

Effects of maternal exposure to di(2-ethylhexyl)phthalate (DEHP) during pregnancy on susceptibility to neonatal asthma

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ARTICLE INFO

Article history:

Received 1 October 2013

Revised 9 December 2013

Accepted 12 December 2013

Available online 24 December 2013

Keywords:

Di(2-ethylhexyl)phthalate (DEHP)

Maternal exposure

Pregnancy

Offspring

Asthma

ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) is used as a plasticizer and is widely dispersed in the environment. In this study, we investigated the effects of maternal exposure to DEHP during pregnancy on neonatal asthma susceptibility using a murine model of asthma induced by ovalbumin (OVA). Pregnant BALB/c mice received DEHP from gestation day 13 to lactation day 21. Their offspring were sensitized on postnatal days (PNDs) 9 and 15 by intraperitoneal injection of 0.5 µg OVA with 200 µg aluminum hydroxide. On PNDs 22, 23 and 24, live pups received an airway challenge of OVA for 30 min. Offspring from pregnant mice that received DEHP showed reductions in inflammatory cell count, interleukin (IL)-4, IL-13, and eotaxin in their bronchoalveolar lavage fluid and in total immunoglobulin E and OVA-specific IgE in their plasma compared with offspring from pregnant mice that did not receive DEHP treatment. These results were consistent with histological analysis and immunoblotting. Maternal exposure to DEHP reduces airway inflammation and mucus production in offspring, with a decrease in inducible nitric oxide synthase (iNOS) in the lung tissue. This study suggests that maternal exposure to DEHP during pregnancy reduces asthmatic responses induced by OVA challenge in offspring. These effects were considered to be closely related to the suppression of Th2 immune responses and iNOS expression.

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Introduction

Asthma can have variable symptoms, including reversible airway obstruction, wheezing, coughing, tightness of the chest, and breathlessness (Holgate, 2008; Roh et al., 2008; Selgrade et al., 2008). In particular, the prevalence of allergic asthma in children has increased markedly in recent decades and has become an important problem (Liu et al., 2002). Asthma is triggered in the response to allergens, such as pollens, air pollutants, and house dust mites. In addition, maternal exposures to materials such as tobacco smoke, diesel exhaust, industrial chemicals, and ozone during pregnancy have been linked in epidemiological studies to asthma and other respiratory diseases in children (Hertz-Picciotto et al., 2008; Margolis et al., 2009; Wigle et al., 2008). In in vivo experiments, maternal exposure to organic compounds, tobacco smoke, and diesel exhaust during pregnancy has been found to cause increased neonatal asthma susceptibility (Fedulov et al., 2008; Midoro-Horiuti et al., 2010; Rehan et al., 2012).

Di(2-ethylhexyl) phthalate (DEHP) is used as a plasticizer in a wide range of consumer products, including vinyl floors, food wrapping, cosmetics, and toys. Currently, DEHP is the most commonly used

phthalate, accounting for approximately 50% of the market for PVC plasticizers in the European Union (Andrade et al., 2006). DEHP is widely dispersed in the environment. Thus, many people are exposed to DEHP in considerable doses via various routes. It is well known that a major route of exposure is the ingestion of contaminated food, other materials and water (Christiansen et al., 2010). In particular, children are considered to be more susceptible because they are more likely to be exposed to DEHP. According to Guo et al. (2012), children's exposure to DEHP through sucking or chewing on toys or other materials can reach 85 µg/kg/day. Because DEHP is able to cross the placenta and pass into breast milk, exposure to DEHP during gestation and lactation has been of particular concern (Andrade et al., 2006; Shae, 2003). Various epidemiological studies suggest a link between exposure to DEHP and the risk of asthma development. One study showed that DEHP administration led to a significant increase in the production of immunoglobulin (Ig) G1 and IgE (Dearman et al., 2008). A similar study found that DEHP might promote an aggravated asthmatic response involving increased immunological and inflammatory mediators, and pathophysiological changes of lung tissue (Larsen et al., 2007).

In the current study, we investigated the effects of maternal exposure to DEHP during the pregnancy on the development of neonatal asthma using a murine model of asthma induced by ovalbumin (OVA). We measured the levels of Th2 cytokines, chemokine, IgE, proinflammatory protein, and detected histopathological changes in offspring.

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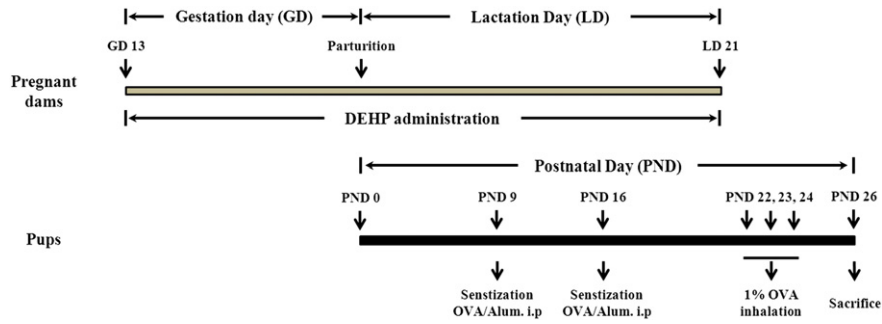


Fig. 1. Experimental procedure.

Materials and methods

Animal and DEHP treatment. Pregnant BALB/c mice (gestational day (GD) 13) were purchased from Central Lab. Animal (Seoul, Korea). The mice were kept in a room maintained at $23 \pm 3^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$ under a controlled 12 h light/dark cycle. The animals were housed in a polycarbonate cage during the experimental period. The pregnant dams were allowed standard rodent chew containing 2500 ppm of DEHP (Sigma, St. Louis, MO, USA) from GD 13 to weaning. The morning on which parturition was complete was designated as postnatal day (PND) 0. All pups were examined as soon as possible on the day of birth to determine the number of live pups

in each litter. To minimize the influence of the number of live pups, the size of each litter was adjusted on PND 2 by eliminating extra pups to give three males and three females per litter whenever possible. Live pups were divided into eight groups: (1) the “CPM” group, comprising males that received only a PBS challenge; (2) the “DEPM” group, males that received maternal exposure to DEHP and a PBS challenge; (3) the “COM” group, males that received only OVA challenge; (4) the “DEOM” group, males that received maternal exposure to DEHP and an OVA challenge; (5) the “CPF” group, females that received only a PBS challenge; (6) the “DEPF” group, females that received maternal exposure to DEHP and a PBS challenge; (7) the “COF” group, females that received only an OVA challenge; and (8) the “DEOF” group, females

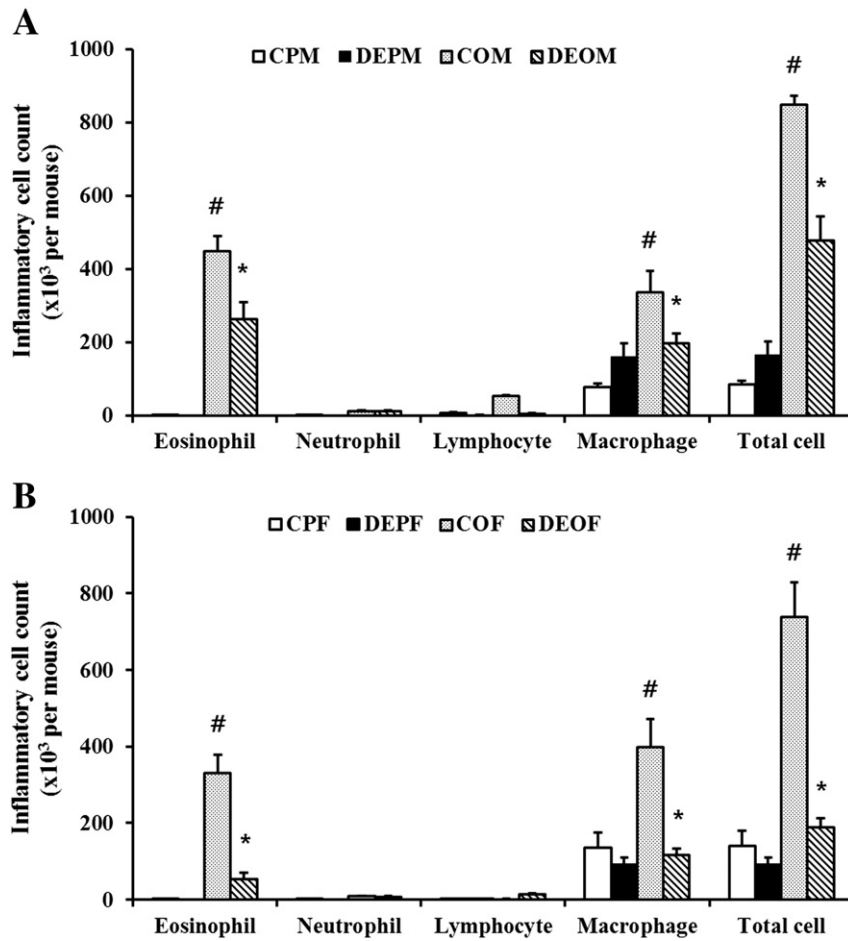


Fig. 2. Maternal exposure of mice to DEHP during pregnancy reduces the recruitment of inflammatory cells in the bronchoalveolar lavage fluid (BALF) of offspring. (A) Group of male offspring, (B) group of female offspring. CPM, males that received only a PBS challenge; DEPM, males that received maternal exposure to DEHP and a PBS challenge; COM, males that received only an OVA challenge; DEOM, males that received maternal exposure to DEHP and an OVA challenge; CPF, female that received only a PBS challenge; DEPF, females that received maternal exposure to DEHP and a PBS challenge; COF, females that received only an OVA challenge; DEOF, females that received maternal exposure to DEHP and an OVA challenge. Values are the means $\pm$ SD ($n = 6$). #Significantly different from CPM or CPF mice, $P < 0.05$; *significantly different from COM or COF mice, $P < 0.05$.

that received maternal exposure to DEHP and an OVA challenge. The Institutional Animal Care and Use Committee of Chungnam National University approved the protocols for the animal study, and the mice were cared for in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and Korean national animal welfare law.

OVA-induced allergic asthma. OVA sensitization and airway challenge were performed as described previously (Lee et al., 2011). In brief, live pups were sensitized on PNDs 9 and 15 by an intraperitoneal injection of 0.5 µg of OVA emulsified in 200 µg aluminum hydroxide in 20 µL PBS buffer (pH 7.4). On PNDs 22, 23 and 24, live pups received an airway challenge with OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). These procedures are illustrated schematically in Fig. 1.

Following the OVA challenge, bronchoalveolar lavage fluid (BALF) samples were obtained from the mice and processed, and inflammatory cells were counted as described previously (Lee et al., 2011). In brief, live pups were euthanized by an intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharm., Seoul, Korea) 48 h after the last challenge, and a tracheostomy was performed. To obtain BALF, ice-cold PBS (0.33 mL) was infused into the lung three times and withdrawn via tracheal cannulation (total volume 1.0 mL). Total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells by Trypan blue staining. To determine differential cell counts, cells in 100 µL of BALF were centrifuged onto slides using a Cytospin apparatus (Hanil Science Industrial, Seoul, Korea) (200 g, 4 °C, 10 min). The slides were dried, and the cells were fixed and stained using Diff-Quik® staining reagent (B4132-1A; IMEB, Deerfield, IL, USA), according to the manufacturer's instructions. The supernatant obtained from BALF was stored at –70 °C for biochemical analysis.

Measurement of cytokines, chemokine, and IgE. The levels of IL-4, IL-13, and eotaxin in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA) according to the manufacturer's protocols. The levels of total IgE in BALF and plasma were also measured using an ELISA, as follows. Microtiter plates were coated with anti-IgE antibodies (anti-mouse IgE; 10 g/mL; Serotec, Oxford, UK) in PBS-Tween 20, and incubated with a BALF or plasma sample. The plates were then washed four times, and 200 µL of o-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated for 10 min in the dark, and the absorbance was then measured at 450 nm.

Immunoblotting. Equal amounts of total lung protein (30 µg) were heated at 100 °C for 5 min, loaded onto 8% SDS-PAGE gels, and electrophoresed. The proteins were then transferred to a nitrocellulose membrane (at 100 V for 2 h), and the membrane was blocked for 1 h with Tris-buffered saline containing 0.05% Tween-20 (TBST) plus 5% skim milk and then incubated with anti-inducible nitric oxide synthase (anti-iNOS; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA) overnight at 4 °C. The membrane was washed three times with TBST and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The membrane was again washed three times with TBST, and was then developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Uppsala, Sweden). For quantitative analysis, densitometric band values were determined using Chemi-Doc (Bio-Rad, Hercules, CA).

Histology. After BALF samples were obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 4 µm thickness, and stained with hematoxylin (Sigma MHS-16) and eosin (Sigma HT110-1-32) solution and periodic acid–

Schiff reagent (PAS; IMEB, San Marcos, CA) to estimate inflammation and mucus production, respectively.

Image capture and photomicrography. Photomicrographs were obtained using a Photometric Quantix digital camera running MS Windows software, and montages were assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but were not otherwise manipulated.

Statistical analysis. Data are expressed as means ± standard deviation (SD). Statistical significance was determined using an analysis of variance (ANOVA) followed by a multiple comparison test with Dunnett adjustment. $P < 0.05$ was considered significant.

Results

Suppression of OVA-induced inflammatory cell eosinophilia in BALF

Among male mice, the COM group showed a significant increase in the inflammatory cell count in their BALF compared with the CPM group, whereas the DEOM group exhibited a significant reduction compared with the COM group. The inflammatory cell count in BALF did not significantly differ between the CPM and DEPM groups (Fig. 2A). These findings are consistent with those of female mice: the COF group showed a significant increase in the inflammatory cell count in their BALF compared with the CPF group, whereas the DEOF group exhibited a significant reduction compared with the COF group (Fig. 2B).

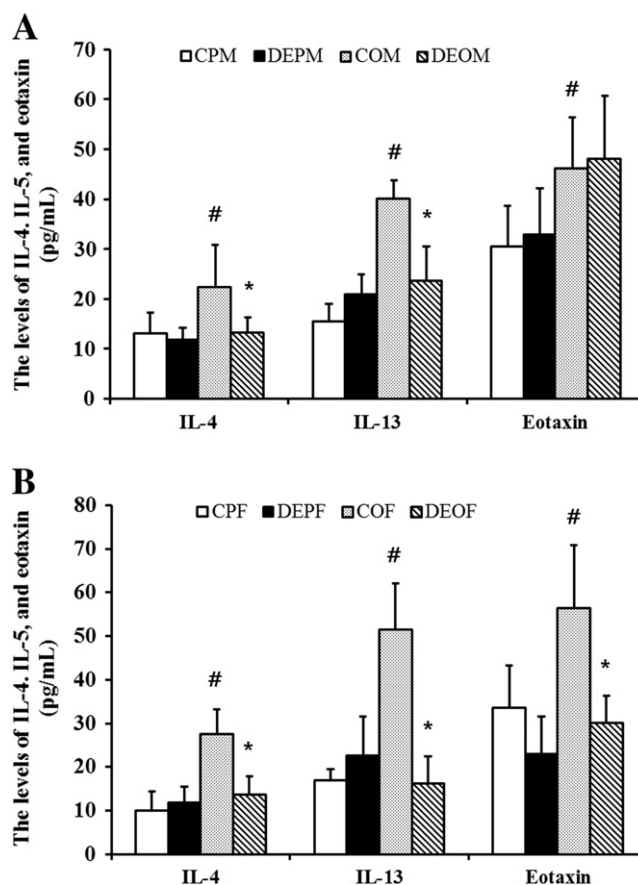


Fig. 3. Maternal exposure of mice to DEHP during pregnancy decreases the levels of IL-4, IL-13, and eotaxin in the bronchoalveolar lavage fluid (BALF) of offspring. Values are the means ± SD (n = 6). [#]Significantly different from CPM or CPF mice, $P < 0.05$; ^{*}significantly different from COM or COF mice, $P < 0.05$.

Reduced levels of IL-4, IL-13, and eotaxin in BALF

The levels of IL-4, IL-13, and eotaxin in BALF did not significantly differ between the CPM and DEPM groups. However, the COM group exhibited significant increases in these factors compared with the CPM group. On the other hand, the DEOM group had decreased the levels of these factors in their BALF compared with the COM group (Fig. 3A). These reductions were also clearly observed in female mice. Similar to male mice, there was no significant difference between the CPF and DEPF groups. The COF group showed marked increases in the levels of IL-4, IL-5, and eotaxin in their BALF compared with the CPF group. By contrast, the DEOF group exhibited a significant reduction in these factors compared with the COF group (Fig. 3B).

Reduction in total IgE and OVA-specific IgE levels in plasma

As shown in Fig. 4A, the levels of total IgE and OVA-specific IgE in plasma were much higher in the COM group than in the CPM group. However, the DEOM group had significantly lower levels than the COM group. As observed for IgE and OVA-specific IgE in the plasma of male mice, the levels in female mice from the COF group were much higher than in the CPF group, whereas the levels in the DEOF group were significantly lower than in the COF group (Fig. 4B).

Reduction in iNOS expression in lung tissue

The COM group had significantly increased iNOS expression in their lung tissue compared with the CPM group. However, the DEOM group had significantly decreased iNOS expression in their lung tissue compared with the COM group (Fig. 5). In female mice, iNOS expression in lung tissue was markedly increased in the COF group, whereas the levels in the DEOF group were significantly reduced when compared with the COF group as well as the results of male group.

Attenuation of airway inflammation and mucus production

We observed a marked increase in the infiltration of inflammatory cells into the peribronchiolar and perivascular lesions in lung tissue sections from mice in the COM group compared with mice in the CPM group (Fig. 6A). By contrast, the DEOM group exhibited less infiltration of inflammatory cells into peribronchiolar and perivascular lesions compared with the COM group. These changes were consistent with those seen in female mice (Fig. 6B).

Lung sections from mice in the COM group exhibited overproduction of mucus on PAS staining (Fig. 7A). Sections from mice in the DEOM group showed mild mucus production in the bronchial airway compared with mice in the COM group. Similarly, the COF group showed mucus overproduction, whereas the DEOF group exhibited

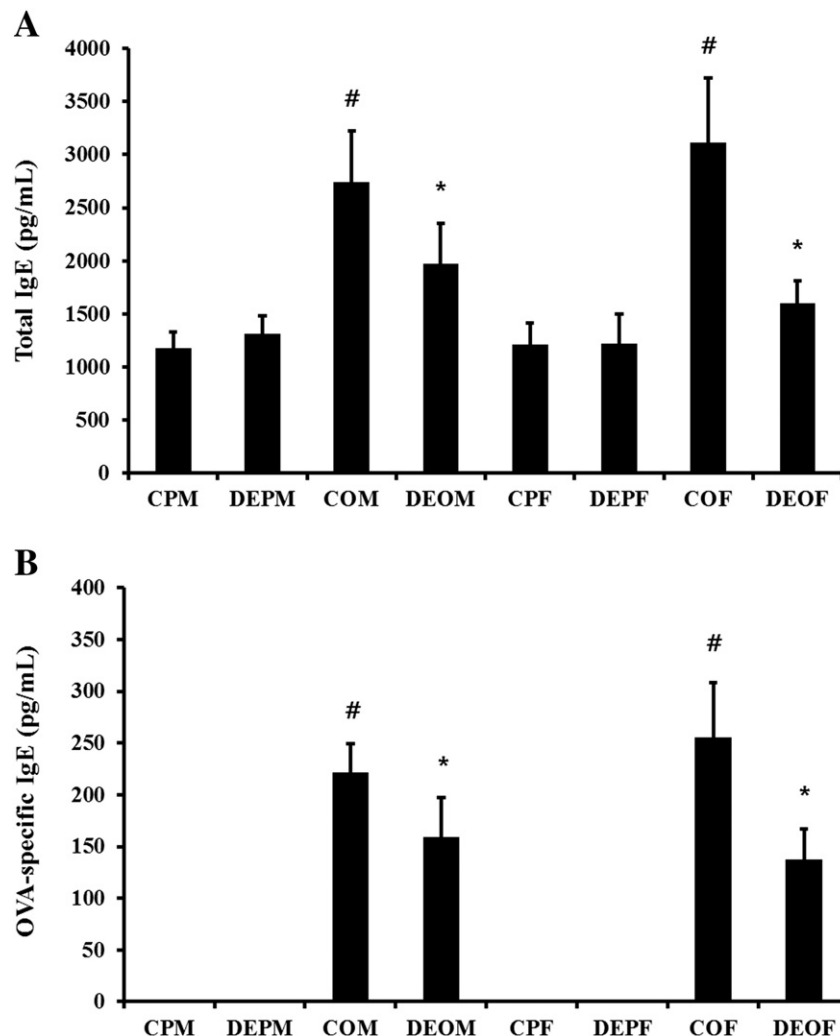


Fig. 4. Maternal exposure of mice to DEHP during pregnancy reduces the levels of IgE and OVA-specific IgE in the serum of their offspring. (A) Group of male offspring, (B) group of female offspring. Values are the means $\pm$ SD ($n = 6$). #Significantly different from CPM or CPF mice, $P < 0.05$; *significantly different from COM or COF mice, $P < 0.05$.

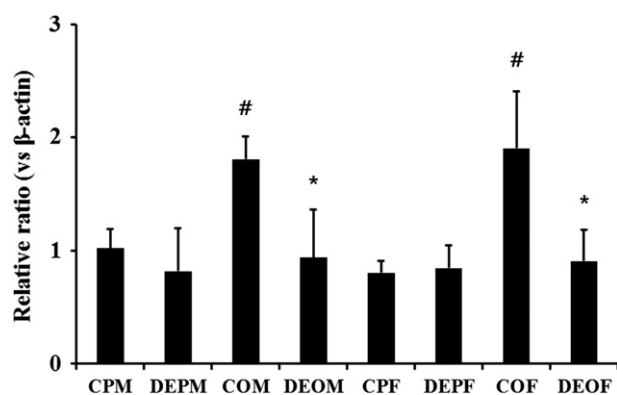


Fig. 5. Maternal exposure of mice to DEHP during pregnancy reduces the expression of iNOS protein in the lung tissues of their offspring. The expression of iNOS is expressed as a relative ratio to β -actin. Values are the means $\pm$ SD ($n = 6$). #Significantly different from CPM or CPF mice, $P < 0.05$; *significantly different from COM or COF mice, $P < 0.05$.

the reduction in mucus production compared with the COF group (Fig. 7B).

Discussion

In this study, we evaluated the effects of maternal exposure to DEHP on the development of asthma in offspring using a murine model of asthma induced by OVA. Maternal exposure to DEHP significantly reduced the increase in inflammatory cell counts and proinflammatory cytokine levels induced by OVA challenge in both male and female offspring. In addition, maternal exposure to DEHP was associated with the suppression of airway inflammation and mucus production in the lung tissue of offspring with asthma induced by OVA compared with the OVA-challenged offspring of mice not exposed to DEHP; the former showed a reduction in iNOS expression in lung tissue.

Exposure to various chemicals during pregnancy can promote the development of asthma in offspring (Fedulov et al., 2008; Wigle et al., 2008). Previous studies demonstrated that maternal exposure to chemicals enhances allergic sensitization and airway inflammation and responsiveness in a model of asthma induced by OVA (Midoro-Horiuti et al., 2010; Rehan et al., 2012). Indeed, in humans, maternal exposure to chemicals such as those found in smoke, organic compounds, and air pollutants during pregnancy increases the risk of asthma in childhood (Shaheen et al., 2005). We hypothesized

that maternal exposure to DEHP during pregnancy could increase the risk of asthma in the offspring of mice; however, we instead found that maternal exposure to DEHP during pregnancy reduces asthmatic responses in the offspring. Airway inflammation is a very important step in the development of asthma (Busse and Rosenwasser, 2003). Th2 cells activated by various allergens produced proinflammatory mediators, such as IL-4, IL-5, and IL-13. Elevated levels of proinflammatory mediators induce the infiltration of inflammatory cells into the airway. These inflammatory cells in turn produce proinflammatory mediators and chemoattractants, aggravating airway inflammation. In this study, maternal exposure to DEHP decreased the levels of Th2 cytokines, which was associated with a reduction in the inflammatory cell count in BALF and in IgE levels in serum. These findings indicate that maternal exposure to DEHP inhibits the development of asthma in offspring through the inhibition of Th2 immune response.

iNOS plays an important role in airway disease, including allergic asthma (Islam et al., 2010). Recent studies have shown that iNOS expression is closely related to Th2 cytokine release in attenuating the asthmatic response, including airway constriction, inflammatory cell infiltration, and remodeling processes (Chibana et al., 2008; Moon et al., 2008). In the current study, maternal exposure to DEHP significantly decreased airway inflammation via downregulation of iNOS overexpression in offspring. Without OVA challenge, maternal exposure to DEHP did not show any changes compared with normal control. There is convincing evidence that maternal exposure to DEHP did not cause the development of asthma in offspring. Indeed, our findings suggest that maternal exposure to DEHP reduces neonatal asthma susceptibility. Our findings are contrary to those of previous studies (Bornehag et al., 2004; Jaakkola et al., 2006). DEHP exposure was previously found to promote and aggravate allergic asthma with histological and physiological changes (Guo et al., 2012). DEHP also increased the CD3 expression and IL-4 production in splenocytes from atopic prone NC/Nga mice (Koike et al., 2009). On the other hand, some previous studies are consistent with ours (Larsen and Nielsen, 2009; Larsen et al., 2007). Previous studies demonstrated that coexposure to DEHP and OVA reduced the IgG1 level and reduced the IgE/IgG1 ratio. As described above, contrary findings were considered to be related to the route and dosage of DEHP. Larsen et al. (2007) reported that airborne DEHP is only able to increase serum IgG1 and lung inflammatory cell levels in high concentration. Intraperitoneal injection of DEHP at low concentrations induces a reduction in IgG1 level. Moreover, no observed adverse effect level (NOAEL) of DEHP is 5 mg/kg according to Christiansen et al. (2010). In this study, consumption of DEHP by pregnant dams was calculated to be 3.88 mg/kg/day during pregnancy

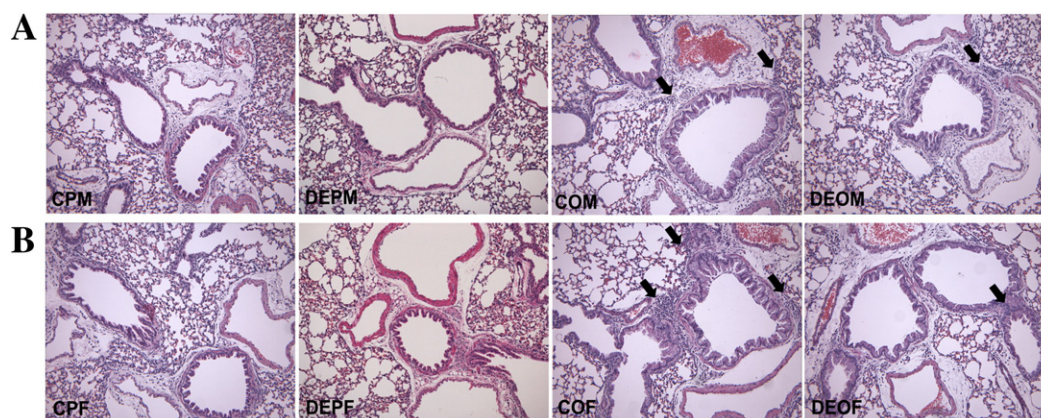


Fig. 6. Maternal exposure of mice to DEHP during pregnancy attenuates airway inflammation in offspring. (A) Group of male offspring, (B) group of female offspring. The arrows indicate inflammatory infiltration.

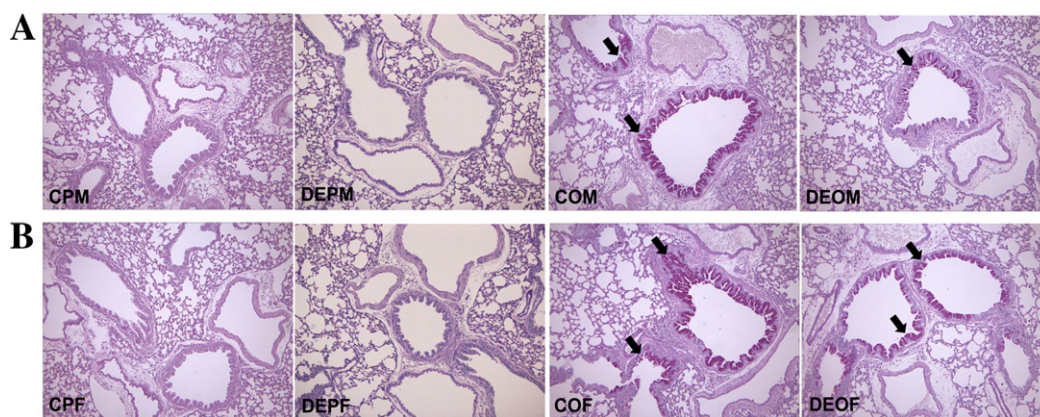


Fig. 7. Maternal exposure of mice to DEHP during pregnancy decreases mucus production in the lung tissue. (A) Group of male offspring, (B) group of female offspring. The arrows indicate mucus production.

and 4.78 mg/kg/day during lactation, which is lower than NOEL of DEHP. Therefore, we consider the antiasthmatic effects of DEHP to be distinct from toxicity induced by DEHP exposure.

Conclusion

This current study is one of very few investigating the correlation between maternal exposure to DEHP during pregnancy and asthma susceptibility in offspring. This study suggests that maternal exposure to DEHP during pregnancy attenuates asthmatic responses induced by OVA challenge in offspring. These effects were considered to be closely related to the suppression of Th2 immune responses and iNOS expression.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2011-0014591).

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