

NO₂ inhalation enhances asthma susceptibility in a rat model

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Abstract Nitrogen dioxide (NO₂) is a major air pollutant. Epidemiologic studies have found that NO₂ exposure is associated with an increased risk of asthma. Nevertheless, the potential molecular mechanisms remain unclear. In this study, we investigated the effect of NO₂ inhalation on the occurrence of allergic airway inflammation and its underlying mechanisms. Firstly, male Wistar rats were exposed to 2 and 5 mg/m³ NO₂ (28 days, 5 h/day). The results showed that NO₂ exposure could induce pulmonary inflammatory response, mucus formation, and Th1/Th2 imbalance in the lung of normal rats, resulting in allergic asthma-like features. Secondly, male Wistar rats were exposed to 5 mg/m³ NO₂ (42 days, 5 h/day), sensitized with ovalbumin (OVA), challenged with aerosolized OVA, and characterized in asthma models. Results showed that NO₂ exposure aggravated lung inflammation in the OVA-sensitized rats, accompanied by the increase in inflammatory cell infiltration, mucus hypersecretion, and collagen deposition. Furthermore, NO₂ exposure promoted the increase in the expression of mucin gene (MUC5AC) and pro-inflammatory factors [interleukin (IL)-1β, intercellular adhesion molecule-1 (ICAM-1), and IL-6] as well as serum OVA-specific immunoglobulin E (IgE) production. Taken together, we established that NO₂ exposure promotes allergic airway inflammation and increases the asthma susceptibility. The

underlying mechanisms involve the promotion of activation of interleukin-4/signal transducer and activator of transcription-6 (IL-4/STAT6) pathway [IL-4 receptor (IL-4R) α, janus kinase (JAK) 1, JAK 3, and STAT6] and related transcription factor [T cell-specific protein-tyrosine kinase (Lck), extracellular-regulated kinase (ERK)1/2, and nuclear factor-κB (NF-κB)]. In particular, the imbalance of Th1/Th2 cell differentiation [IL-4, interferon (IFN)-γ, GATA-binding protein-3 (GATA-3), and T-box expressed in T cells (T-bet)] plays a pivotal role in NO₂-induced inflammatory responses. These findings may provide a better understanding of mechanism of NO₂-associated respiratory diseases.

Keywords Nitrogen dioxide · Airway inflammation · Allergic asthma · Th1/Th2 differentiation · IL-4/STAT6 pathway

Introduction

Nitrogen dioxide (NO₂) is a major air pollutant generated by the combustion process that exists widely in both indoor and outdoor environments (Hedberg et al. 1989; Krishna et al. 1998). In the outdoors, peak concentrations can reach 4 parts per million (ppm) in highly trafficked areas during rush hour (Chauhan et al. 1998), while indoor levels may peak at 2–4 ppm in ferries, ice-skating rinks, and kitchens with gas cooking stoves (Bascom 1996; Krishna et al. 1998). In daily life, exposure to relatively low levels of NO₂ may pose a major risk to human health (Liu et al. 2009; Svendsen et al. 2012; Santus et al. 2012; Yeatts et al. 2012). Epidemiological studies have shown that NO₂ exposure is related to increases in hospital admission, morbidity, and mortality of asthma (Liu et al. 2009; Svendsen et al. 2012; Santus et al. 2012; Yeatts et al. 2012). An increase of 50 µg/m³ NO₂ (1 h maximum) was linked to a 2.6% increase in asthma admissions as well as

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a 1.3% increase in general mortality in 15 European cities (Touloumi et al. 1997; Chauhan and Johnston 2003). The inhalation of NO₂ has the potential to exert a “pro-allergic” effect on the bronchial epithelium of healthy human airways (Pathmanathan et al. 2003) and could augment the allergic inflammation in asthmatics (Barck et al. 2005). A limited number of studies have linked NO₂ inhalation to epithelial damage, airway inflammation, hyperresponsiveness, and functional changes in the lungs of rodents (Proust et al. 2002; Hussain et al. 2004; Poynter et al. 2006; Bevelander et al. 2007). So far, the role of NO₂ for asthma is still unclear, and the exact mechanisms underlying the adverse pulmonary effects are not well understood.

Asthma is a chronic inflammatory respiratory disease characterized by inflammation of airway, airway hyperresponsiveness (AHR), and hypersecretion of mucus as main characteristics (Kim et al. 2013). As indicated by the cumulative evidence, asthma is closely related to T helper type (Th)1/Th2 imbalance and their characteristic cytokine secretion (Guo et al. 2014). Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13, are thought to drive the accumulation of serum immunoglobulin E (IgE), eosinophils in the lungs of asthmatic patients, thus further inducing AHR (Foster et al. 2001; Guo et al. 2014), whereas Th1 cells release interferon (IFN)- γ and inhibit the development of Th2 (Kuo et al. 2001; Ji et al. 2014). The Th1 differentiation and Th2 differentiation are also thought to be regulated by transcription factors T-box expressed in T cells (T-bet) and GATA-binding protein-3 (GATA-3), respectively (Szabo et al. 2000; Hirahara et al. 2008). Moreover, interleukin-4/signal transducer and activator of transcription-6 (IL-4/STAT6) pathway plays a pivotal role in the pathogenesis of allergic asthma (Zhu et al. 2004; Oh et al. 2010; Ashino et al. 2014). IL-4R $\alpha^{-/-}$ or STAT6 $^{-/-}$ mice fail to produce mucus hypersecretion, goblet cell hyperplasia, and AHR (Grunig et al. 1998; Kuperman et al. 1998; Cohn et al. 1999). In addition, extracellular-regulated kinase (ERK)1/2, T cell-specific protein-tyrosine kinase (Lck), and nuclear factor- κ B (NF- κ B) may participate in asthmatic inflammation (Hart et al. 1998; Yamashita et al. 2005; Kemp et al. 2010).

The aim of this study was to confirm the role of NO₂ pollution on pulmonary injuries and enhance the understanding of possible mechanism of asthma susceptibility promoted by NO₂ inhalation. In the present study, firstly, we established NO₂ exposure model by treating rats with 2 and 5 mg/m³ NO₂ for 28 days to determine the possibility that exposure to NO₂ may promote airway inflammation and increase the susceptibility to asthma. Secondly, we developed a rat model of asthma, consisting of 3 weeks of 5 mg/m³ NO₂ exposure, followed by OVA sensitization and aerosol challenge. Pathological alteration, OVA-specific IgE, mucin gene (MUC5AC), and the pro-inflammation factors [IL-1 β , IL-6, and intercellular adhesion molecule (ICAM)-1], Th1/Th2 cytokines, transcription factors, and IL-4/STAT6 pathway were

investigated. The results will offer the base for theory of early diagnosis, assessment of risk, and clinical therapy for high-risk people in polluted areas.

Materials and methods

Animals

Adult male Wistar rats (about 6 weeks old) with a body weight of 150–157 g were purchased from Experimental Animal Center, Academy of Military Medical Sciences of Chinese PLA (Beijing, China). The animals were housed in a standard animal room (24 $\pm$ 2 °C) with a relative humidity of 50 $\pm$ 5% and a 12-h light/dark cycle. The animals were received food and water ad libitum.

All animal experiment procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of the People's Republic of China. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Shanxi University (No. HZ20140503).

NO₂ exposure

For the first section (Fig. 1a, experiment 1), animals were arbitrarily divided into three groups and each consisted of six rats. Two groups were exposed to 2 and 5 mg/m³ NO₂ for 28 days (days 0–27, 5 h/day), respectively. For the same time period, control group was exposed to clean, filtered air.

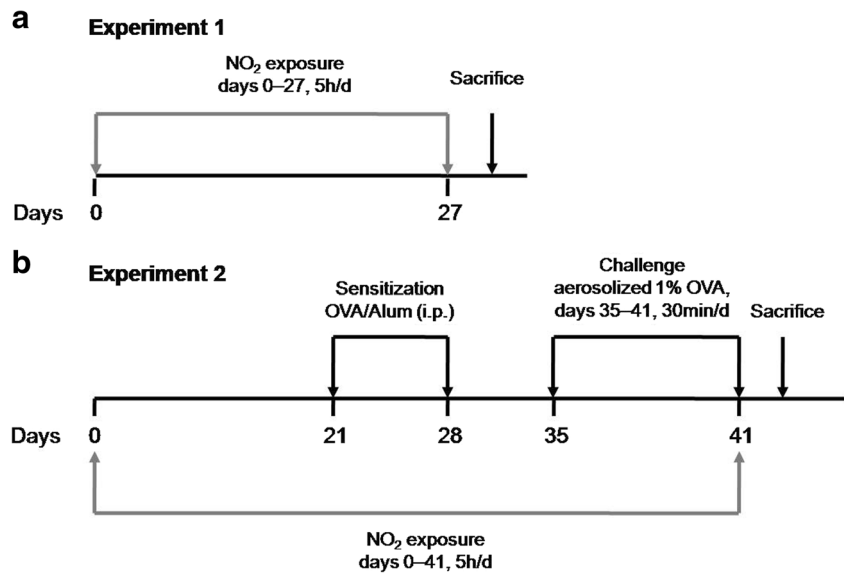
For ovalbumin model of allergic asthma (Fig. 1b, experiment 2), animals were randomly assigned into three groups of 10 animals each: control group, OVA group (model group), and NO₂ + OVA group. The rats in NO₂ + OVA group were exposed to NO₂ (5 mg/m³) for 42 days (days 0–41, 5 h/day). For the same time period, control group and OVA group of rats were exposed to clean, filtered air.

High-purity liquid NO₂ (99.99%) for exposure was purchased from Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd., Beijing, China. As described previously, dynamic inhalation method was used for exposure (Han et al. 2013). The NO₂ concentration in the chamber was maintained at constant levels and was measured every 30 min by the method described by Kumie et al. (2009).

OVA sensitization/challenge

As shown in Fig. 1b, according to a previous method reported by Chapman et al. (2011), the rats in group of OVA and NO₂ + OVA were sensitized by intraperitoneal (i.p.) injection with 100 mg of OVA (grade V; Sigma-Aldrich), dispersed in 100 mg aluminum hydroxide (alum, Sigma-Aldrich) in 1 ml normal saline after exposed to NO₂ for 21 days (day 21). A

Fig. 1 The experimental protocol. **a** Male Wistar rats were exposed to 2 and 5 mg/m³ NO₂ or clean air for 28 days (days 0–27, 5 h/day), respectively. **b** Male Wistar rats were exposed to 5 mg/m³ NO₂ or clean air for 42 days (days 0–41, 5 h/day). Rats were sensitized by intraperitoneal (i.p.) injection with ovalbumin/aluminum (OVA/alum) or saline on days 21 and 28 and then were challenged with aerosolized 1% OVA or saline for 7 days (days 35–41, 30 min/day). Rats were sacrificed within 24 h after the last time exposure or challenge



booster injection of this OVA–alum mixture was given 7 days later (day 28). After 7 days, the rats were challenged with aerosolized 1% OVA in 1 ml normal saline for 7 days (days 35–41, 30 min/day). The controls received normal saline following the same protocol. Rats were sacrificed within 24 h after the last challenge, and samples (lung tissue, serum, and bronchoalveolar lavage fluid) were collected for analyses.

Bronchoalveolar lavage and cell differentials

Bronchoalveolar lavage fluid (BALF) was obtained according to the method reported by Liu et al. (2008). The trachea was surgically exposed and cannulated, and then, 3 ml of sterile normal saline was instilled three times into the lung. Aliquots were pooled from individual animals. BALF was centrifuged at 1000 rpm for 10 min, and the cell pellets were then resuspended in 1.0 ml normal saline. Total cell count was performed using a hemacytometer. Smears were stained with Wright–Giemsa, and differential cell counts (macrophage, neutrophil, lymphocyte, and eosinophil) in BALF were determined microscopically by counting ≥ 500 cells/slide.

Serum OVA-specific IgE and IL-4 analysis

After rats were sacrificed, blood was collected into serum separator tubes, centrifuged at 2500 rpm for 20 min, and frozen at -80°C for analysis. Lung tissues were homogenized and centrifuged. The supernatant was collected for IL-4 determination. OVA-specific serum IgE levels in serum and IL-4 content in lung tissue were measured by ELISA kit (Xi Tang Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's guidelines. Absorbance values were read at 450 nm on a Thermo model 3001 microplate reader. OVA-specific serum IgE and IL-4 contents were quantified using

standard curve. The sensitivities of the ELISA kits were 8 ng/ml for OVA-specific serum IgE and 16 pg/ml for IL-4. The coefficient of variation (CV) of ELISA kits was $< 10\%$ (intra-assay and inter-assay) for OVA-specific serum IgE and $< 10.2\%$ (intra-assay and inter-assay) for IL-4.

Histopathology and transmission electron microscopy

Lungs were removed and fixed in 10% formalin. After fixation, the tissues were embedded in paraffin and cut into 4- μm sections. Sections were affixed to slides, stained with hematoxylin and eosin (HE), and examined by light microscopy with $\times 400$ magnification (Olympus, Japan).

For transmission electron microscopy (TEM), about 1-mm³ slices of the lungs were fixed with 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, samples were post-fixed in 2.5% osmium tetroxide for 1 h, dehydrated with ethanol, and embedded in resin. Then, 70–80-nm-thickness sections were cut and routinely stained for contrast with uranyl acetate and lead citrate. Images were taken using transmission electron microscope (JEOL, JEM1011, Japan).

Quantitative RT-PCR

Lung tissue was homogenized in TRIzol (Invitrogen, USA), and total RNA was extracted according to the manufacturer's instructions. Residual genomic DNA was removed by treatment with DNase I. RNA was converted to cDNA using reverse transcription kit (TaKaRa, China) according to the manufacturer's recommendations. RT-qPCR was performed using SYBR Premix Ex Taq II kit (TaKaRa, China). GAPDH serves as the internal control, and the relative quantification of target

genes was measured. The sequences of PCR primers are shown in Table 1.

Western blotting

Proteins were isolated from lung tissues using ice-cold lysis buffer with protease and phosphatase inhibitors. The protein concentration was measured by Bradford method. Proteins (30 μ g) were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% BSA in Tris-buffered saline (TBST, 0.1% Tween 20) and then incubated with first antibodies p-NF- κ B p65 (Santa Cruz Biotechnology), p-ERK1/2, p-Lck, p-STAT6, ERK1/2, STAT6 (Affinity Biotechnology), and β -actin (Cell Signaling Technology) at dilution of 1:500 (for p-ERK1/2, p-Lck, p-STAT6, ERK1/2, STAT6), 1:100 (for p-NF- κ B p65), or 1:5000 (for β -actin) at 4 °C overnight. The blots were washed in TBST, incubated with secondary antibody (IRDye 800CW, LI-COR) at dilution of 1:5000, and then visualized with LI-COR Odyssey infrared fluorescence instrument.

Statistical analysis

Data were presented as mean $\pm$ standard error (SE). One-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) test was used to determine significant differences between groups, using SPSS 17.0 software. Differences were considered significant when $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

To determine the possibility that NO₂ exposure may promote allergic airway inflammation and increase susceptibility to allergic asthma, we firstly established exposure model by treating rats with 2 and 5 mg/m³ NO₂ for 28 days immediately followed by the investigation of pathological features and the levels of pro-inflammatory factors, MUC5AC, and Th1/Th2 cytokines in the lung of normal rats.

NO₂ exposure induces pulmonary inflammatory response, mucus formation, and Th1/Th2 imbalance in the lung of normal rats

HE staining was used to examine the histopathologic changes in the lung tissues of normal rats exposed to NO₂. As Fig. 2a indicated, exposure to NO₂ induced airway inflammation in the lung demonstrated by an increase in airway epithelial cell damage, infiltration of inflammatory cells, and visible mucus secretion compared with the control.

We also investigated the levels of IL-1 β , ICAM-1, and mucus-associated gene MUC5AC in the lungs after NO₂ exposure. As expected, consistent with the histopathologic result, NO₂ exposure markedly enhanced the messenger RNA (mRNA) expression of MUC5AC, IL-1 β , and ICAM-1 in a dose-dependent manner in the lung tissues of rats compared to the control (1.47-fold, 4.68-fold of control for MUC5AC, $P < 0.01$; 1.56-fold, 1.80-fold of control for IL-1 β , $P < 0.05$; 1.62-fold, 1.91-fold of control for ICAM-1, $P < 0.01$) (Fig. 2b). As shown in Fig. 2c, compared with control rats, 2 and 5 mg/m³ NO₂ exposure increased the levels of IL-4 and GATA-3 with a concentration-dependent property in the lung (1.12-fold, 1.59-fold of control for IL-4, $P < 0.01$; 1.28-fold, 1.36-fold of control for GATA-3, $P < 0.01$), whereas decreased the mRNA expression of IFN- γ and T-bet (0.81-fold, 0.66-fold of control for IFN- γ , $P < 0.05$; 0.78-fold, 0.61-fold of control for T-bet, $P < 0.05$).

According to the above results, we found that NO₂ inhalation could cause lung inflammation and injury, as well as mucus formation. At the same time, upregulation of GATA-3 and IL-4 levels and reduction of IFN- γ and T-bet expression occurred in the lung, which indicates that the imbalance of Th1/Th2 was induced by NO₂. Interestingly, results obtained here are in many ways similar to the clinical phenomenon observed from asthmatics. Taken together, the above results demonstrate that exposure to NO₂ stimulates the generation of Th2 lung inflammation, resulting in allergic asthma-like features.

NO₂ exposure exacerbates lung inflammation and Muc5ac expression in the lung of OVA-sensitized/challenged rats

In histopathologic examination (Fig. 3a), few inflammatory cells were observed in control group. In contrast, we found representative pathological features of inflammation (perivascular and peribronchial inflammation) and alterations of structure in the lungs of OVA or the combined NO₂ plus OVA exposure rats, which were observed in asthmatic. The pathological changes, which include inflammatory cell infiltration in peribronchial and perivascular spaces, airway wall thickness and epithelial folding, and hyperplasia of basement membrane, are commonly linked to asthma in humans. In addition, compared with OVA-challenged group, the histopathologic changes were severer in the NO₂ plus OVA group.

Consistent with histopathologic and inflammatory changes, evaluation of the mRNA level of MUC5AC in lung tissue demonstrated marked increase in the lung of rat subjected to OVA (Fig. 3b), relative to the control. Moreover, an obvious enhancement of MUC5AC expression was observed in the lung of rats subjected to NO₂ plus OVA compared with OVA exposure alone.

Table 1 Primer sequences used for real-time RT-PCR

Gene	Accession no.	Sequence	
GAPDH	NM_017008.4	Forward primer	5'-ATG TAT CCG TTG TGG ATC TGA C-3'
		Reverse primer	5'-CCT GCT TCA CCA CCT TCT TG-3'
T-bet	NM_005109.3	Forward primer	5'-GGG CCC CCT CTC CTT TTG ATA A-3'
		Reverse primer	5'-CGC CCC GTG TCT GTT TTC TAA GTT-3'
GATA-3	NM_133293.1	Forward primer	5'-GTC TTC AGC CCA CCA CCC CAT TAC-3'
		Reverse primer	5'-GCC GGT TCT GCC CAT TCA TTT TAT-3'
ICAM-1	NM_012967.1	Forward primer	5'-TTC AAC CCG TGC CAG GC-3'
		Reverse primer	5'-GTT CGT CTT TCA TCC AGT TAG TCT-3'
IFN- γ	NM_138880.2	Forward primer	5'-CGC CGC GTC TTG GTT TTG-3'
		Reverse primer	5'-GCT GAT GGC CTG GTT GTC TTT C-3'
IL-1 β	NM_031512.2	Forward primer	5'-GCC TCA AGG GGA AGA ATC TAT ACC-3'
		Reverse primer	5'-GGG AAC TGT GCA GAC TCA AAC T-3'
IL-6	NM_012589.2	Forward primer	5'-TCTGTTGTGGGTGGTATCCTCTG-3'
		Reverse primer	5'-TCCAGCCAGTTGCCCTTCTTG-3'
IL-4R α	NM_133380.2	Forward primer	5'-GGC TGC GGC TGT TGC TAT GAT GAA-3'
		Reverse primer	5'-AAC CCC GGC CTT AGC ACA GAC CTC-3'
JAK1	NM_053466.1	Forward primer	5'-GAT CCA GTG GCG GCA AGA AAC C-3'
		Reverse primer	5'-AGC GGG GGA GCC ACA TCA GTA-3'
JAK3	NM_012855.2	Forward primer	5'-GGA GAG TGA GGC GCA TGT GAA GAT-3'
		Reverse primer	5'-CAG ATT GGC GGG AGA AGA TGT TGT-3'
STAT6	NM_001044250.1	Forward primer	5'-GCG GAA GAC AGC TGC CTA ACT CA-3'
		Reverse primer	5'-TCC CCG ATT GCC CAT AAG AAG A-3'
Muc5ac	XM_003749022.2	Forward primer	5'-CTG CCG TCC CAA CCA AGA AGT C-3'
		Reverse primer	5'-TGG CCA TCA CGC TCC TAA TCA G-3'

NO₂ exposure increases levels of OVA-induced peribronchial collagen deposition and eosinophilic inflammation

The detailed ultrastructure of the lung was studied by using TEM. As is shown in Fig. 4, the interalveolar areas were filled with inflammatory cells, infiltrate, and exudates in the lungs of OVA-challenged group. Also, hyperplasia of alveolar type II cells and collagen deposition could be observed (Fig. 4c, d), which were few in the control group (Fig. 3a, b). Furthermore, compared to the OVA group, rats subjected to NO₂ plus OVA (Fig. 4e, f) showed obvious influx of inflammatory cells into the lung parenchyma, thickened sub-epithelial basement membrane as well as increased levels of transverse and longitudinal collagen fibrils, which seem to be more densely packed in lung tissues.

NO₂ exposure increases leukocytic infiltration in the lung of OVA-sensitized/challenged rats

As anticipated (Table 2), the total numbers of leukocytes, lymphocytes, neutrophils, and eosinophils in BALF in rats exposed to OVA were increased compared to control.

Interestingly, rats subjected to OVA after exposure to NO₂ showed dramatic increases in the proportion of neutrophils and eosinophils, with concomitant decrease in macrophages. These results suggested that NO₂ exposure strongly promoted leukocytic infiltration in the lung following subsequent OVA challenge.

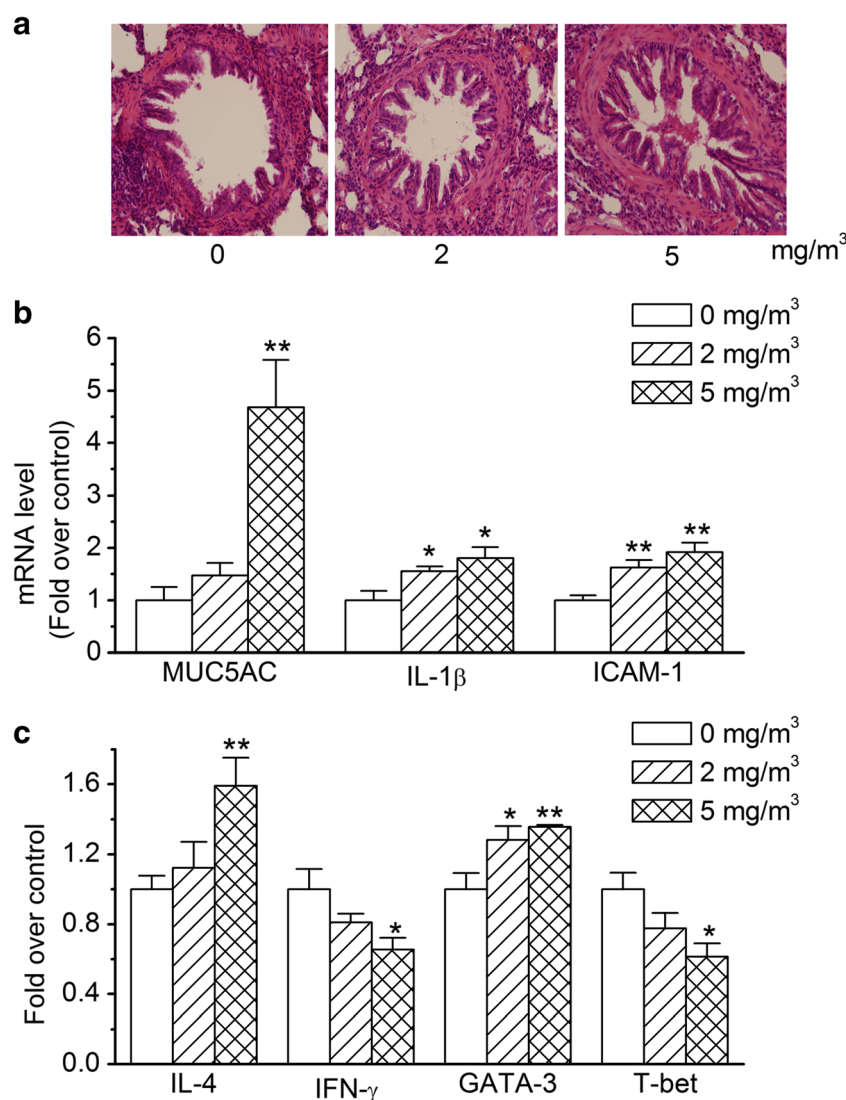
NO₂ exposure increases OVA-specific IgE production

Elevation of serum IgE level is a hallmark of the Th2-dominant inflammatory response (Liu et al. 2008). Results indicated that compared with control, the levels of OVA-specific IgE in serum were markedly elevated in rats exposed to OVA or NO₂ plus OVA. Interestingly, rats subjected to NO₂ plus OVA demonstrated a markedly increase in OVA-specific IgE level, compared with exposure to OVA alone (Fig. 3c).

NO₂ exposure promoted imbalanced mRNA levels of Th1 and Th2 cytokines

As is shown in Fig. 4, in OVA group rats, Th2 cytokine (IL-4) (Fig. 5a) and several pro-inflammatory factors (IL-1 β , ICAM-1, and IL-6) (Fig. 5b–d) were significantly

Fig. 2 Effects of NO₂ exposure on lung histological changes (a), mRNA expression of MUC5AC and pro-inflammatory cytokines (IL-1 β and ICAM-1) (b), and levels of Th1/Th2 cytokines (IL-4, IFN- γ , GATA-3, and T-bet) (c) in normal rats. H&E staining, magnification $\times 400$, only a representative picture is shown for each group. Total RNA was extracted from each lung, and the mRNA expression of MUC5AC, IL-1 β , ICAM-1, IFN- γ , GATA-3, and T-bet was determined by RT-PCR as described in “Materials and methods” section. The level of IL-4 was measured by ELISA. Value in each treated group was expressed as a fold increase compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means $\pm$ SE ($n = 6$); * $P < 0.05$, ** $P < 0.01$ versus control group



elevated compared to control, while Th1 cytokine (IFN- γ) was markedly decreased (Fig. 5e). As expected, NO₂ exposure dramatically promoted the increase in the level of IL-4, IL-1 β , ICAM-1, and IL-6 and the decrease in the level of IFN- γ in NO₂ plus OVA group rats. The results suggested that NO₂ exposure could promote the imbalance of Th1/Th2 cytokines. Also, these results indicated that NO₂ exposure is the predominant risk factor in driving the lung toward Th2 polarization.

NO₂ exposure increases the activation and expression of GATA-3, ERK1/2, LCK, and NF- κ B

As shown in Fig. 6, compared to control group, mRNA level of GATA-3 (Fig. 6a) and activation of Lck, ERK1/2, and NF- κ B (Fig. 6c–e) in OVA group were markedly increased, whereas the level of T-bet was significantly

suppressed (Fig. 6b). Under this condition, in NO₂ plus OVA group, the increase in the mRNA expression of GATA-3 and the activation of p-Lck, p-ERK1/2, and p-NF- κ B were markedly promoted by NO₂ treatment as well as the decrease in the mRNA expression of T-bet, but total level of ERK1/2 was not apparently affected.

NO₂ exposure enhances activation of IL-4/STAT6 pathway

As shown in Fig. 7, in the lungs of OVA group, mRNA expression of IL-4R α , JAK1, JAK3, and STAT6 and protein levels of STAT6 and p-STAT6 were increased compared to the control group. Interestingly, in the lungs of NO₂ plus OVA group, expression of IL-4R α , JAK1, JAK3, and STAT6 and activation of p-STAT6 were significantly elevated as compared with OVA group.

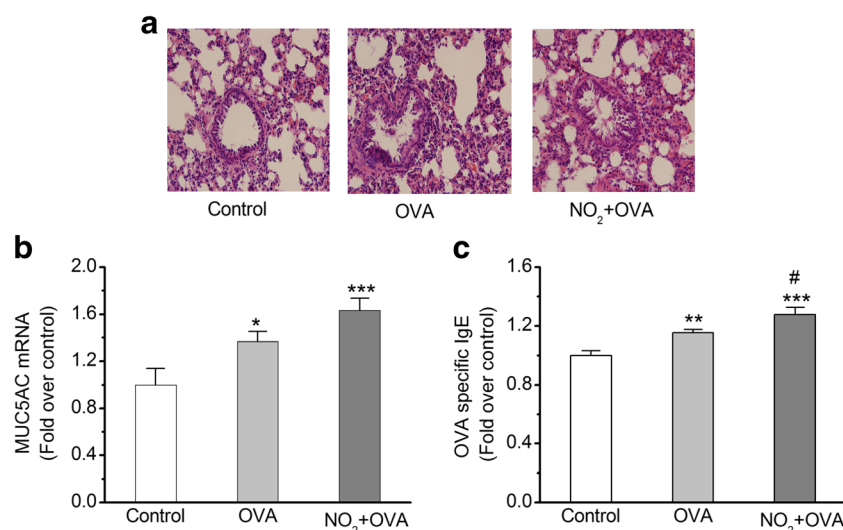


Fig. 3 Effects of NO₂ exposure on lung histological changes (a), MUC5AC mRNA expression (b), and serum OVA-specific IgE level (c) in experimental rats. H&E staining, magnification $\times 400$, only a representative picture is shown for each group. Total RNA was extracted from each lung, and the mRNA expression of MUC5AC was determined by RT-PCR as described in “Materials and methods” section.

The level of OVA-specific IgE in serum was measured by ELISA. Value in each treated group was expressed as a fold increase compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means $\pm$ SE ($n = 10$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group and # $P < 0.05$ versus OVA group

Discussion

NO₂ is a primary air pollutant that could cause health problems to humans while present in both indoor and outdoor air,

notably to those who live in a heavily polluted area (Hedberg et al. 1989; Krishna et al. 1998). Currently, national ambient air quality standards for NO₂ in China is 0.04 mg/m³ annual arithmetic mean (Zhao et al. 2016). The ambient levels of NO₂

Fig. 4 Transmission electron micrographs of the lungs taken from control group (a, b), OVA group (c, d), and NO₂ + OVA group (e, f). Lung sections were prepared as described in “Materials and methods” section. Site of collagen bundles (arrow); alveolar type II epithelial cells (II). EOS eosinophils. Magnification, $\times 6000$. Data are representative of three rats/group. Bar = 5 μ m

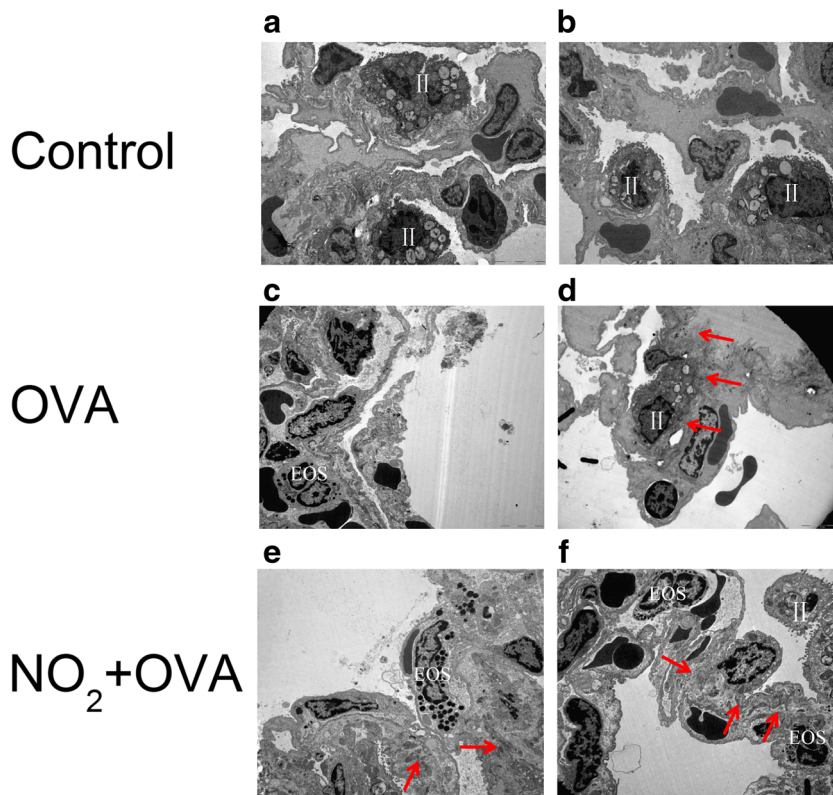


Table 2 Leukocytic composition of bronchoalveolar lavage fluid cells

Leukocyte sub-type	Control	OVA	NO ₂ + OVA
Total BALF leukocytes	$8.2 \pm 2.31 \times 10^4$	$45.6 \pm 7.56 \times 10^4$	$53.8 \pm 6.23 \times 10^4$
Macrophages (%)	17.3 ± 1.54	14.5 ± 3.25	$9.5 \pm 1.45^{*#}$
Lymphocytes (%)	73.5 ± 8.59	84.8 ± 7.12	$90.3 \pm 6.34^*$
Eosinophils (%)	0 ± 0.00	$0.3 \pm 0.11^*$	$1.2 \pm 0.35^{***##}$
Neutrophils (%)	2.2 ± 0.78	$3.9 \pm 0.61^*$	$5.2 \pm 1.62^{*#}$

Bronchoalveolar lavage fluid (BALF) was collected within 24 h after the last OVA challenge. The total cells and differential cells in BALF were counted as described in “Materials and methods” section. Data were expressed as means $\pm$ SE ($n = 10$)

* $P < 0.05$, *** $P < 0.001$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus OVA group

vary with traffic densities and meteorological conditions. In urban areas' heavy motor vehicle traffic, NO₂ concentration in outdoor air can reach up to 4 ppm, which markedly exceeds the standard (Chauhan et al. 1998). In this study, NO₂ concentrations of 2 and 5 mg/m³ (approximately 1.33 and 2.66 ppm), which may be considered to be beyond the normal level in the environment, were used with several aspects taken into consideration. Firstly, animals were exposed to regular periods of extended exposure with recess periods between exposures (i.e., 5 h/day, for 42 days, with 19 h between exposures), whereas in daily life, exposure to air pollutants for individuals is often long term or even lifelong. This may model an occupational exposure of individuals to the toxic gas. Secondly, rat is an obligate nose breather, and a part of the inhaled NO₂ is trapped in the nasal passages. Although NO₂ levels in this

study were higher than that in ambient air, actually, NO₂ concentration that reaches the lung tissue may be far lower than 2 mg/m³. Thirdly, concentrations of NO₂ used in rodent studies vary widely (0.2–170 ppm), as do the durations of exposure (5 h–28 days) (Bevelander et al. 2007), while studies in human have been performed using 2 ppm (Pathmanathan et al. 2003). Therefore, in this study, NO₂ concentration of 5 mg/m³ was chosen to represent a sub-chronic, moderate exposure to elicit minor lung damage without pain or distress, and our data may substantially reflect the adverse effect of long-term or lifelong NO₂ exposures on human health.

Epidemiological studies, which include in vivo and in vitro studies, have proved that NO₂ exposure was associated with asthma severity, disease exacerbation, and lung inflammatory response (Proust et al. 2002; Hussain et al. 2004; Poynter et al.

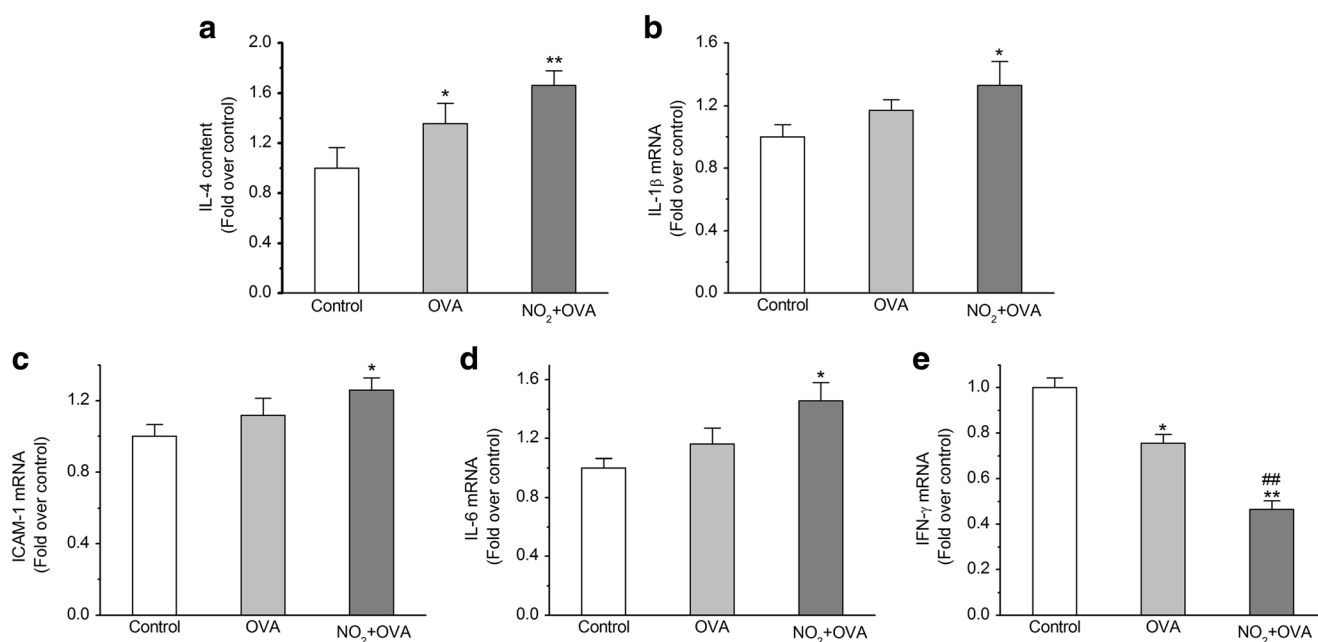


Fig. 5 Effects of NO₂ exposure on IL-4 level (a) and mRNA expression of IL-1β (b), ICAM-1 (c), IL-6 (d), and IFN-γ (e) in lung tissues of experimental rats. The level of IL-4 in lung homogenate was measured by ELISA. Total RNA was extracted from each lung, and the mRNA expressions of IL-1β, ICAM-1, IL-6, and IFN-γ were determined by

RT-PCR as described in “Materials and methods” section. Value in each treated group was expressed as a fold increase compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means $\pm$ SE ($n = 10$); * $P < 0.05$, ** $P < 0.01$ versus control group and # $P < 0.05$, ## $P < 0.01$ versus OVA group

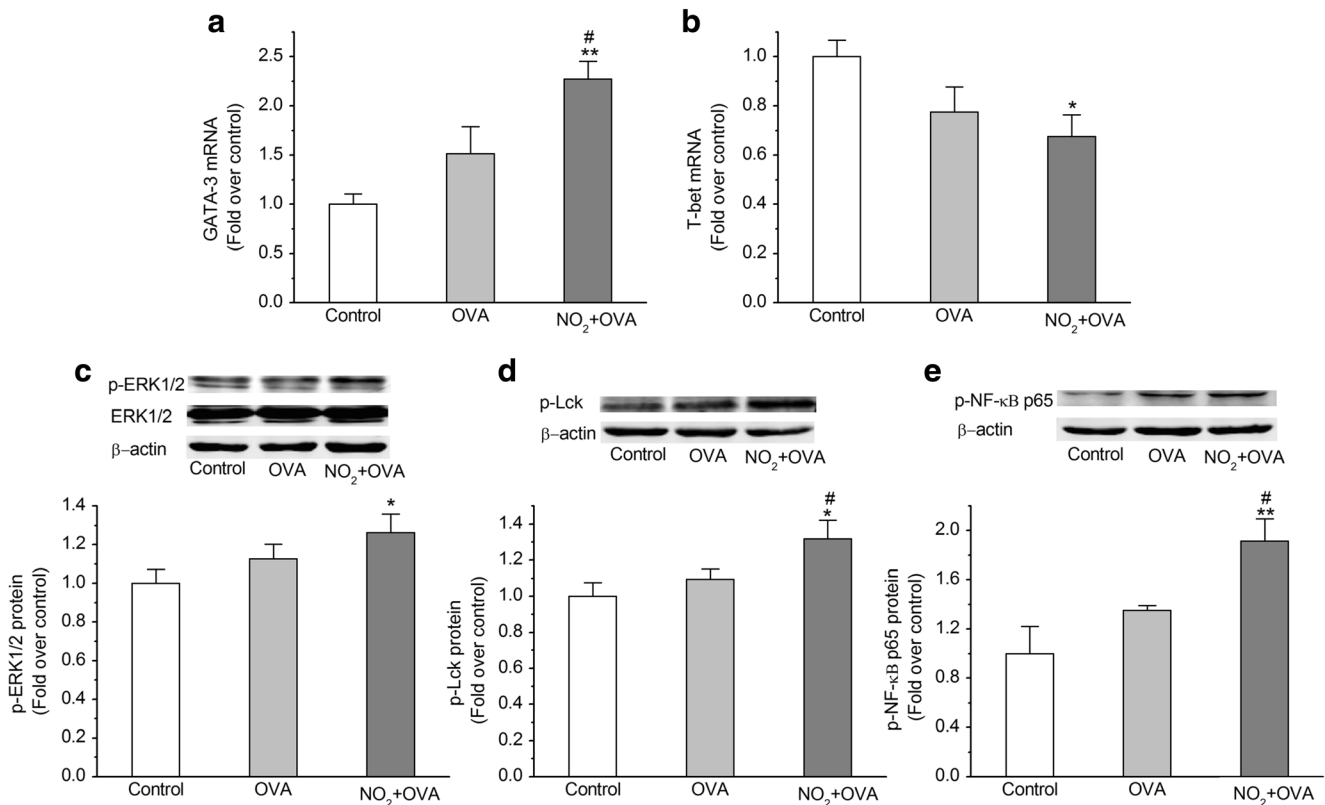


Fig. 6 Effects of NO₂ exposure on mRNA expressions of GATA-3 (a) and T-bet (b) and protein expressions of ERK1/2, p-ERK1/2 (c), p-Lck (d), and p-NF-κB p65 (e) in lung tissues of experimental rats. Total RNA was extracted from each lung, and the mRNA expressions of GATA-3 and T-bet were determined by RT-PCR as described in “Materials and methods” section. The levels of ERK1/2, p-ERK1/2, p-Lck, and p-NF-κB

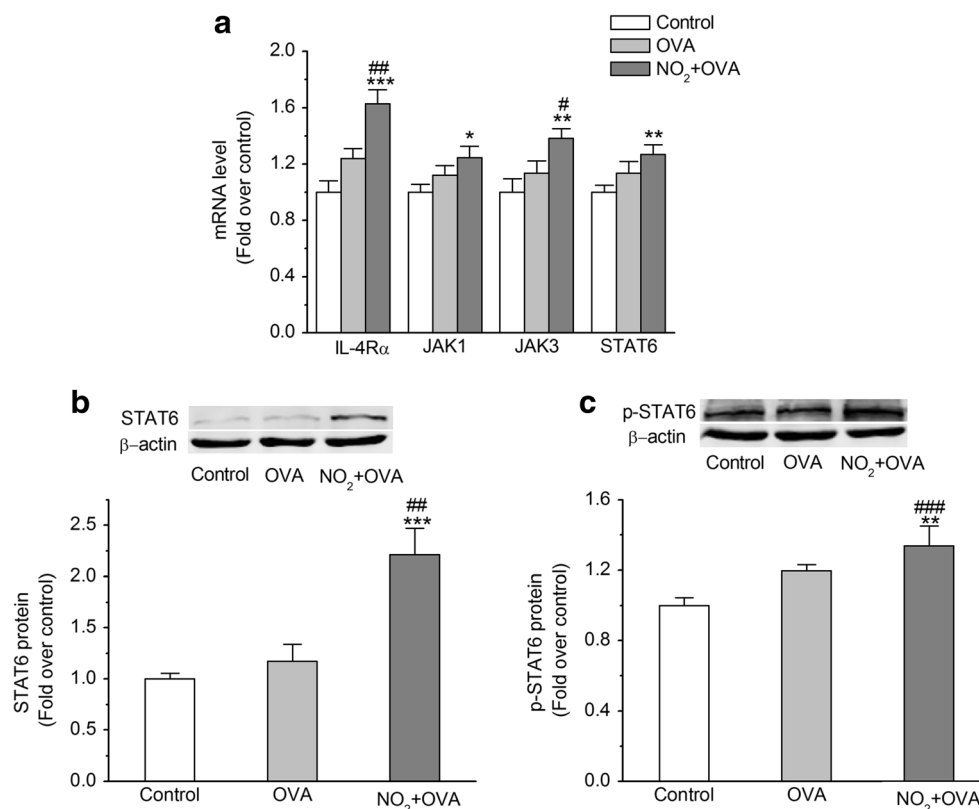
p65 in lung tissues were measured by western blot. Value in each treated group was expressed as a fold increase compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means ± SE (*n* = 10); **P* < 0.05, ***P* < 0.01 versus control group and #*P* < 0.05 versus OVA group

2006; Bevelander et al. 2007; Liu et al. 2009; Santus et al. 2012; Svendsen et al. 2012; Yeatts et al. 2012). However, the exact mechanisms underlying the allergic inflammatory response to NO₂ exposure are poorly understood. To investigate whether NO₂ inhalation could enhance allergic airway inflammation and the mechanisms involved, we set up NO₂ exposure model and developed an ovalbumin-induced rat model of asthma, respectively.

Airway inflammation is one of the hallmark features of allergic asthma and is related to higher level of allergen-specific IgE and increased number of inflammatory cells (Guo et al. 2014). The present study showed that exposure to NO₂ induced increases in airway epithelial cell damage, infiltration of inflammatory cells, and visible mucus excretion as well as mRNA expression of ICAM-1 and IL-1β in the lungs of normal rats. Furthermore, inhalation of NO₂ also could aggravate allergic airway inflammation, demonstrated by an increase in histopathologic changes and elevated expressions of pro-inflammatory factors (IL-1β, ICAM-1, and IL-6) in the lungs as well as increased leukocytic infiltration (lymphocytes, neutrophils, and eosinophils) in BALF and serum levels of OVA-specific IgE. Airway inflammation is

generally accompanied by mucus secretion, which contributes to airway obstruction. Clinical studies also showed that mucus hypersecretion is a primary characteristic of asthma. In patients with acute and chronic asthma, mucus was excessively produced by hyperplastic goblet cells (Ordonez et al. 2001). In our experiments, the expression of mucus-associated gene MUC5AC was enhanced by the inhalation of NO₂ in the lungs of normal and OVA-sensitized rats. In addition, TEM observation provided further evidences that inflammatory cells and infiltrate, collagen deposition, and thickness of air-blood barrier were increased as well as thickened sub-epithelial basement membrane in the lungs of rats exposed to NO₂ plus OVA. It has been showed that characteristic structural changes such as increase in collagen deposition and hypertrophy and hyperplasia of smooth muscle were considered as the characteristics of the tissue alteration in asthmatic patients and animals (Barnes 2008a; Oberholzer and Pretorius 2009). The results were consistent with previous literature that shows that NO₂ exposure can induce eosinophilic inflammation and metaplasia of mucus cell in the lung of mouse, as well as OVA-specific IgE and Th2 immune response (Bevelander et al. 2007). Therefore, combined with previous

Fig. 7 Effects of NO₂ exposure on mRNA expressions of IL-4R α , JAK1, JAK3, and STAT6 (a) and protein expressions of STAT6 (b) and p-STAT6 (c) in lung tissues of experimental rats. Total RNA was extracted from each lung, and the mRNA expressions of IL-4R α , JAK1, JAK3, and STAT6 were determined by RT-PCR as described in “Materials and methods” section. The protein levels of STAT6 and p-STAT6 in lung tissues were measured by western blot. Value in each treated group was expressed as a fold increase compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means $\pm$ SE ($n = 10$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group and # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus OVA group



investigations, our study gives strong evidence that exposure to NO₂ before OVA-sensitized/challenged could enhance allergic airway inflammation and allergic asthma-like features, which may contribute to the development of asthma and the augment of susceptibility to allergic asthma.

The mechanisms by which exposure to NO₂ injures the lungs and promotes allergic airway inflammation and the susceptibility to allergic asthma remain to be determined. Allergic asthma is mainly regulated by Th1/Th2 cells via the secretion of characteristic cytokines (Guo et al. 2014). IL-4, a key Th2 cytokine, plays an important role in Th2 cell differentiation from Th0 cells, as well as IgE production by switching of B cells (Barnes 2008b; Ji et al. 2015). In contrast, IFN- γ , a principal Th1 cytokine, has been shown to be critical in repressing Th2 immune responses and IgE synthesis to IgE synthesis to suppress the progress of asthma (Guo et al. 2014). Accordingly, ratio of Th1/Th2 cells can be assessed by ratio of IFN- γ /IL-4, and cytokine level is a significant indicatrix in the assessment of asthma. Our study shows that exposure to NO₂ distinctly elevated the level of IL-4 and suppressed the expression of IFN- γ in the lungs of normal rats and NO₂ plus OVA group rats. This result suggests that inhalation of NO₂ could aggravate the Th1/Th2 imbalance. Earlier studies suggested that Th1 and Th2 cells are out of balance in patients with allergic asthma (Shi et al. 2011). However, an elevation of Th17 cells, the absence of Treg cells, and Th17/Treg imbalance are associated with moderate to severe asthma (Shi et al.

2011), suggesting that an imbalance in Th17/Treg may also play an important role in the pathogenesis of asthma (Ji et al. 2014; Wan and Wei 2015; Lim et al. 2016). Further work is required to clarify the relationship between NO₂ exposure and Th17/Treg and assess which of these cells plays a more important role in allergic asthma induced by NO₂.

GATA-3 is the Th2 master transcription factor by promoting Th2 cytokine expression (Hirahara et al. 2008). On the contrary, T-bet is to act as a prime regulator for IFN- γ production and Th1 cell differentiation. T-bet-deficient cells of both in vivo and in vitro appeared severe deficiencies in Th1 cell differentiation (Szabo et al. 2000), and lack of T-bet can spontaneously induce airway hypersensitivity in T-bet^{-/-} mice (Finotto et al. 2002). IFN- γ has been suggested to enhance the expression of T-bet, which leads to a potential positive feedback loop during Th1 differentiation (Afkarian et al. 2002). Meanwhile, the induction of GATA-3 inhibits the differentiation of Th1 cell, not only by generating IL-4 but also by restraining the Th1 cell master transcription factor T-bet (Ho and Glimcher 2002). T-bet/GATA-3 balance is also regarded as a marker of the balance of Th1/Th2 cytokine (Chakir et al. 2003). In this study, consistent with the change of IL-4 and IFN- γ , we observed that exposure to NO₂ strongly promoted the increase in GATA-3 expression, as well as the suppression of T-bet level in normal rats and OVA-sensitized rats. Combined with the above study, the results imply that exposure to NO₂ promoted the Th1/Th2 imbalance and Th2 polarization, which may contribute to explain

the increased risk for allergic asthma among individuals long term exposed to NO₂.

Activation of IL-4/STAT6 pathway is important in the pathogenesis of asthma. IL-4 signals through IL-4R α , JAK1/3, and STAT6 to regulate Th2-related genes and IgE class switching (Zhu et al. 2004). As a type I receptor, IL-4R α confers ligand binding specificity and signal transduction for IL-4 (Oh et al. 2010). STAT6 is the primary signal transducer, and activation of STAT6 plays a crucial role in IL-4-mediated Th2 differentiation and IgE secretion (Daniel et al. 2000). STAT6^{-/-} mice showed almost no reaction to IL-4 and IL-13 and failed to develop Th2 immune responses, airway hyperresponsiveness, and eosinophilia following allergen sensitization (Akira 1999). JAK1/3 play key role in the initiation of inflammation, which have been identified as effective targets in the therapy of various inflammatory diseases (Ashino et al. 2014). Hence, we investigated the level of IL-4R α , JAK1, JAK3, and STAT6, and the results showed that NO₂ exposure also significantly elevated the expression of IL-4R α , JAK1, JAK3, and STAT6 and activation of p-STAT6 in OVA-sensitized rats. The results showed that the activation of IL-4/STAT6 pathway was promoted by NO₂ exposure. In addition to STAT6, many other factors may induce the activation of GATA3 during Th2 polarization. Previous studies showed that Lck controls GATA3 and IL-4 production, and Lck^{-/-} Th2 cells exhibited lower levels of IL-4 and GATA3 and a higher expression of T-bet and IFN- γ (Kemp et al. 2010). Moreover, activated ERK inhibits ubiquitination and degradation of GATA3 in Th2 cells (Yamashita et al. 2005), thereby increasing the level of activated GATA3. In contrast, activated Lck and ERK negatively regulate T-bet expression (Yamashita et al. 2005; Kemp et al. 2010). Multiple pro-inflammatory genes, which may cause allergic asthma, can be induced by NF- κ B (Choi et al. 2004). Our results showed that activation of Lck, ERK1/2, and NF- κ B was markedly promoted by NO₂ treatment. In a word, these results suggest that NO₂ exposure mainly promotes the IL-4/STAT6 pathway activation and related transcription factor and consequently enhances Th1/Th2 imbalance, which was partly related to NO₂-aggravated airway inflammation and mucus hypersecretion. Thus, Th2-dependent IL-4/STAT6 pathway might represent a promising target for therapeutic interventions of inflammatory diseases in polluted area.

Conclusion

The present study demonstrates that NO₂ exposure enhanced allergic airway inflammation and asthma susceptibility in rats. The underlying mechanism may attribute to the modulation of Th1/Th2 imbalance via promoting the IL-4/STAT6 signaling pathway. Our results suggest that NO₂ exposure may be a risk factor in the pathogenesis of asthma, and these findings could be of help in risk assessment of NO₂ on human, early

diagnosis of asthma, and clinical therapy by targeting the signaling pathways in NO₂-polluted areas. Further work is required to explain the role of NO₂ on asthma and its exact mechanisms.

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