

Original Article



Maternal Diisononyl Phthalate Exposure Activates Allergic Airway Inflammation via Stimulating the Phosphoinositide 3-kinase/Akt Pathway in Rat Pups*

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Abstract

Objective To evaluate the effect of diisononyl phthalate (DINP) exposure during gestation and lactation on allergic response in pups and to explore the role of phosphoinositide 3-kinase/Akt pathway on it.

Methods Female Wistar rats were treated with DINP at different dosages (0, 5, 50, and 500 mg/kg of body weight per day). The pups were sensitized and challenged by ovalbumin (OVA). The airway response was assessed; the airway histological studies were performed by hematoxylin and eosin (HE) staining; and the relative cytokines in phosphoinositide 3-kinase (PI3K)/Akt pathway were measured by enzyme-linked immunosorbent assay (ELISA) and western blot analysis.

Results There was no significant difference in DINP's effect on airway hyperresponsiveness (AHR) between male pups and female pups. In the 50 mg/(kg·d) DINP-treated group, airway response to OVA significantly increased and pups showed dramatically enhanced pulmonary resistance (RI) compared with those from controls ($P < 0.05$). Enhanced Akt phosphorylation and NF- κ B translocation, and Th2 cytokines expression were observed in pups of 50 mg/(kg·d) DINP-treated group. However, in the 5 and 500 mg/(kg·d) DINP-treated pups, no significant effects were observed.

Conclusion There was an adjuvant effect of DINP on allergic airway inflammation in pups. Maternal DINP exposure could promote OVA-induced allergic airway response in pups in part by upregulation of PI3K/Akt pathway.

Key words: Allergic airway inflammation; Asthma; DINP; Maternal exposure; PI3K/Akt

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INTRODUCTION

Allergic asthma is a chronic airway disorder characterized by chronic eosinophilic airway inflammation, reversible airway obstruction, and non-specific airway hyperresponsiveness (AHR). A great deal of evidence indicated that these inflammatory responses are mediated by T-helper type 2 (Th2) and

T-helper type 1 (Th1) cells together with mast cells, bronchial epithelial cells, and eosinophils, as well as a number of inflammatory cytokines and chemokines^[1-2]. Epidemiological studies have shown that the prevalence of allergic disease, such as asthma, increased among children and adolescents during the past 30 years, in parallel with the period of increasing production of chemicals in industrialization^[1]. This suggests that the prevalence

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of allergic airway disease might be associated with the exposure to environmental contaminants^[3-7].

Diisononyl phthalate (DINP) is one plasticizer used in soft poly vinyl chloride (PVC) materials, which is a substitute for di-2-ethylhexyl phthalate (DEHP), a reproductive toxicological chemical. Humans are exposed to DINP via ingestion, inhalation, and dermal contact. Ingestion is considered to be the major route, accounting for over 90% of total DINP intake^[8]. Moreover, as the restriction of DEHP use in Europe, DINP has replaced DEHP to be the most commonly used plasticizer. It has been reported that the use of DINP and diisodecyl phthalate (DIDP) is now threefold higher than the use of DEHP in Europe^[9]. A human biomonitoring study showed a doubled DINP exposure from 1988 to 2003, and with the recent change in use pattern from DEHP to DINP in Europe, the risk of human DINP exposure may be rapidly increasing^[10].

Allergic asthma and allergy have been reported in industrial workers and children exposed to phthalates and PVC materials, suggesting a possible association between chronic phthalate exposure and immune-mediated disease^[11-16]. Phthalate plasticizers and metabolites have been shown to enhance or suppress the effect of immunogens, which means these chemicals have adjuvant effects on anaphylactic reaction^[17-18]. Several *in vitro* and animal studies have examined the consequences of DINP exposure on mast cell degranulation, eosinophilic inflammation, and regulatory T cells, the results suggest that the developing immune system might be a particular sensitive target of DINP^[19-20].

However, there are limited data determining co-effect of prenatal and postnatal DINP exposure on the developing immune system. So we designed this *in vivo* study to evaluate the effect of DINP on the development of allergic asthma in rats and the underlying mechanism. Our hypothesis is that maternal DINP exposure during the critical period of fetal immune system and respiratory system development could affect allergic inflammation response in pups, and modulate the severity of the effect through the phosphoinositide 3-kinase/akt pathway.

METHODS

Chemicals

Hen egg ovalbumin (OA grade V, >98%, CAS No. 9006-59-1, Cat. No. A5503) was from Sigma (St. Louis, Mo., USA). Diisononyl phthalate, CAS No 68515-48-0, AR, purity 99% was from Sinopharm Chemical

Reagent Co., Ltd (Shanghai, China). Aluminium hydroxide (CAS No 21645-51-2, AR, >97%) was from Shanghai Meixing Chemical Co., Ltd (Shanghai, China).

Animals

Specific-Pathogen Free (SPF) female Wistar rats at age of 50-60 d, obtained from Animal Center of Fudan University, Shanghai, China, were housed in plastic cages in an air-controlled room at 21±3 °C with relative humidity of 55%±15%. The animals were maintained on a 12 h light/12 h dark cycle and had free access to tap water and standard laboratory animal feed. Nulliparous rats were mated overnight and checked in the next morning for sperm in the vaginal smear. The morning when sperm was detected in the vaginal smear was defined as gestational day (GD) 0.5, and postnatal day (PND) 0.5 was the day after birth.

DINP Treatment

Pregnant rats were randomly assigned to treatment groups (5-6 animals) and administrated once a day by oral gavage with vehicle (corn oil from supermarket), 5, 50, or 500 mg DINP/kg bw from GD 7 to PND 21. According to Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals, a part of litters were culled on PND 4 to balance provision of nutrition^[21-22].

Allergic Airway Inflammation Model^[23-24]

On PND 22, 23, and 37, the pups ($n=6-8$ per group) in groups of corn oil and DINP treatment were sensitized by subcutaneous injection of 1 mg OVA and 22.5 mg Al(OH)₃ suspended in 0.5 mL saline. The rats were challenged with 1% OVA aerosol for 30 min on PND 44 and 45. In addition, 6 pups in the control group were sensitized and challenged with saline, which were used as negative control model. Every group had the same quantity of male and female pups.

Measurements of Respiratory Function

The method for the invasive respiratory function measurements used in this study has been described previously^[25]. Briefly, the pups were anesthetized 24 h after the last aerosol challenge, and were placed in a supine position and warmed with an incandescent lamp. At the upper part of the trachea, a T-shape incision was made and a T-shape cannula, which was directly attached to a heater-controlled pneumotachograph (Fleisch model 000, Hans

Rudolph, Kansas City, Mo., USA), was gently inserted into the trachea. Tidal flow was determined by a pneumotachograph connected to a differential pressure transducer (Auto Tran, model 600D-011, ± 2 cm H₂O). To measure the transpulmonary pressure, a water-filled PE-90 tube was inserted into the esophagus to the level of the mid-thorax (lower one-third of the esophagus) and coupled to a pressure transducer (PT14MX, Jialong Teaching Equipment, Shanghai, China). The pneumotachograph tidal flow signal was integrated with time to obtain the tidal volume. The OVA-induced airway AHR, typically reflected by high pulmonary resistance (RI) or low dynamic compliance (C_{dyn}), were calculated over a complete respiratory cycle using an integration method over flows, volumes and pressures and were continuously recorded with the software for physiology experiments. The respiratory parameters were averaged in 60-s segments, and the results of RI and C_{dyn} were expressed as a percentage of the corresponding baseline value.

Bronchoalveolar Lavage Fluid (BALF) Analysis

After the measurements of respiratory function, BALF was collected according to Guo et al.^[26]. Briefly, tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold saline of 1ml was instilled into the trachea and right lung through the tracheal cannula, the chest of rat was gently massaged and rinsed for approximately 1 min, and then the liquid was withdrawn. The process was repeated for 3 times and the total quantity of BALF was 2.4-2.6 mL for each sample. BALF cytokine levels were determined as described previously^[27]. According to the instructions, the levels of IL-13 and IFN- γ in BALF were detected using ELISA kits (Abcam, Cambridge, MA, USA).

Histopathological Analysis

After the measurements of respiratory function, the complete left lung was collected from the animal. About 1/3 of the left lungs were fixed in 10% neutral formalin, paraffinized, cut into 5 μ m sections, and stained with hematoxylin and eosin (HE) for examining cell infiltration. Analyses of cell infiltration were performed using blind method.

Western Blot Analysis

About 2/3 of the left lungs were lysed in ice-cold lysis buffer (150 mmol/L NaCl; 50 mmol/L Tris-HCl,

pH 7.4; 1 mmol/L EDTA; 5 g/mL leupeptin; 5 g/mL aprotinin A; 1 mmol/L PMSF; 0.1% SDS; 1% sodium deoxycholate; 1% Triton X-100; 1 mmol/L NaF). After centrifugation (15 min; 3000 \times g; 4 °C), the supernatants were removed, total protein was determined, separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes. The blots were probed with the appropriate primary antibody, i.e. rabbit anti-Akt (1:1000; Cell Signalling Technology, Massachusetts, USA), rabbit anti-phospho-Akt1 (Ser473) (1:1000; Cell Signalling Technology, Massachusetts, USA), mouse anti-GAPDH (1:5000; Abcam, Cambridge, MA, USA), respectively, followed by the incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. The staining was visualized using ECL reagents (Beyotime, Haimen, China). The densitometric evaluation was carried out using Quantity One software version 4.6 (Bio-Rad), and the relative quantity of an individual protein is expressed as the ratio of the gray scale respective to that of GAPDH.

Assay for Nuclear Factor-kappa B (NF- κ B)

Nuclear factor-kappa Bp65 (NF- κ Bp65) DNA-binding activity in lung tissue samples was evaluated to measure the degree of NF- κ B activation. Analysis was performed according to the manufacturer's protocol for a commercial ELISA kit (NF- κ Bp65 Transcription Factor Assay Colorimetric, cat. no. KHO0371, Invitrogen Corporation Carlsbad, CA, USA).

Statistical Analysis

Data are presented as means \pm standard deviation (SD). Comparisons between groups were made using SAS 9.1 software after datasets passed normality testing. Statistical significance was determined using a two-tailed Student's *t*-test or a one-way ANOVA, which was followed by the Bonferroni *post-hoc* test when more than two treatments were compared. A value of $P < 0.05$ was used to indicate statistically significant differences.

RESULTS

Establishment of OVA-induced Allergic Airway Inflammation Model

In the corn oil group, the OVA-treated pups showed a significant increase in AHR compared with the saline-treated control pups ($P < 0.05$), suggesting

higher airway responsiveness was found in OVA-treated pups (Figure 1A-B). Figure 1C and 1D showed the different inflammatory responses while pups were treated with OVA or saline. The intense infiltration of inflammatory cells into the lungs was observed near the bronchioles of pups sensitized and challenged with OVA (Figure 1D). In contrast, few inflammatory cell infiltrations were observed in the lungs of pups sensitized and challenged with saline only (Figure 1C). The results showed that we successfully established OVA-induced allergic airway inflammation models. In addition, no significant sex specific differences in susceptibility to OVA treatment was found in rat pups.

Maternal DINP Exposure Promotes OVA-induced Allergic Response in Rat Pups

The airway response to OVA with or without maternal DINP treatment was evaluated in rat pups. Figure 2A showed the effect of maternal DINP exposure on the development of AHR in OVA-treated pups. In the 50 mg/(kg-d) DINP-treated group, the

airway response to OVA significantly increased and pups showed dramatically enhanced RI compared with those from controls ($P<0.05$). However, in the 5 and 500 mg/(kg-d) groups, DINP-treated pups did not show any significant effect on RI and Cdyn (data not shown) compared with the control group. There was no significant difference on DINP's effect on AHR between male pups and female pups. In contrast to the corn oil-treated group, the pups from 50 and 500 mg/(kg-d) DINP-treated dams showed significant heavier eosinophil infiltration (Figure 2B-E). These findings suggested that DINP treatment during GD 7 to PND 21 had an adjuvant effect on AHR and allergic airway inflammation in pups.

DINP Treatment Increased Th2 Cytokine Levels, Enhanced PI3K/Akt Activation and NF- κ B Translocation

The imbalance in Th1/Th2 expression is critical to the pathogenesis of allergic asthmatic inflammation. The results from the current study showed that OVA inhalation in sensitized pups increased the IL-13

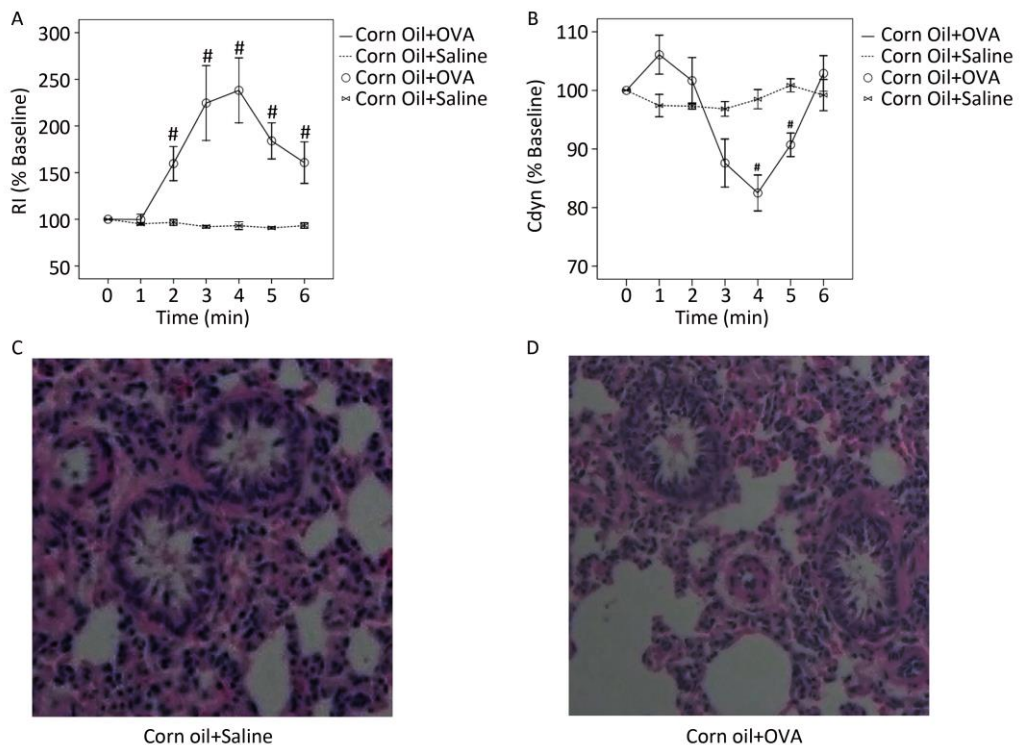


Figure 1. Airway responsiveness and airway inflammation in corn oil+OVA group and corn oil+saline groups. (A) lung resistance (RI, $n=6-8$ pups); Data are shown as mean \pm SD. (B) dynamic compliance (Cdyn, $n=6-8$ pups); Data are shown as mean \pm SD. (C) Transverse section of lung tissue in the corn oil+saline group; H&E, $\times 100$. (D) Transverse section of lung tissue in the corn oil+OVA group; H&E, $\times 100$. #Significant difference from group of corn oil+saline induced model, $P<0.05$.

(one Th2 cytokine) level in BALF compared with saline aerosol control (Figure 3A). In addition, the level of IFN- γ , a Th1 cytokine, significantly decreased in OVA-challenged pups (Figure 3B). The IL-13 level in 50 mg/(kg-d) DINP-treated group was significantly higher than that from corn oil+OVA controls (Figure 3A). Consistently, lower IFN- γ level was found in 50 mg/(kg-d) DINP-treated group (Figure 3B). These findings indicated that DINP exposure during gestation and lactation could enhance the Th2-predominant immune activity in the OVA-induced allergic airway inflammation model pups.

Th2 immune responses might be regulated by the PI3K/Akt pathway in animal models of allergic asthma^[28-29]. To explore the critical role of PI3K/Akt signaling pathway in the proinflammatory mechanism of DINP's effect in OVA-challenged pups, we examined the phosphorylation of Akt, an important step of phosphorylation cascade in PI3K/Akt signaling pathway, in lung tissues obtained 24 h after the last OVA or saline aerosol challenge. The express levels of Akt phosphorylation (ser⁴⁷³) increased in OVA aerosol challenged pups and DINP-treated pups compared with those from saline

aerosol controls. As shown in Figure 3C, 50 mg/(kg-d) DINP treatment enhanced the express of Akt phosphorylation compared with the corn oil group. However, no significant increase was found in 50 mg/(kg-d) DINP treatment group. Besides, PI3K/Akt pathway activation has been shown to promote NF- κ B DNA binding activity^[30]. The pups in the 50 mg/(kg-d) DINP-treated group had higher levels of NF- κ B activity in lung tissues compared with those from controls, which were consistent with results of Akt (Figure 3D).

DISCUSSION

DINP is ubiquitous in environment and human tissues. Given the increasing concern about the correlation between DINP exposure and asthma attack, we hypothesize that DINP might act as an adjuvant and DINP exposure during critical period of immune system and respiratory system development might induce allergic response in pups. A series of experiments, e.g. analysis of the immune response and cytokines in PI3K/Akt signaling pathway of pubertal rats from DINP-treated dams, were designed in this study.

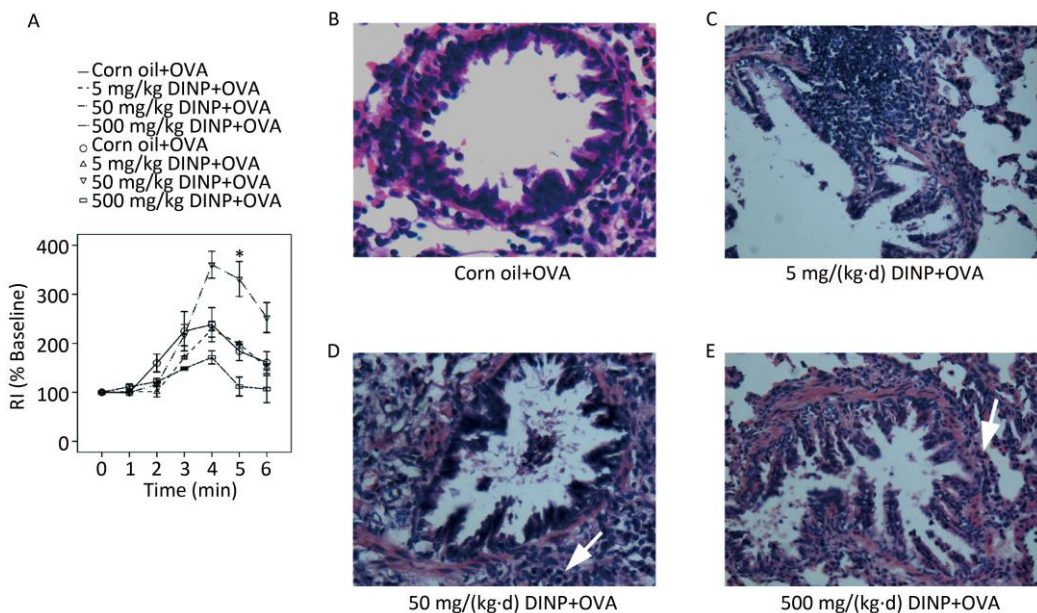


Figure 2. Effects of DINP treatment during gestation and lactation on OVA-induced AHR and airway inflammation in rat pups. (A) lung resistance (RI, $n=6-8$ pups); Data are shown as mean \pm SD. (B) histopathological observation of the lung in corn oil+OVA group; H&E, $\times 200$. (C) Transverse section of lung tissue in the 5 mg/(kg-d) DINP+OVA group; H&E, $\times 200$. (D) Transverse section of lung tissue in the 50 mg/(kg-d) DINP+OVA group; H&E, $\times 200$. (E) Transverse section of lung tissue in the 500 mg/(kg-d) DINP+OVA group; H&E, $\times 200$. *Significant difference from the corn oil+OVA induced model group, $P<0.05$. \rightarrow : eosinophil infiltration.

According to the method from Morokata et al. and Nakajima et al., we established the allergic airway inflammation model in rats^[23-24]. The increased inflammatory infiltration and AHR in OVA-challenged pups showed allergic airway inflammation, which was the basis of exploring the impact of maternal DINP exposure on pups' allergic inflammation. Although sex specific differences were shown in study in the effects of maternal exposure to bisphenol A (BPA), one plasticizer and environmental endocrine disruptor, on allergic lung inflammation^[31], no significant sex specific differences was found while maternal DINP treatment affected allergic lung inflammation in pups. In the 50 mg/(kg-d) DINP-treated group, the Akt (ser⁴⁷³) phosphorylation and NF- κ B translocation in lung tissues was significantly activated in pups. The level of IL-13 in BALF was increased, and the IFN- γ concentration was reduced. No significant effects were found in 5 and

500 mg/(kg-d) DINP-treated groups. However, we did not count inflammatory cell in BALF, which limited our result's interpretation.

Previous studies showed that the activation and immune responses of eosinophils, T and B lymphocytes, bronchial epithelial cells, and mast cells might be regulated by PI3K/Akt signaling pathway^[32-37]. Activation of the PI3K/Akt pathway resulted in a pronounced augmentation of NF- κ B, the vital regulator of proinflammatory gene expression in allergic asthma, which mediates the synthesis of Th2 cytokines^[38-40]. Otherwise, Nashed et al.^[35] and Takeda et al.^[36] have demonstrated that using mice deficient in p110 δ or p110 γ PI3K could show a major drop in Th2 cytokines and chemokines upon allergen sensitization and challenge, which means that PI3K/Akt pathway and downstream NF- κ B translocation play important roles in the expression of Th2 cytokines in allergic airway disease.

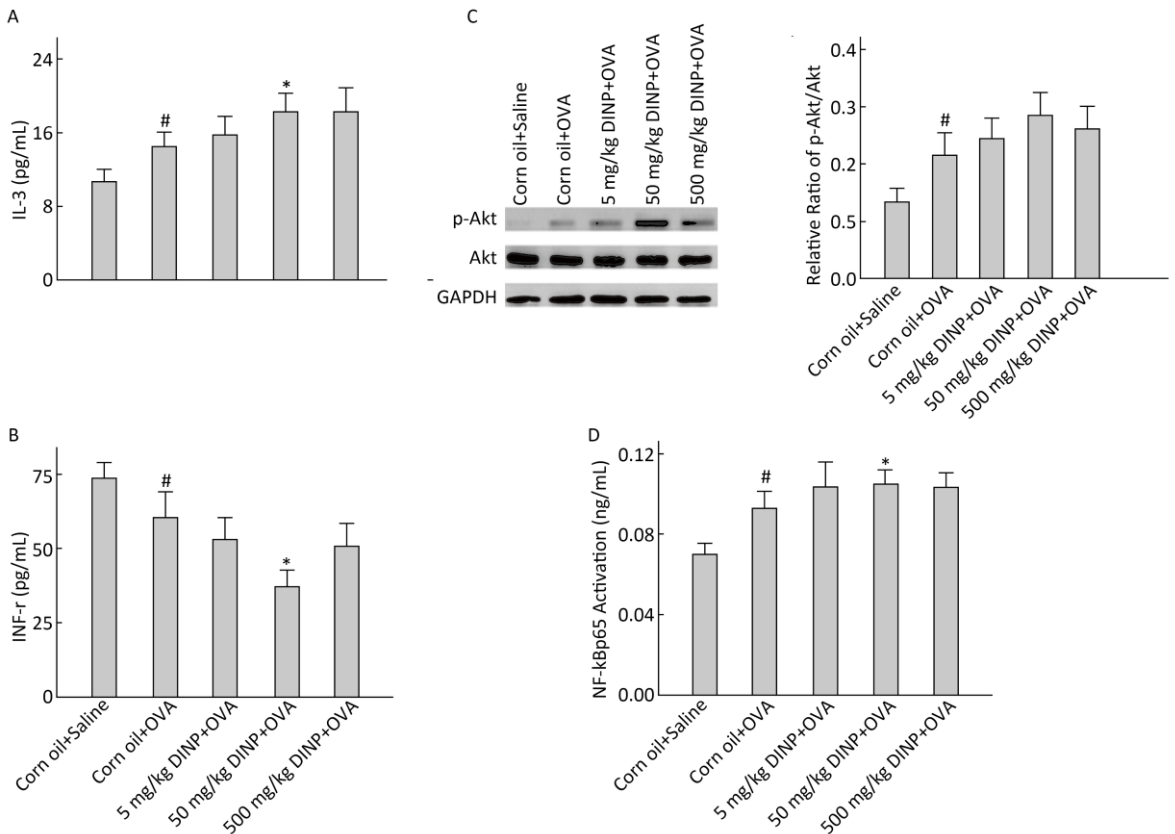


Figure 3. Effects of DINP on OVA-induced Th2 and Th1 cytokines expression, Akt activation, and the downstream NF- κ B translocation in rat pups. (A-B, D) Values are shown as mean \pm SD ($n=6-8$ pups). (C) The experiments were repeated for three times with similar pattern of results ($n=3$ pups); Data are shown as mean \pm SD. [#]Significant difference from group of corn oil+saline induced model, $P<0.05$. ^{*}Significant difference from group of corn oil+OVA induced model, $P<0.05$.

Our finding in this study was consistent with the previous studies and suggested that the adjuvant effect of DINP on pups' allergic airway inflammation might be due to the stimulation of PI3K/Akt pathway and the downstream NF- κ B activity.

According to Nakajima et al., estrogen receptor (ER) plays a role in BPA's effects on immune events in asthma^[24]. In our study we found that relatively low DINP exposure at 50 mg/(kg-d) could have adjuvant effects on pups' allergic airway inflammation instead of high exposure at 500 mg/(kg-d), which might be due to the existence of different ER subtypes on mast cells. Mast cells, in lung tissues, express ER α and ER β , with ER β being more abundant than ER α ^[41-45]. In general, environmental estrogens induce mast cell degranulation and enhance the release of allergic mediators via ER α , while possess a negative effect on allergic asthma via ER β ^[46-47]. However, ER α may be a more important regulator of the allergic asthma, resulting in an adjuvant effect on degranulation with a relatively low concentration of environment estrogen^[48]. But when the environment estrogen reaches a high level, both the ER α and ER β play a role in the degranulation of mast cells, leading to a non-significant or an immunosuppressive effect on allergic inflammation. DINP is an environmental estrogen, which has been demonstrated to work by binding to estrogen receptors^[49]. These findings suggest that DINP may play functions by binding to the different subtypes of ER and result in the various effects on allergic airway inflammation. In addition, studies have revealed that lower level of phthalate exposure showed adjuvancy and higher level showed no obvious or suppressive effects on allergic airway disease, which are consistent with our results^[50-53].

DINP treatment during gestation and lactation at 50 mg/(kg-d) by oral gavage was found to have an adjuvant effect to induce allergic airway inflammation in rat pups. Using the data from the animal study, we calculated the reference dose (RfD) for humans to be 500 μ g/(kg-d). This value is derived by applying a 100-fold safety factor to account for possible differences between animals and humans and for differences in the sensitivity among individuals. Studies have shown that phthalates can cross the placental barrier, so fetuses are exposed^[54]. According to Heudorf et al.^[55], the maximum exposure to DINP in 0-12-month old infants was up to 135.02 μ g/(kg-d). Therefore, the RfD recommended in our study is close to the human exposure levels, which indicates maternal DINP

exposure might have potential adverse effects to cause allergic disorders in childhood.

CONCLUSION

During in utero and lactation exposure, DINP acts as an adjuvant. 50 mg/(kg-d) DINP treatment could promote allergic airway responses, enhance Akt activation, and downstream NF- κ B translocation and Th2 cytokines synthesis in pups, suggesting this adjuvant affected upregulation of PI3K/Akt pathway and the downstream NF- κ B activity.

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