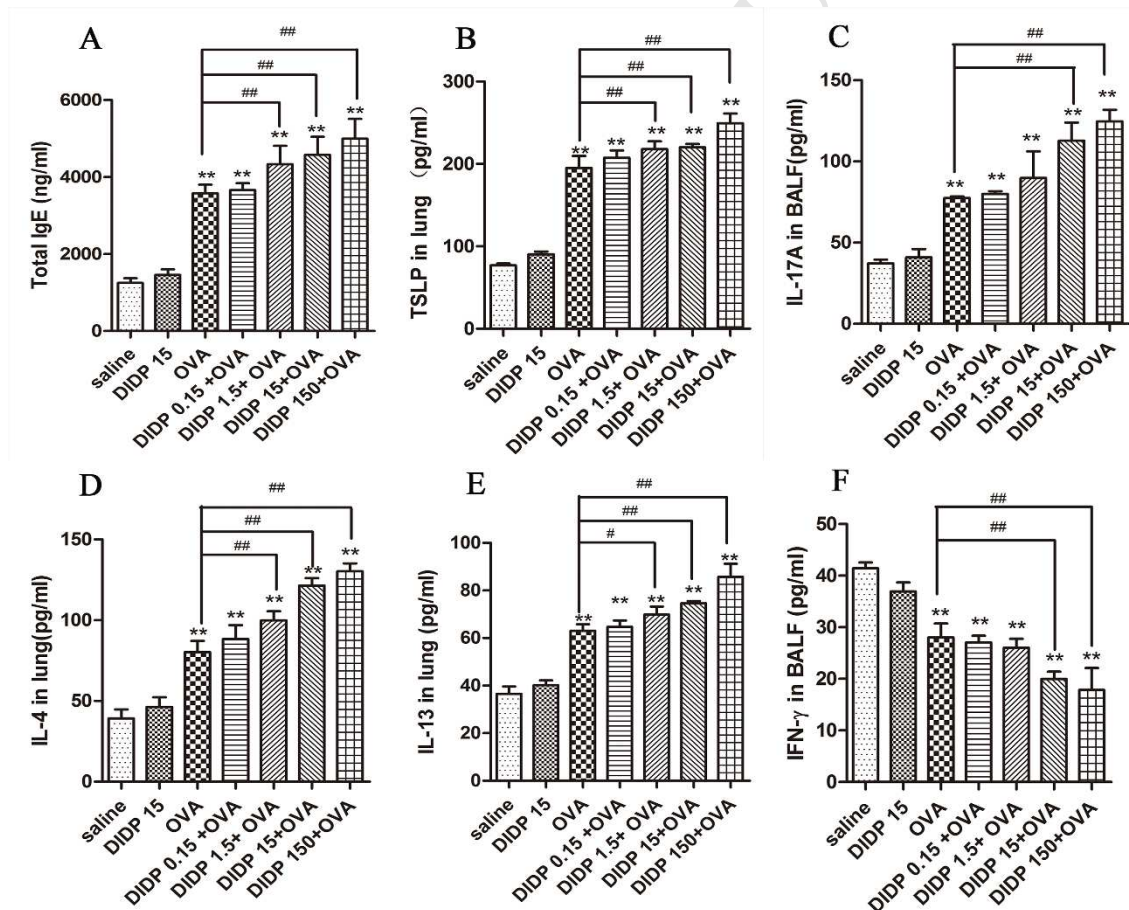


Fig.3.DIDP exacerbating the Th2 and Th17 immune response. (A) The changes in total-IgE in serum. (B) The expression level of TSLP in the lung. (C) The level of IL-17A in BALF. (D) The expression level of IL-4 in the lung. (E) The expression level of IL-13 in the lung. (F) The expression level of IFN- γ in BALF. $n=7$, Group data were expressed as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$, significant difference compared with the saline group; #

$p < 0.05$ and $## p < 0.01$, significant difference compared with the OVA group.

As shown in Fig.3, except for the DIDP 15 group, IL-4, IL-13 and IFN- γ (Fig.5D, E and F) expression was significantly different from the saline group ($P < 0.01$). Interestingly, exposure to DIDP induced a sharp increase in the levels of the Th2 cytokines, IL-4 and IL-13, and a marked decrease in the levels of the Th1 cytokine, IFN- γ . Compared with the OVA group, the IL-4 and IL-13 in the DIDP (1.5, 15, 150)+OVA groups showed a significant or very significant increase ($p < 0.05$ or 0.01), while the IFN- γ in the DIDP (15, 150)+OVA group was very significantly lower than that in the OVA group ($p < 0.01$). With increasing DIDP exposure, these changes in the levels of Th1 and Th2 cytokines were more obvious.

Similarly, the expression of IL-17A in the group exposed to DIDP alone showed no significant difference from the saline group (Fig.3C), while a marked increase was seen in the OVA group ($p < 0.01$). Importantly, exposure to DIDP induced a sharp increase in the levels of IL-17A in the presence of sensitization with OVA. Compared with the OVA group, the levels of IL-17A in the DIDP 15+OVA group and the DIDP 150+OVA group showed very significant increases ($P < 0.01$).

These results indicate that exposure to DIDP not only exacerbated a predominant Th2-type response but also Th17-type immunity.

3.3 DIDP aggravated oxidative stress

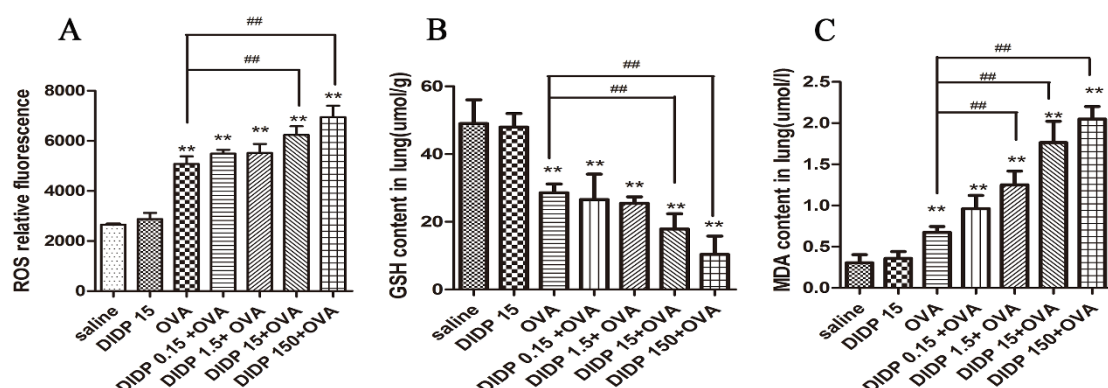


Fig.4. The impact of exposure to DIDP on oxidative stress (A) ROS in lung tissue. (B) GSH concentrations in lung tissue. (C) MDA concentrations in lung tissue. Group data were expressed as mean $\pm$ SD. * $p < 0.05$ and

** $p < 0.01$, significant difference compared with the saline group; # $p < 0.05$ and ## $p < 0.01$, significant difference compared with the OVA group. (n=5).

The impact of exposure to DIDP on oxidative stress was determined by investigating the changes in ROS, GSH and MDA levels (Fig.4). Exposure to 15 mg/kg/day DIDP alone did not induce significant changes in the levels of ROS, GSH and MDA compared to the saline group (Fig.4A,B and C), while sensitization with OVA elevated the levels of ROS and MDA, and induced a decrease in GSH ($p < 0.01$), compared with the saline group. In the presence of OVA, exposure to DIDP aggravated oxidative stress, which was shown by comparing the levels of ROS, GSH and MDA between those groups exposed to different doses of DIDP in the OVA-treatment groups.

3.4 Blocking oxidative stress alleviated asthmatic lesions

To assess the role of oxidative stress induced by DIDP on the development of allergic asthma, mice were treated with antioxidant vitamin E to block oxidative stress. It was found that treatment with vitamin E effectively inhibited the oxidative stress, as seen when comparing the levels of ROS, GSH and MDA between the DIDP 15+OVA group and its vitamin E treatment group (Fig.5A, B,C).

As shown in Fig.5D, treatment with vitamin E alleviated mucosal wrinkling, prevented airway remodeling and induced less inflammatory-cell infiltration. This is seen when comparing the DIDP 15+OVA+VE group with the DIDP 15+OVA group, and the OVA+VE group with the OVA group.

Treatment with vitamin E prevented the DIDP exacerbation of AhR, which is suggested by comparing the changes in Re, Ri and Cldyn in the DIDP 15+OVA group compared with its vitamin E treatment group (Fig.5E).

To further verify the role of oxidative stress on allergic asthma, the production of total-IgE and TSLP were determined after treatment with vitamin E to block oxidative stress (Fig.5F and 5G). It was shown that the production of total-IgE and TSLP in the DIDP 15+OVA+VE group decreased sharply, compared with DIDP15+OVA ($p < 0.01$).

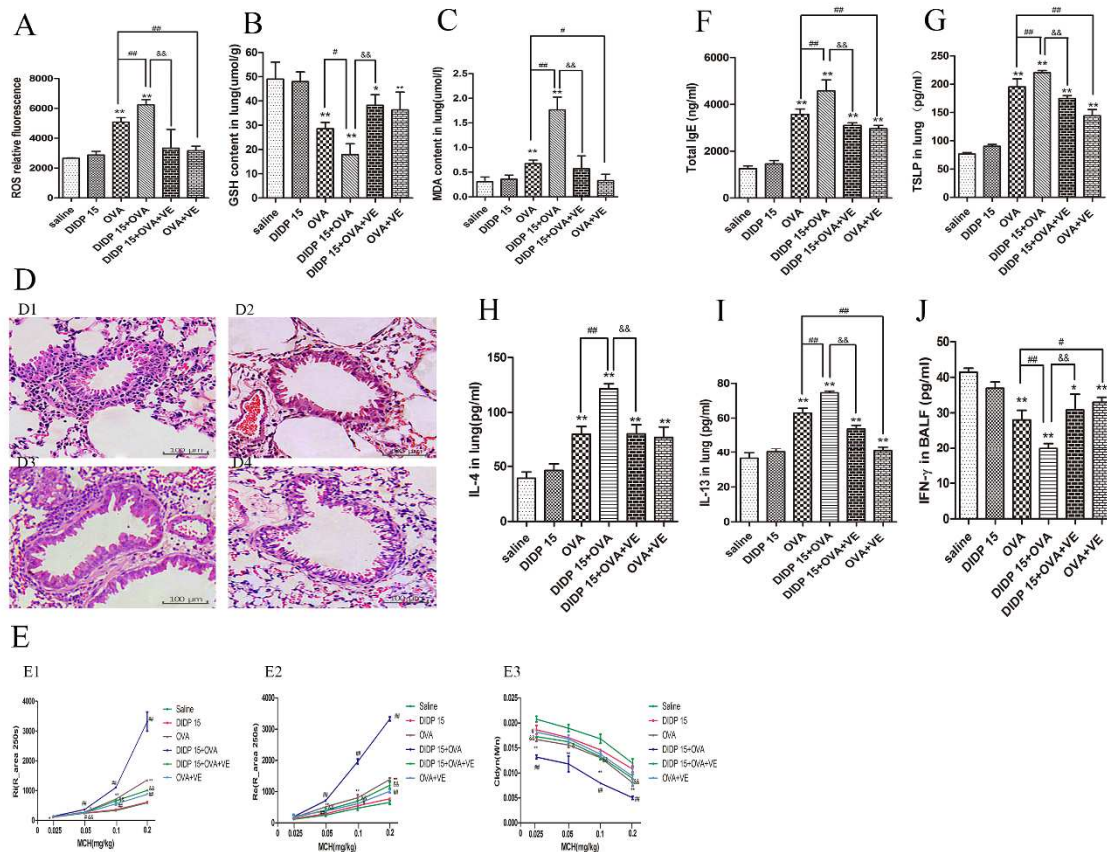


Fig.5. Blocking oxidative stress alleviated asthmatic lesions. (A) ROS in lung tissue. (B) GSH concentrations in lung tissue. (C) MDA concentrations in lung tissue. (D) H&E staining showing the changes in the bronchial lumen. (D1) DIDP 15 +OVA. (D2) DIDP 15+OVA+VE. (D3) OVA. (D4) OVA+VE. (E) The impact of blocking oxidative stress on Airway hyperresponsiveness. (E1) inspiratory resistance (Ri). (E2) expiratory resistance (Re). (E3) lung dynamic compliance (Cldyn). (F) The level of total-IgE in serum. (n=7) (G) The level of TSLP in lung. (n=5) (H) The level of IL-4 in lung. (I) The level of IL-13 in lung. (J) The level of IFN- γ in BALF. Group data were expressed as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$, significant difference compared with the saline group; # $p < 0.05$ and ## $p < 0.01$, significant difference compared with the OVA group; &#p $p < 0.05$ and &#p $p < 0.01$, significant difference compared with the DIDP 15 +OVA group.

After treatment with vitamin E, the expression of Th1 and Th2 cytokines were investigated (Fig.5H, I, J). We saw that blocking oxidative stress by the administration of vitamin E prevented the increase of IL-4 and IL-13 induced by DIDP exposure, when comparing the DIDP 15+OVA+VE group with the DIDP 15+OVA ($p < 0.01$). This indicated that the predominant Th2 response was

inhibited by blocking oxidative stress.

3.5 Inhibiting MAPK alleviated Th17 type asthmatic lesions

To clarify the underlying mechanism of DIDP exposure on Th17 type asthmatic lesions, SB203580 was used to block the p38 MAPK signaling pathway. Immunohistochemistry for phospho-p38 in the lung tissue showed that sensitization with OVA induced an increase in phospho-p38 expression, which distributed along the tracheal mucosa or tracheal periphery, when comparing the saline group to the OVA group (Fig. 6A, B). Compared with the OVA group, exposure to 15mg/kg·d DIDP markedly exacerbated the levels of phospho-p38, while administering SB203580 effectively decreased the expression of phospho-p38 ($p<0.01$). Importantly, Phospho-p38 MAPK in the DIDP 15+OVA+SB203580 group was effectively inhibited, which was shown by comparing with the DIDP 15+OVA group ($p<0.01$).

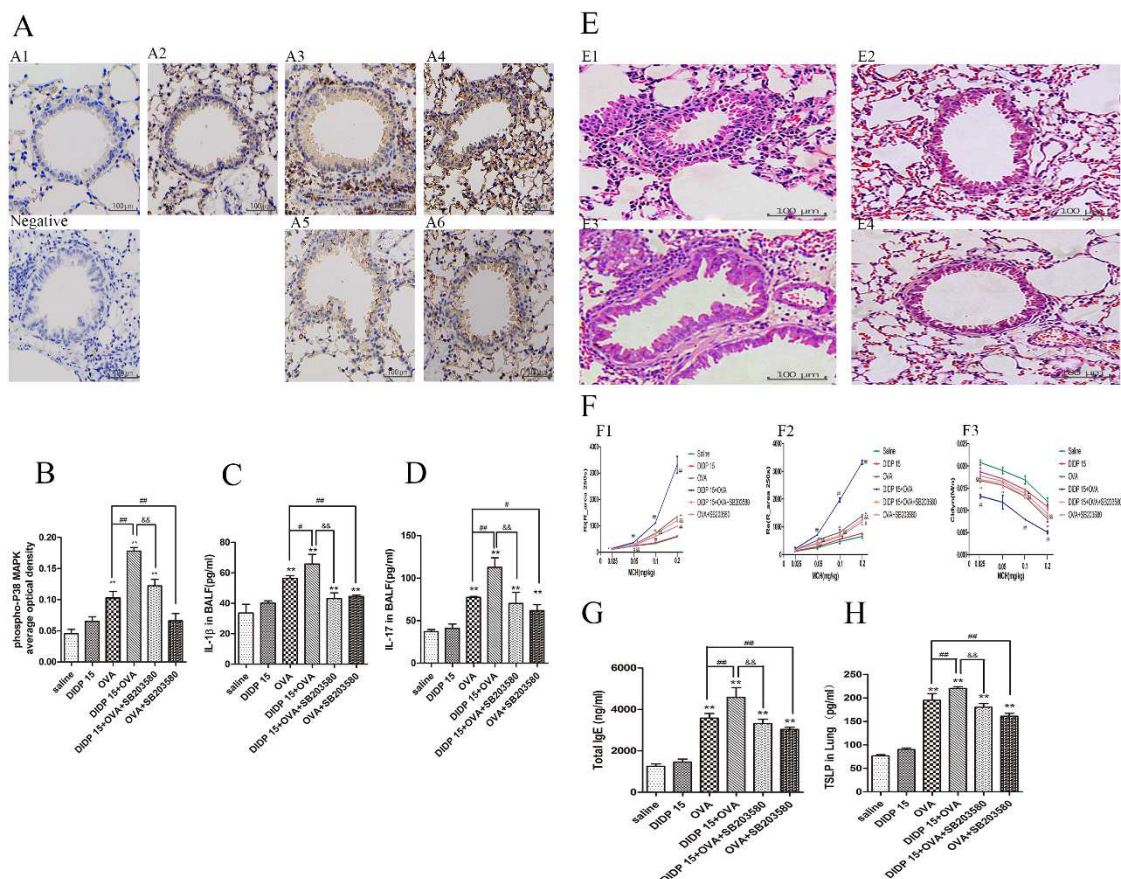


Fig.6. Inhibiting MAPK alleviated Th17 type asthmatic lesions (A) Immunohistochemistry for phospho-p38 in the lung tissue. A1-A6 represent different exposure groups (saline group, DIDP 15, OVA, DIDP 15+OVA, OVA+SB203580, DIDP 15+OVA+SB203580,). (B) The expression scores of phospho-p38. (C) Expression of

IL-1 β in BALF. Expression of IL-17A in BALF. (E) H&E staining showing the changes after administering SB203580 in the lung. (E1) DIDP 15 + OVA. (E2) DIDP 15 + OVA+SB203580.(E3)OVA.(E4) OVA+SB203580. (F) The impact of administering SB203580 on airway hyperresponsiveness. (F1) Inspiratory resistance (Ri). (F2) Expiratory resistance (Re). (F3) Lung dynamic compliance (Cldyn). (G)The expression level of total IgE in serum. (H)The expression level of TSLP in the lung. Group data were expressed as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$, significant difference compared with the saline group; # $p < 0.05$ and ## $p < 0.01$, significant difference compared with the OVA group; & $p < 0.05$ and && $p < 0.01$, significant difference compared with the DIDP 15+OVA group.

After administering SB203580, the histopathological changes were examined by H&E staining of the lung tissue (Fig.6E). Treatment with SB203580 effectively inhibited the airway remodeling induced by DIDP exposure. It was shown that the tracheal mucosal folds became more regular and thinner, there was less infiltration of inflammatory cells and the alveolar structure was more complete in the DIDP 15+OVA+SB203580 group (Fig.6E2), compared with the DIDP 15+OVA group (Fig.6E1).

Analyzing the changes in Re, Ri and Cldyn for each group after administering SB203580, suggested that the AhR induced by DIDP exposure was effectively inhibited by blocking the p38 MAPK signaling pathway (Fig.6F). It was shown that the Re and Ri (Fig.6F1, F2) in the groups treated with SB203580 decreased sharply ($p < 0.05$ or 0.01), while Cldyn (Fig.6F3) increased markedly, when comparing the OVA+SB203580 group with the OVA group, and the DIDP 15+OVA+SB203580 group with the DIDP 15+OVA group.

The ELISA test for total-IgE and TSLP showed that total-IgE and TSLP (Fig.6G and H) in the SB203580-treatment groups decreased significantly ($p < 0.01$), when comparing the OVA+SB203580 group with the OVA group, and the DIDP 15+OVA+SB203580 group with the DIDP 15+OVA group.

It was shown that the levels of IL-1 β and IL-17A were induced to increase by exposure to 15 mg/kg·d DIDP and OVA sensitization (Fig.6C, D). However, the elevation in the levels of IL-1 β and IL-17A were inhibited when SB203580 was administered. These results suggest that blocking

the p38 MAPK signaling pathway could effectively inhibit Th17 type asthmatic lesions induced by DIDP exposure and OVA sensitization.

4. Discussion

Phthalates are classified as Endocrine Disrupting Chemicals (EDCs) impairing fertility problems, respiratory diseases, childhood obesity and neuropsychological disorders. As a high-molecular-weight phthalate, DIDP is widely used as an alternative to DEHP. Although DIDP showed less reproduction or development toxicity than DEHP, present study suggests that DIDP is associated with the occurrence of asthma (Bertelsen et al., 2013) and high systolic blood pressure in children and adolescents, which similar to the HMW phthalates DiNP and DEHP (Katsikantami et al., 2016). Phthalate levels in indoor air were shown to be related to particle size fraction (Rakkestad et al., 2007). Particle size fraction seem to play a different role on asthma control and severity (Veremchuk et al., 2018). The acceptable daily intake (ADI) of DIDP was considered to be 150 µg/kg/day, a figure proposed by the United States Consumer Products Safety Commission in 2010 (Kransler et al., 2013). Our results have shown that DIDP exposure aggravated allergic asthma in the presence of OVA, and that exposure to 15 mg/kg/day markedly exacerbated airway remodeling and promoted the occurrence of AhR.

The main features of airway remodeling include epithelial cell detachment, an increase in mucus secretion, sub-epithelial fibrosis and smooth muscle cell proliferation (Sumi and Hamid, 2007). In this study, exposure to DIDP exacerbated airway remodeling in asthmatic model mice. As the exposure dose of DIDP increased, the airway remodeling was more severe. Consistent with the histological analysis, DIDP exposure also resulted in an increase of AhR induced by OVA sensitization. The occurrence of AhR is closely related to airway remodeling. Bronchial wall thickening increased leading to airway collapse and airway stenosis, thereby promoting AhR (Elias et al., 1999).

Several studies in the past have shown that airway remodeling can be explained as being the

consequence of Th2 inflammation and reactive oxygen species (ROS) (Elias et al., 1999; Sumi and Hamid, 2007). In this study, exposure to DIDP aggravated the level of oxidative stress induced by the OVA sensitization and challenge as shown by the changes in the levels of ROS, GSH and MDA, and exacerbated the levels of Th2 cytokines, IL-4 and IL-13. Exposure to DIDP may cause damage to epithelial cells, resulting in increased ROS levels, triggering airway inflammation. IL-4 may cause the generation of extensive mucus metaplasia (Sumi and Hamid, 2007). IL-13 can not only increase the secretion of mucus, but can also promote sub-epithelial fibrosis, and may even promote the proliferation of smooth muscle airway cells.

Allergic asthma is characterized by the elevation of IgE and Thymic stromal lymphopoietin (TSLP). In this study, DIDP exposure, together with OVA sensitization resulted in an increase in TSLP, IgE and Th2 cytokine expression (IL-4 and IL-13), and a concomitant decrease in the Th1 cytokine (INF- γ). It was suggested that mechanical damage and the expression of inflammatory cytokines (TNF- α , IL-1 α , IL-4 and IL-5) acted on bronchial epithelial cells which secrete TSLP (Redhu and Gounni, 2012). It has been reported that TSLP plays an important role in the process of asthma induced by DEHP (You et al., 2016), which strengthens the immune response of Th2 cells and induces B lymphocytes to produce IgE, and induce the activation of dendritic cells (DCs) (Suarez et al., 2008). In the indirect effect of DCs, the naive T cells differentiate into Th2 cells, which lead to a Th2 cytokine (IL-4 and IL-13) increase and inhibited expression of IFN- γ . The increased IL-4 and IL-13 can induce B lymphocyte synthesis of IgE (Gould and Sutton, 2008).

Oxidative stress plays a key role in asthma. It has been shown to cause many pathophysiological changes associated with asthma, such as increased lipid peroxidation, decreased glutathione, increased airway reactivity and secretion, and increased vascular permeability (Nadeem et al., 2003). Excessive ROS can induce lipid peroxidation and results in oxidative stress (Hamza and El-Shenawy, 2017). DIDP has been shown to aggravate allergic dermatitis through promoting the accumulation of ROS (Shen et al., 2017). In this experiment, the levels of ROS and MDA in asthmatic mice were seen to increase, while the level of GSH decreased. These changes were more

significant with the increase in the DIDP exposure dose. DIDP exposure may cause large cells such as epithelial cells, to produce large amounts of ROS. Accumulated ROS oxidizes the lipid composition within the cell, causing the MDA to rise while consuming glutathione (GSH) and recruiting inflammatory cells. ROS may act as a signal to promote the expression of inflammatory factors such as IL-4, IL-13, IL-1 β and the activation of NF- κ B signal pathways (Kang et al., 2016; Shen et al., 2017). The activation of NF- κ B can enhance the level of TSLP (Lee and Ziegler, 2007). Inflammatory cells also induce the accumulation of ROS and development of allergic asthma (Qu et al., 2017).

Vitamin E (Jaiswal et al., 2014), Vitamin C (Jaiswal et al., 2017), Curcumin (Jaiswal et al., 2016) and Resveratrol (Hamza and El-Shenawy, 2017) have been reported to be potent ameliorative antioxidants against oxidative stress. In our experiments, we saw that the level of oxidative stress was effectively blocked after treatment with antioxidant Vitamin E, and the airway remodeling and AhR induced by DIDP exposure and OVA sensitization were inhibited. This inhibition of oxidative stress also prevented the elevation of Th2 cytokines and IgE exacerbated by DIDP exposure in asthmatic mice. Clinical studies have also found that moderate amounts of vitamin E can help to control asthma. The effectiveness of vitamin E as an antioxidant is associated with its molecular structure of phenolic hydroxyl groups. Phenolic hydroxyl groups can provide hydrogen atoms to scavenge free radicals (Lu et al., 2011), reduce ROS levels in asthmatic mice, inhibit the inflammatory response, and relieve airway remodeling. In addition, vitamin E can activate the body's antioxidant system, for example vitamin E intake can inhibit Nrf2 down-regulated expression (Dworski et al., 2011). Recent studies have found that synthetic vitamin E metabolites can inhibit the release of LPS-induced pro-inflammatory mediators and nitric oxide at the transcriptional level, such as blocking nitric oxide synthase, epoxide Synthase-2 and IL-1 β (Galli et al., 2017).

This study suggested that exposure to DIDP not only promoted a predominant Th2 response, but also induced Th17-type immunity. Studies have found that Th17 participated in airway

remodeling, the key to the effect seemed dependent on IL-17 secreted by Th17 cells (Zhao et al., 2013). Th17/IL-17 has been proven to not only increase the secretion of mucus but also promote the proliferation of airway smooth muscle fibrosis, or even adjust smooth muscle function (Halwani et al., 2010; Wang et al., 2010a).

In this experiment, exposure to DIDP promoted the elevation of the expression of IL-1 β , phospho-p38 MAPK and IL-17A. It was reported that when exposed to diesel exhaust particulates, the level of IL-17 in childhood asthma patients significantly increased (Brandt et al., 2013). Reports have suggested that IL-1 β is important for promoting Th17 cell differentiation and proliferation (Newcomb and Peebles, 2013). It has been shown that in the presence of IL-1 β , naive T cells can be induced to differentiate into Th17 cells. Further studies found that when IL-1 β was present, IL-17A was abundantly expressed, because Th17 cells expressed higher levels of IL-1R (Liu et al., 2017). This indicated that the expression of IL-17A is closely associated with IL-1 β . Studies have proved that IL-1 β can activate the p38 MAPK pathway, meanwhile p38 MAPK pathways activate stable mRNA of IL-1 β , increasing its transcription (Khorasanizadeh et al., 2017; Wuyts et al., 2003). Under the influence of ATP, p38 MAPK was activated to produce phospho-p38 MAPK (Coulthard et al., 2009). The activation of p38 MAPK was reported to promote the production of IL-17 (Tang et al., 2016). SB203580 is analogous to ATP and competes for the ATP binding site, which induces the phosphorylation of p38 (Coulthard et al., 2009). In this study, treatment with SB203580 effectively inhibited the activation of p38 MAPK, and markedly prevented the elevation of IL-1 β and IL-17A induced by DIDP exposure and OVA sensitization. The results of this study confirmed that the expression of IL-17A was induced by the p38 MAPK pathway. Importantly, treatment with SB203580 relieved airway remodeling and AhR, suggesting that SB203580 may function as a drug and p38 MAPK / IL-17 may act as a target to control asthmatic lesions. IL-17A participated in airway remodeling and IL-1 β can promote the expression of TSLP (Comeau and Ziegler, 2010) and induce the production of IgE. Blocking the p38 MAPK pathway inhibited the expression of IL-17A and IL-1 β . This may be a way to inhibit the production of TSLP and total-IgE.

In conclusion, this study demonstrated that DIDP exposure aggravated allergic asthma in the presence of OVA. It was suggested that exposure to DIDP exacerbated the Th2 and Th17 response through aggravating oxidative stress and the activation of the p38 MAPK pathway. These findings provide a scientific basis for the clinical treatment of allergic asthma. It expands understanding in the adverse effect and underlying mechanisms about DIDP exposure impact the development of allergic asthma.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2017YFC0702700), the Fundamental Research Funds for the Central Universities (CCNU17ZDJC05).

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Highlights

- Exposure to DIDP aggravated Th2 and Th17-mediated allergic asthma.
- DIDP exacerbates the Th2 response through aggravating oxidative stress.
- DIDP induced Th17-type immunity by activation of p38 MAPK pathway.