

Expression levels of neuroimmune biomarkers in hypothalamus of allergic mice after phthalate exposure

Tin-Tin Win-Shwe^{a*}, Rie Yanagisawa^a, Eiko Koike^a, Hiroshi Nitta^a and Hirohisa Takano^b

ABSTRACT: Previously, we demonstrated that maternal exposure to phthalates enhances atopic dermatitis in male mouse offspring. However, whether phthalate exposure affects neuroimmune biomarkers in allergic mice has not yet been studied. Di-(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalate (DINP) are environmental chemicals that are commonly used as plasticizers. This study was designed to investigate the expression levels of neuroimmune biomarkers in the hypothalamus of a murine model of allergic asthma after phthalate exposure throughout juvenility until adulthood. Six-week-old C3H/HeJ Jcl male mice were treated with DEHP or DINP (0, 0.02, 0.4 or 8 nmol per body per week) and ovalbumin (OVA; 1 µg per body per 2 weeks) for 7 weeks intratracheally. On the day after the completion of the phthalate and OVA treatment, the hypothalamus from each mouse was collected, and the mRNA expression levels of neuroimmune biomarkers were examined using a real-time RT-PCR analysis. The mRNA expression levels of the proinflammatory cytokines interleukin (IL)-1 β and tumor necrosis factor (TNF)- α , the chemokine CCL3, the transcription factor nuclear factor (NF)- κ B, the oxidative stress marker heme-oxygenase (HO)1, a nerve growth factor, and the microglia marker Iba1 were remarkably up-regulated in the hypothalami of mice treated with 8 nmol of DEHP in the presence of the allergen. However, no significant changes were observed, except for reductions in the TNF- α and CCL2 mRNA levels, in mice exposed to DINP combined with the allergen. This study is the first report to show that high-dose DEHP exposure throughout juvenility until adulthood may induce neuroinflammation by modulating neuroimmune biomarkers in the hypothalami of allergic mice. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: phthalates; neuroimmune biomarkers; hypothalamus; allergy; mouse

Introduction

The incidence of asthma and allergy is increasing worldwide in response to various causative factors. One possible factor is exposure to environmental chemicals, such as pesticides, solvents, phthalates and air pollutants. Many epidemiological studies have indicated that exposure to environmental chemicals enhances or aggravates underlying asthma or allergic diseases by modulating immune responses (Bornehag *et al.*, 2004; Jaakkola *et al.*, 1999, 2004, 2006). Our previous study has indicated that nose-only exposure to low levels of toluene aggravates airway inflammatory responses in an allergic mouse model by modulating the number of inflammatory cells and by enhancing the plasma levels of nerve growth factor (NGF) (Win-Shwe *et al.*, 2007). Furthermore, the long-term exposure of male C3H/HeN mice to toluene has increased the mRNA expression levels of NGF and TrkA in the lungs of ovalbumin (OVA)-immunized mice (Fujimaki *et al.*, 2009). Moreover, recent studies have indicated that early exposure to environmental chemicals may lead to metabolic disorders (e.g. obesity and diabetes; Migliarini *et al.*, 2011) and mental illness (e.g. schizophrenia; Leonard *et al.*, 2012) in later life. The potential causative factors for these lifestyle-related diseases may be disturbances in higher brain functions, such as the dysregulation of the neuroimmune system or neuroinflammation.

Because the nervous and immune systems of children may be more sensitive to chemical-induced alterations than those of adults (Dietert and Piepenbrink, 2006), the identification of

neuroimmune functions in adults following early chemical exposure is important. Common adverse outcomes of developmental immunotoxicity are immunosuppression, autoimmunity, allergic responses to chemicals or allergens and dysregulated inflammation (Dietert, 2009). Experimental evidence has indicated that the increased risk of mental illnesses, such as autism, autism spectrum disorders, schizophrenia, Parkinson disease and Alzheimer disease, in offspring exposed to toxicants during early life may be due to immune or inflammatory dysregulation (Dietert and Dietert, 2008; Hertz-Picciotto *et al.*, 2008; Ozawa *et al.*, 2006). Although adverse health effects from developmental immunotoxicity in response to various toxic chemicals, such as endocrine disruptors (e.g. bisphenol A, polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), heavy metals (e.g. lead, mercury) and tobacco smoke are well documented (Dietert, 2009), little is known about phthalate-induced neuroimmune dysfunction during early exposure.

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Phthalate esters including di-(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalate (DINP) are widely used as plasticizers in a variety of polyvinyl chloride-based consumer products including infant toys, food wraps, cosmetics, flooring and wall coverings, and surgical and medical devices such as tubing, blood bags and dialysis equipment (Cadogan and Howick, 1996; Latini, 2000; Heudorf *et al.*, 2007). Therefore, humans are daily exposed to phthalates through ingestion, inhalation and dermal contact, especially in children (NTP-CERHR, 2006; Schettler, 2006).

The nervous and immune systems communicate with each other in a bidirectional manner. Immune responses in the central nervous system (CNS) involve not only the activation of resident cells (including microglia and astrocytes), but also the infiltration of circulating immune cells (including monocytes, neutrophils and T cells). Both activated resident cells and infiltrating cells express, release and/or respond to cytokines, which function in the CNS as both regulators of immunity and modulators of neuronal and glial function (Aloe *et al.*, 2002). Proper communication between neurons, immune cells and neurotrophins is potentially responsible for the regulation and control of neuroimmune cross-talk. In the brain, the hypothalamus is the region that regulates the immune system, reproductive functions, thermoregulation, appetitive behavior and metabolic functions (Guijarro *et al.*, 2006). However, to the best of our knowledge, little is known regarding the effect of phthalates on neuroimmune biomarkers in the hypothalamus. Therefore, to evaluate neuroimmune interactions after exposure to phthalates, we examined potential neuroimmune biomarkers, such as the levels of proinflammatory cytokines, transcription factors, an oxidative stress marker, and a neurotrophin as well as and microglia activation.

Individuals with allergic diseases are thought to be more susceptible to environmental chemical exposure. We hypothesized that phthalate exposure may induce neuroinflammation via the NF κ B pathway and may trigger proinflammatory cytokine production. Neuroinflammation may signal neurons and glia cells to produce neurotrophins to compensate for neurodegeneration. According to our and other studies, proinflammatory cytokines and neuropeptides are suggested to be potential neuroimmune biomarkers (Cizza *et al.*, 2008; Marques *et al.*, 2010; Win-Shwe *et al.*, 2012). In addition, microglia are the major immune cells resident in the CNS (Giulian, 1992) and are thought to be the principal source of brain immune mediators. Moreover, transcription factor and oxidative stress marker are potential candidates to detect the mechanism of neuroinflammatory response. Taken together, proinflammatory cytokines (IL-1 β , TNF- α) and chemokines (CCL2, CCL3), neuropeptides (nerve growth factor, NGF) and inflammatory response-related transcription factor (NF κ B), oxidative stress marker (heme oxygenase 1, HO1) and microglia marker (ionized calcium binding adaptor molecule 1, Iba1) genes were selected to examine the effect of phthalate exposure in the present study. In the present study, we developed an allergic mouse model using OVA immunization and investigated whether exposure to phthalates affects neuroimmune biomarkers in the hypothalamus.

Materials and Methods

Animals

Five-week-old C3H/HeJ Jcl male mice were purchased from Charles River Japan (Osaka, Japan), and six-week-old mice were used in the experiments. Animals were given a commercial diet

(CE-2; Japan Clea Co., Tokyo, Japan) and water *ad libitum* and were housed in cages under controlled environmental conditions (temperature, 22–26 °C; humidity, 40–69%; lights on 07:00–19:00 h). This study was approved by the Ethics Committee of the Animal Care and Experimentation Council of the National Institute for Environmental Studies, Japan.

Dose of Phthalates

We selected relevant doses of phthalates that approximated human exposure levels. According to data from the Ministry of Environment Japan, the predicted maximal indoor exposure to DEHP is 0.18 μ g (0.46 nmol) kg^{-1} per day (for mice, 0.08 nmol/25 g per week). Therefore, we considered a dose that was 100 times the daily exposure level to be a high dose (8 nmol), a dose that was 1:20 of the high dose to be a moderate dose (0.4 nmol), and a dose that was 1:400 of the high dose to be a low dose (0.02 nmol). Since no data regarding the maximal indoor exposure to DINP was available, we used the same doses as those used for DEHP.

Exposure to Phthalates

DEHP or DINP (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and diluted with phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Carlsbad, CA, USA) for each dose (final concentration of DMSO, 0.1%). Although inhalation exposure was the means of administration closest to the actual route of exposure in humans or animals during normal life, we decided to use an intratracheal administration route in the present study to enable the adjustment of the desired dose to the target organ. Animals were allotted to five groups for each phthalate exposure: a control group (vehicle alone), an OVA group, an OVA + phthalate (0.02 nmol) group, an OVA + phthalate (0.4 nmol) group, and an OVA + phthalate (8 nmol) group. The experimental protocol is shown in Fig. 1. DEHP (0, 0.02, 0.4, or 8 nmol per mouse) in experiment 1 or DINP (0, 0.02, 0.4 or 8 nmol per mouse) in experiment 2 was administered by intratracheal instillation once per week for 7 weeks. To create a murine model of allergic asthma, OVA (1 μ g per mouse) was administered by intratracheal instillation biweekly for seven weeks. The compounds (OVA and p.o. phthalates) were inoculated in 0.1 ml aliquots through a polyethylene tube under anesthesia with 4% halothane (Takeda Chemical Industries Ltd, Osaka, Japan).

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) and the determination of the cell counts in BAL fluid were conducted as previously reported (Takano *et al.*, 1997). Briefly, the trachea was cannulated 24 h after the last intratracheal administration following the collection of blood. The lungs were lavaged with 1 ml of sterile saline at 37 °C, which was instilled three times using a syringe. The recovered fluid aliquots were pooled. No differences in the volumes of collected fluids were observed between the different groups. The lavage fluid was centrifuged at 300 *g* for 10 min at 4 °C to recover the alveolar free cells. The total cell count was determined using a fresh fluid specimen and a hemocytometer. Differential cell counts were prepared using the Autosmear (Sakura Seiki Co., Tokyo, Japan) and were stained with Diff-Quik (International Reagents Co., Kobe, Japan). A total of 500 cells were counted under a microscope (AX80; Olympus, Tokyo, Japan).

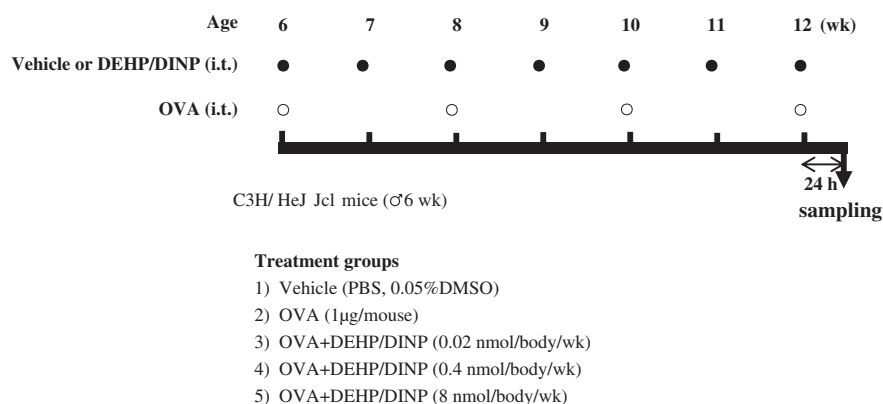


Figure 1. Experimental protocol for exposure to DEHP and DINP in a murine model of allergic asthma (i.t. = intratracheal instillation).

Analysis of Inflammatory Mediator Gene Expressions in the Hypothalamus

Total RNA extraction from the hypothalamic tissues and quantitative estimation of the expression levels

Twenty-four hours after the final exposure, we collected the total hypothalami from all the mice and examined various neuroimmune biomarkers. To collect the total RNA from the hypothalamic samples (total hypothalamus), we used a previously described protocol (Win-Shwe *et al.*, 2006). Briefly, total RNA extraction from the hypothalamic samples was performed using the BioRobot EZ-1 and EZ-1 RNA tissue mini kits (Qiagen GmbH, Germany). Genomic DNA contaminants were removed from each sample during total RNA extraction by treating the tissue extract with RNase-free DNase I (Qiagen GmbH, Germany), a step that is included in the robotic processing of the total RNA. Then, the purity of the total RNA was examined and the quantity was estimated using the ND-1000 NanoDrop RNA Assay protocol (NanoDrop, USA). Total RNA samples producing an $A_{260/280}$ nm value between 1.8 and 2.0 in the ND-1000 NanoDrop RNA Assay protocol were used for the cDNA synthesis.

Reverse Transcription of RNA to cDNA and Quantitative Real-time PCR

Next, we performed first-strand cDNA synthesis from the total RNA using SuperScript RNase H⁻ Reverse Transcriptase II (Invitrogen, USA), according to the manufacturer's protocol. Briefly, 500 ng of total RNA was used as the template and was reverse-transcribed to cDNA using SuperScript RNase H⁻ Reverse Transcriptase II (Invitrogen, USA) and random hexamer oligo (dN)₆-priming in a final reaction volume of 40.0 µl supplemented with RNase Out (Invitrogen, USA) in the presence of 5 mM of MgCl₂; the sample was then digested with RNase H (Invitrogen, USA) to remove the RNA template from the cDNA:RNA hybrid molecule. Next, we examined the hypothalamic mRNA expressions of 18S, IL1-β, TNF-α, CCL2, CCL3, NFκB, HO1, NGF and Iba1 using a quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) method and the ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., USA). The hypothalamic cDNA samples were diluted serially several-fold and were used as standards. Briefly, we conducted the reactions in a final volume of 25 µl containing either 5 µl of each of the 5-fold diluted cDNA samples or each of the diluted standards, 12.5 µl of 2× Master Mix (QuantiTect SYBR Green PCR Kit), and 2.5 µl of 20× probe-primer mix for each gene from the

QuantiTect Gene Expression Assay Systems (Qiagen GmbH, Germany) and 5 µl of RNase-free water. SYBR Green I-based PCR was performed using a dissociation protocol under the following conditions: one initial cycle of enzyme activation for 15 min at 95 °C followed by 20–35 cycles of amplification consisting of initial denaturation at 94 °C for 15 s, annealing at 59 °C for 30 s, elongation at 72 °C for 30 s, and finally one cycle of the melting curve analysis protocol. The tissue 18S rRNA level was used as the internal control. Data were analyzed using the comparative threshold cycle method. Then, the relative expression levels of mRNA were individually normalized to the 18S rRNA content in the respective samples and were expressed as the mRNA signals per unit of 18S rRNA expression.

Statistical Analyses

All the data were expressed as the mean ± standard error (SE). The statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo Inc., Tokyo, Japan). The dose–response data were analyzed using a one-way analysis of variance with a *post-hoc* analysis using the Bonferroni–Dunn method. Differences were considered significant at $P < 0.05$.

Results

Body Weight and Brain Weights

To determine the general toxicity of DEHP or DINP, we measured the body and brain weights of the mice at the time of sampling. We found that the body weights and brain weights were not significantly different between the control (vehicle) and the DEHP- or DINP-treated groups (data not shown).

Cellular Profile in BAL Fluid

To confirm the allergic response in our animal model, we examined the cellular profile in the BAL fluid. OVA exposure significantly induced the infiltration of neutrophils, eosinophils and monocytes into the lung ($P < 0.01$; $P < 0.05$, Table 1); however, no significant effects of DEHP or DINP were observed. These findings indicate that an allergic response occurred in the present mouse model.

Table 1. Cellular profile for bronchoalveolar lavage fluid following exposure to phthalates

Group	Total cells	Macrophages	Neutrophils	Eosinophils	Lymphocytes	Monocytes
Vehicle	26.5 ± 4.10	25.8 ± 4.04	0.45 ± 0.26	0	0.03 ± 0.01	0.16 ± 0.11
OVA	29.1 ± 2.23	27.1 ± 2.23	0.55 ± 0.20	0.63 ± 0.14**	0.07 ± 0.02	0.74 ± 0.27**
OVA + DEHP 0.02 nmol/body/week	27.4 ± 4.31	24.4 ± 3.98	0.71 ± 0.26	1.14 ± 0.34**	0.12 ± 0.07	0.99 ± 0.30*
OVA + DEHP 0.4 nmol/body/week	27.1 ± 3.68	23.1 ± 2.91	1.26 ± 0.51	1.37 ± 0.26**	0.07 ± 0.02	1.25 ± 0.27**
OVA + DEHP 8 nmol/body/week	29.2 ± 3.58	24.7 ± 2.78	1.40 ± 0.42**	1.44 ± 0.38**	0.09 ± 0.03	1.58 ± 0.50**
Vehicle	22.1 ± 1.97	21.9 ± 1.95	0.10 ± 0.05	0.01 ± 0.00	0	0.05 ± 0.02
OVA	22.8 ± 1.69	21.8 ± 1.64	0.42 ± 0.10**	0.35 ± 0.08*	0.05 ± 0.02	0.23 ± 0.07**
OVA + DINP 0.02 nmol/body/week	23.6 ± 3.25	22.0 ± 3.25	0.59 ± 0.11**	0.67 ± 0.25**	0.07 ± 0.02	0.31 ± 0.05**
OVA + DINP 0.4 nmol/body/week	27.4 ± 1.67	24.1 ± 1.24	0.70 ± 0.20**	1.76 ± 0.79**	0.16 ± 0.07	0.69 ± 0.22**
OVA + DINP 8 nmol/body/week	29.7 ± 2.94*	25.0 ± 2.47	2.05 ± 0.58**	1.42 ± 0.61**	0.14 ± 0.05	1.12 ± 0.28**,,#

Data represent the means ± SE
 * $p < 0.05$ vs the control (vehicle) group.
 ** $p < 0.01$ vs the control (vehicle) group, 9–13 animals per group for di-(2-ethylhexyl) phthalate (DEHP) and 11–14 animals per group for di-isonyl phthalate (DINP). OVA, Ovalbumin.

DEHP Up-regulates the Expression Levels of Neuroimmune Biomarkers in the Hypothalamus

We examined the effects of the intratracheal instillation of DEHP on the expression of inflammatory cytokines and chemokines, a

transcription factor, an oxidative stress marker, a neurotrophin, and a microglia marker in the hypothalami of mice. The mRNA expression levels of the cytokines IL-1 β and TNF- α and the chemokine CCL3 were significantly up-regulated by high-dose DEHP in the presence of the allergen ($P < 0.05$; Fig. 2A, B, D). NF κ B is an

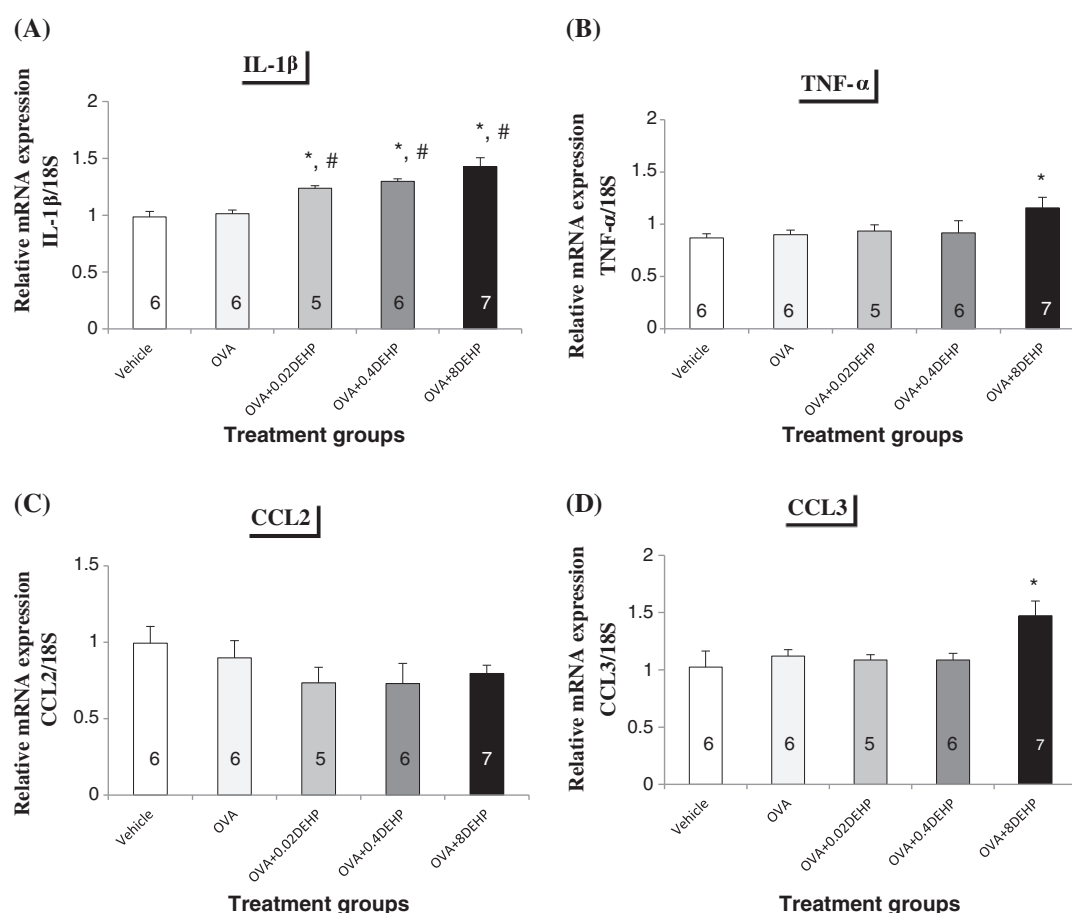


Figure 2. Messenger RNA expression levels of the proinflammatory cytokines (A) IL-1 β and (B) TNF- α and the chemokines (C) CCL2 and (D) CCL3 in the hypothalami of the control (vehicle) and the DEHP-treated allergic mice. Each bar represents the mean $\pm$ SE. The number of animals used was indicated in each bar (* $P < 0.05$ for DEHP-treated group compared with the control (vehicle) group; # $P < 0.05$ for high-dose DEHP-treated group compared with the OVA group).

important transcription factor in the inflammatory response, and its mRNA level was significantly stimulated by high-dose DEHP ($P < 0.05$; Fig. 3A). In addition, the mRNA level of the oxidative stress marker HO1 was significantly increased by high-dose DEHP ($P < 0.05$; Fig. 3B). Next, we also examined the expression of the neurotrophin NGF and found that the NGF mRNA level was up-regulated by high-dose DEHP ($P < 0.05$; Fig. 3C). Microglia are a major type of immune cell in the brain, and we investigated the expression of the microglia marker Iba1 and found that the Iba1 mRNA level was stimulated by high-dose DEHP ($P < 0.05$; Fig. 3D). However, we did not observe any dose-dependent effects of DEHP on neuroimmune biomarkers in the hypothalamus. These findings indicate that a high dose of DEHP can induce a neuroinflammatory response in the hypothalamus of adult mice during juvenile exposure.

DINP Does Not Affect the Expression Levels of Neuroimmune Biomarkers in the Hypothalamus

We next examined the effects of the intratracheal instillation of DINP on neuroimmune biomarkers in the hypothalamus of mice. The mRNA expression levels of IL-1 β , CCL3, NGF and Iba1 were not different between the control (vehicle) and the DINP-treated allergic mice (Fig. 4A, D). However, the mRNA expression levels of TNF- α and CCL2 were suppressed in mice exposed to high-dose DINP in the presence of the allergen (Fig. 4B, C). Moreover, DINP did not affect the mRNA expression levels of NF κ B, HO1,

NGF, or Iba1 in the presence of the allergen (Fig. 5A–D). A summary of the effects of phthalate exposure on the neuroimmune biomarkers is shown in Table 2.

Discussion

Previously, our laboratory has demonstrated that DEHP enhances atopic dermatitis, such as skin lesions related to mite allergen in adult mice (Takano *et al.*, 2006). In addition, we have reported that maternal exposure to phthalates enhances atopic dermatitis in male offspring (Yanagisawa *et al.*, 2008). Regarding DINP, we have shown that DINP aggravates atopic dermatitis related to mite allergen in mice (Koike *et al.*, 2010). We have also reported that DEHP and DINP enhance Th2 responses through the activation of bone marrow-derived dendritic cells and splenocytes from NC/Nga mice (Koike *et al.*, 2009, 2010). Previously, *in vivo* studies have indicated that exposure to phthalates during gestation and lactation affects neuroendocrine and reproductive systems, such as brain aromatase activity levels and hypothalamic sex steroid-regulated gene expression and sexual behavior (Andrade *et al.*, 2006; Lee *et al.*, 2006). Recently, an *in vitro* study demonstrated that DEHP induces apoptosis in neuro-2a cells (Lin *et al.*, 2011), and DEHP's metabolite mono-(2-ethylhexyl) phthalate, or MEHP, has been shown to inhibit cell proliferation and to promote cell differentiation in PC12 cells (Chen *et al.*, 2011). Moreover, maternal exposure to DEHP may influence the feeding behavior in offspring and may trigger

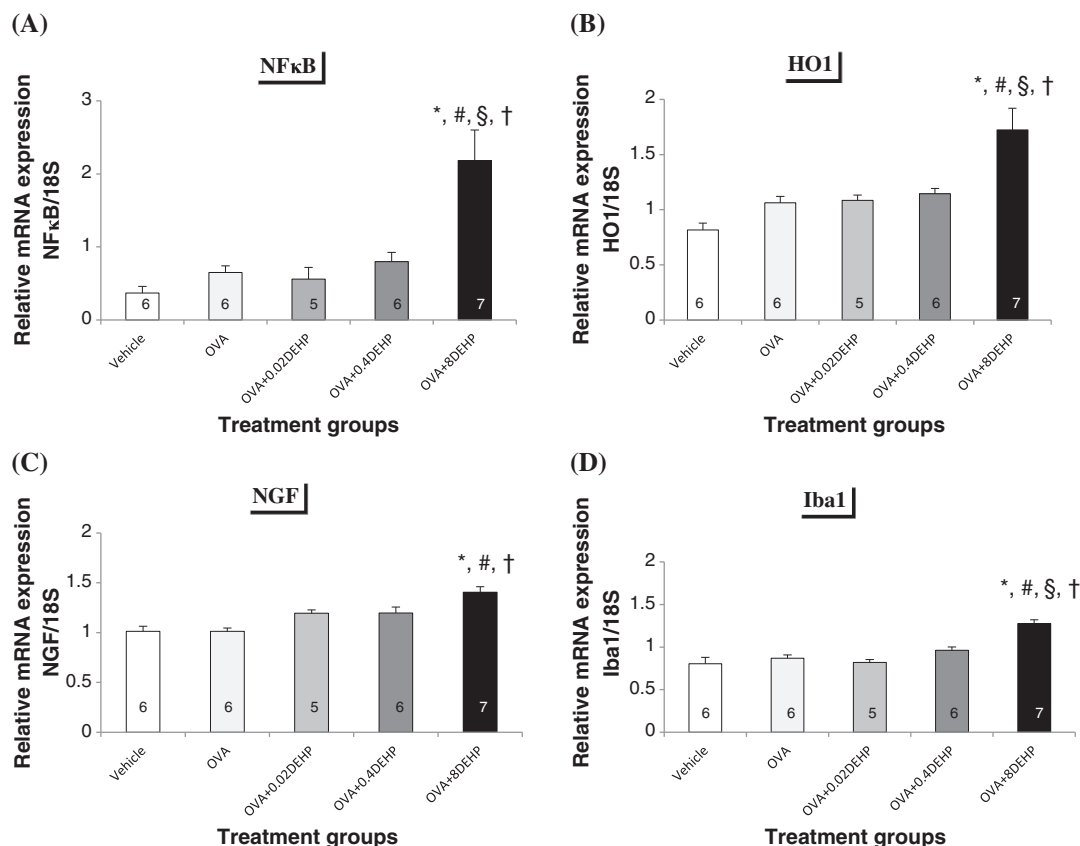


Figure 3. Messenger RNA expression levels of (A) the transcription factor NF κ B, (B) the oxidative stress marker HO1, (C) the neurotrophin NGF, and (D) the microglia marker Iba1 in the hypothalamus of the control (vehicle) and the DEHP-treated allergic mice. Each bar represents the mean $\pm$ SE. The number of animals used was indicated in each bar (* $P < 0.05$ for the DEHP-treated group compared with the control (vehicle) group; # $P < 0.05$ for the high-dose DEHP-treated group compared with the OVA group; § $P < 0.05$ for the high-dose DEHP-treated group compared with the low-dose DEHP-treated group; † $P < 0.05$ for the high-dose DEHP-treated group compared with the moderate-dose DEHP-treated group).

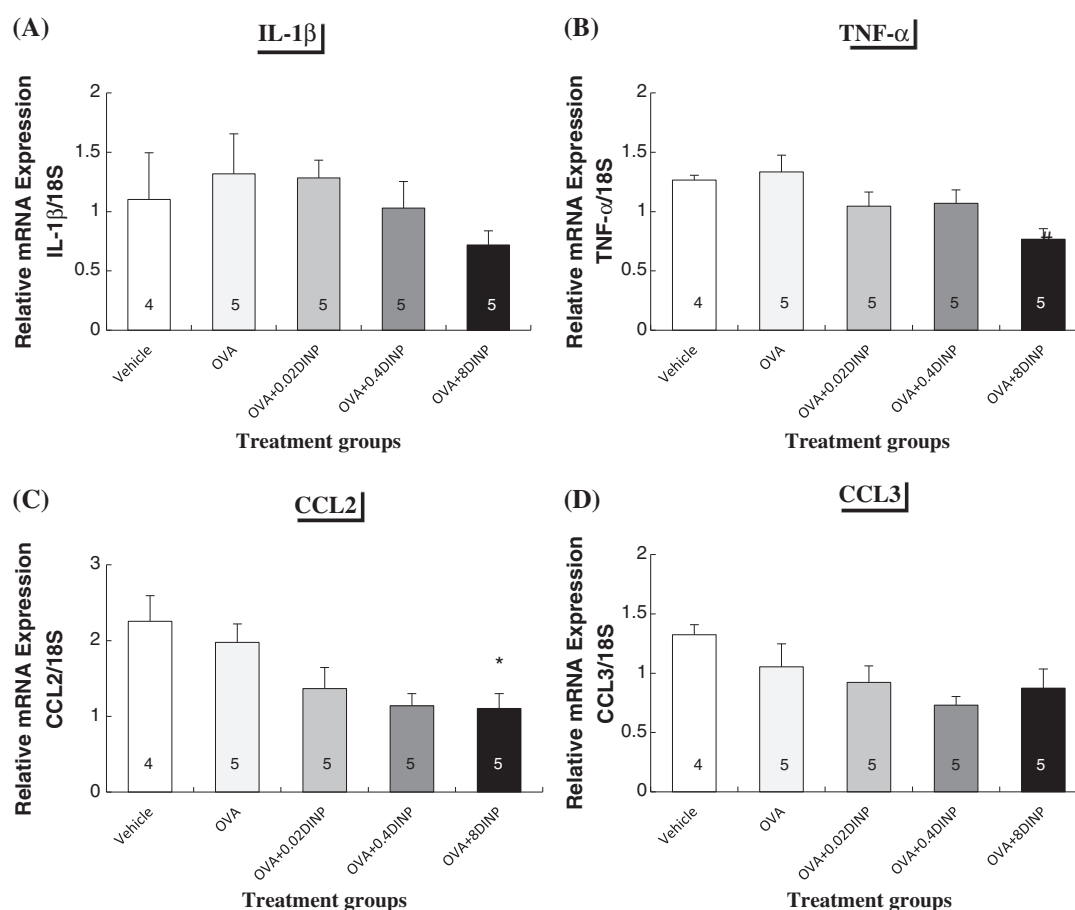


Figure 4. Messenger RNA expression levels of the proinflammatory cytokines (A) IL-1 β and (B) TNF- α and the chemokines (C) CCL2 and (D) CCL3 in the hypothalami of the control (vehicle) and the DNP-treated allergic mice. Each bar represents the mean $\pm$ SE. The number of animals used was indicated in each bar (* $P < 0.05$ for the high-dose DNP-treated group compared with the control (vehicle) group; # $P < 0.05$ for the DNP-treated group compared with the OVA group).

obesity by modulating plasma leptin level (Hayashi *et al.*, 2012). However, whether phthalate exposure affects the neuroimmune system remains uncertain. This uncertainty prompted us to study the effects of phthalates on the response of neuroimmune biomarkers in susceptible animal models, such as allergic mice.

Cytokines are principal biomarkers of neuroinflammation in neuropathology and neurodegenerative process in the nervous system (Franzen *et al.*, 2004; Cacquevel *et al.*, 2004). Previously, we reported that proinflammatory cytokine mRNA levels were up-regulated in the brains of mice exposed to environmental pollutants, such as carbon black nanoparticles, nanoparticle-rich diesel exhaust, and the volatile organic compound toluene (Win-Shwe *et al.*, 2006, 2008a, 2008b, 2010, 2012). In the present study, we detected the expression levels of proinflammatory cytokines and chemokines and found that the mRNA expression levels of IL-1 β , TNF- α and CCL3 increased in parallel to the increase in the transcription factor NF κ B in the hypothalami of allergic mice exposed to high-dose DEHP.

Neurotrophins and their related receptors have been identified as targets for neurotoxins and are known to play a role in bidirectional signaling between cells of the immune and nervous systems. Among the neurotrophins, NGF plays a crucial role in the survival and development of specific peripheral and brain neurons (Chao *et al.*, 2006; Allen and Dawbarn, 2006). Previously, we have demonstrated that exposure to low levels of toluene up-regulate the expression of neurotrophins and their receptors

in the adult mouse hippocampus in a strain-dependent manner and in accordance with the allergic condition (Win-Shwe *et al.*, 2009). The exact mechanism behind the upregulation of neurotrophins in the mouse hypothalamus following DEHP exposure is still unclear. At least in part, the up-regulation of neurotrophins and related receptors in the hypothalami of allergic mice may compensate for or protect against neurodegeneration induced by exposure to high-dose DEHP.

Using PC 12 cells, Bedogni and colleagues have demonstrated that reactive oxygen species (ROS) rapidly generated in the cell cytosol in response to NGF and Rac-1 have a role in survival signaling to Akt and CREB, whereas harmful mitochondrial oxidants produced at the onset of apoptotic death are reduced by the neurotrophin (Bedogni *et al.*, 2003). In the present study, the mRNA expression of the oxidative stress marker HO1 was significantly higher in the hypothalami of mice exposed to DEHP in the presence of the allergen. The expression of HO1 genes might play a role in scavenging ROS through the activation of NGF.

Microglia are the major immune cells resident in the CNS (Giulian, 1992) and are thought to be the principal source of brain immune mediators. Activated microglia generally function as cytotoxic effector cells, releasing several cytotoxic substances including free radicals, excitatory amino acids, arachidonic acid derivatives and cytokines. We detected major immune cell microglia activation and found an increase in the Iba1 mRNA level in the hypothalami of high-dose DEHP-exposed allergic

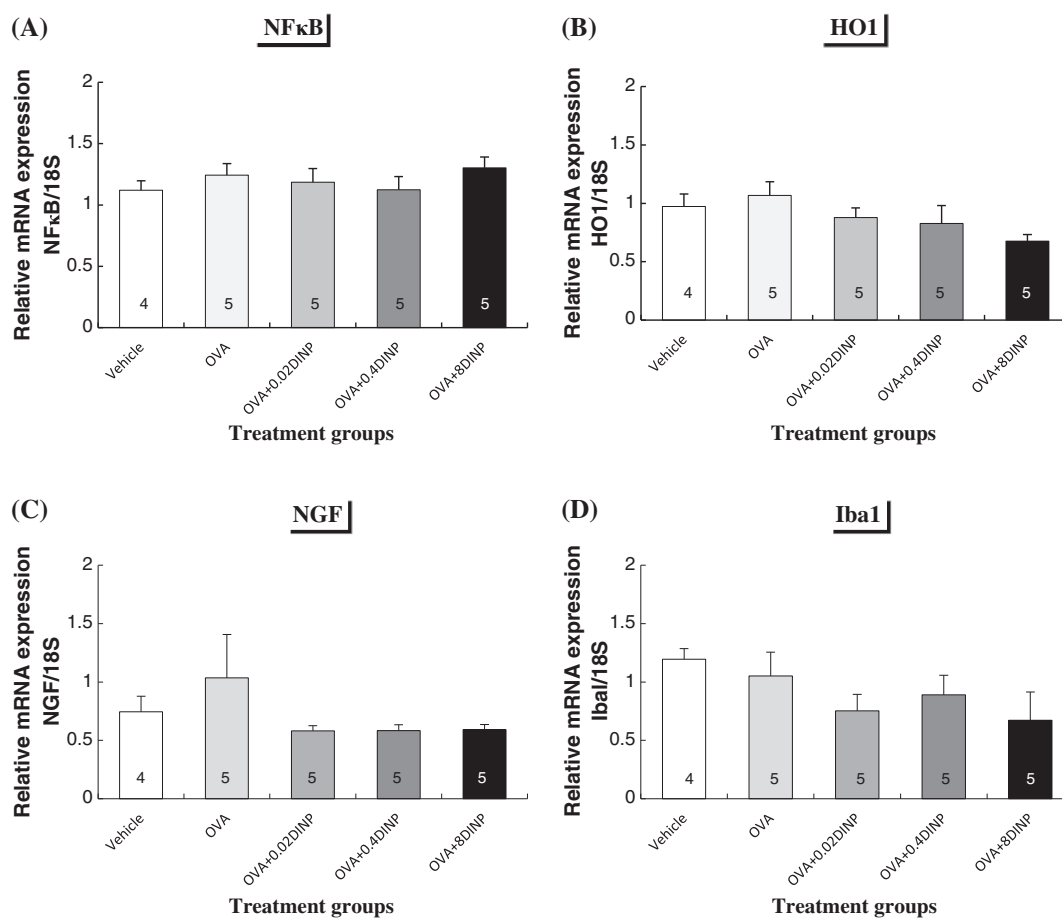


Figure 5. Messenger RNA expression levels of (A) the transcription factor NFκB, (B) the oxidative stress marker HO1, (C) the neurotrophin NGF, and (D) the microglia marker Iba1 in the hypothalami of the control (vehicle) and DNP-treated allergic mice. Each bar represents the mean ± SE. The number of animals used is indicated in each bar.

Table 2. Summarized results of the effects of high dose DEHP or DNP treatment on neuroimmune biomarkers in the hypothalamus of allergic model of mice

Biomarkers		mRNA expression	
		DEHP	DNP
Proinflammatory cytokines	L-1β	↑	→
	TNF-α	↑	↓
	CCL2	→	↓
	CCL3	↑	→
Transcription factor	NFκB	↑	→
Oxidative stress marker	HO1	↑	→
Neurotrophins	NGF	↑	→
Microglia marker	Iba1	↑	→
↑ = increased significantly ↓ = decreased significantly → = not changed.			

mice, compared with that in the corresponding control mice. Inflammatory signals may induce neurotrophin expression in human microglia cells through a pathway involving the transcription factor NF-κB (Heese *et al.*, 1998). Taken together, these results suggest that DEHP exposure during the brain maturation period might trigger neuroinflammation via the NFκB-IL-1β/TNF-α pathway, and the up-regulation of these cytokines might signal neuronal and non-neuronal cells, including microglia, through NFκB-dependent and/or NFκB-independent mechanisms to produce neurotrophic factors.

DNP is currently used as an alternative of DEHP because DEHP is highly toxic to humans, especially to children. DEHP is a chemical form of dioctyl phthalate and has the same core structure as DNP, but has longer, branched side chains attached, each having eight carbon atoms. In the present study, no significant change in allergic mice exposed to DNP was observed except for a decrease in the mRNA expression levels of TNF-α and CCL2. To the best of our knowledge, little is known about the effect of DNP on brain functions. From our findings, we suggest that DEHP and DNP affect hypothalamic neuroimmune biomarkers in allergic mice differently. These differences may be related to either pharmacokinetic or pharmacodynamic properties of the different phthalate structures.

In the present study, the significant responses were observed at a dose which is 100 times the estimated daily exposure in human. Firstly, it may be suggested that a very high level of

phthalate exposure is needed to affect the expression levels of neuroimmune biomarkers. Secondly, duration of exposure (e.g. chronic exposure), route of administration (e.g. inhalation exposure) and exposure schedule (e.g. daily exposure) may influence the dose–response effects.

In conclusion, this study is the first report describing the changes in the expression levels of neuroimmune biomarkers in allergic mice following phthalate exposure throughout juvenility until adulthood. Our findings clearly demonstrated that DEHP exposure induced a neuroinflammatory response in the hypothalami of allergic mice. Further studies are needed to explore the neuroimmune network involved in the behavioral response to phthalate exposure.

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