Acute nitrogen dioxide (NO₂) exposure enhances airway inflammation via modulating Th1/Th2 differentiation and activating JAK-STAT pathway

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HIGHLIGHTS

- Exposure to 5 mg/m³ NO₂ led to pathological alteration in rat lung.
- NO₂ inhalation significantly stimulated MUC5AC expression.
- NO₂ inhalation obviously up-regulated IL-1β, IL-6 and ICAM-1 levels.
- NO₂ inhalation caused the imbalance of Th1/Th2 differentiation ratio.
- NO₂ inhalation activated the JAK-STAT pathway.

ABSTRACT

Nitrogen dioxide (NO₂) is an air pollutant associated with poor respiratory health, asthma exacerbation, and an increased likelihood of inhalational allergies. However, the underlying mechanisms are not clear. In the present study, the airway inflammatory response was first assessed in rats exposed to 5 mg/m³ NO₂ for seven days. The results showed that NO₂ exposure caused the pulmonary pathological alteration, and significantly stimulated MUC5AC expression. Following this, obviously up-regulated changes of pro-inflammatory cytokines (IL-1β, IL-6, and ICAM-1) were observed. Also, NO₂ inhalation induced the imbalance in the ratio of Th1/Th2 differentiation (IL-4, IFN-γ, GATA-3 and T-bet) and the activation of following JAK-STAT pathway (JAK1, JAK3 and STAT6). The findings clarify an important mechanism for NO₂ inhalation being injurious to the lung and augmenting the degree of allergic airway inflammation.

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1. Introduction

NO₂ is a free-radical gaseous component of indoor and outdoor air pollution generated during combustion processes, such as the operation of motor vehicles and biomass burning (Poynter, 2012). Peak levels of up to 0.4 ppm (ppm) are encountered in the outdoors, particularly along kerbsides in downtown areas with heavy motor vehicular traffic. Peak levels in the indoor environment can reach up to 4 ppm in garages, ferries, skating ice rinks, and kitchens with gas cookers (Pathmanathan et al., 2003). The national air standard is less than 0.08 mg/m³ in China, but the level of NO₂ in outdoor air can markedly exceed that standard during heavy traffic or rush hour in urban areas (Chan and Yao, 2008).

NO₂ poses a major problem to human health both in outdoor as well as indoor atmosphere. To date, an increasing body of epidemiologic literatures has indicated that even exposure to typical low level NO₂ can increase the risk of emergency room admissions for acute respiratory diseases and exacerbation of obstructive lung diseases in the general population (Santus et al., 2012). In addition, more and more studies have linked NO₂ exposure with respiratory tract disease and lung cancer (Samoli et al., 2006; Katanoda et al., 2011). An investigation in 17 cities of China showed that a 10 mg/m³ increase in NO₂ concentration was associated with a 2.52% increase
in risk of death from respiratory diseases (Chen et al., 2012). Especially, several epidemiological and experimental studies have shown strong links between exposure to high levels of NO2 and morbidity and mortality of asthma, and NO2 inhalation augmented the degree of allergic airway inflammation and hyperresponsiveness in rodent models of asthma (Poynter et al., 2006).

Asthma is a chronic inflammatory disease, and the typical symptoms include airway hyperresponsiveness (AHR), cough, dyspnea, wheezing and so on (Kim et al., 2013). Among the process, sputum production due to airway mucus hypersecretion is an important component marked by the increased expression of the predominant MUC5AC in the epithelium (Ordóñez et al., 2001). Also, pro-inflammatory cytokines IL-1β, IL-6 and ICAM-1 play a critical role on the human MUC5AC promoter activity (Barnes, 2008a). Asthma is considered as a complex disease, and the generally accepted view of its mechanism is that Th2 differentiation predominantly, resulting in the imbalance in the ratio of TH1/TH2 differentiation (Zhang et al., 1999) and activating the following JAK-STAT signaling pathway (Li et al., 2005; Ashino et al., 2014; Walford and Doherty, 2013).

To data, the precise mechanisms underlying the biological link between NO2 exposure and airway inflammation are not well understood. In the present study, we set up acute exposure model by treating rats with 5 mg/m3 NO2, and determined pulmonary pathological alteration, the expression of MUC5AC and the pro-inflammation (IL-1β, IL-6 and ICAM-1). Then, we further analyzed the markers representing the TH1/TH2 differentiation (IL-4/IFN-γ and GATA-3/T-bet) and the IL-4-JAK-STAT pathway (JAK1, JAK3, STAT6), to clarify the possible mechanism for NO2-induced pulmonary injuries, even exacerbation of asthma.

### 2. Materials and methods

#### 2.1. Animal exposure and treatment protocols

Male Wistar rats, weighing 170–220 g, were supplied by Experimental Animal Center, Academy of Military Medical Sciences of Chinese PLA (Beijing, China). The rats were housed in stainless steel cage (50 × 40 × 25 cm3) under standard conditions (24 ± 2 °C, 50 ± 5% humidity) with a 12-h light/dark cycle. The rats were randomly divided into 2 groups, each group containing 6 male rats. The treatment group was exposed to 5 mg/m3 NO2, and the control group was exposed to clean air. The NO2 gas was diluted with fresh air at the intake port of the chamber to yield desired concentrations; then, it was delivered to the animals through a tube positioned in the upper part of the chamber and distributed homogeneously via a fan. The NO2 concentration within the chambers was measured every 30 min by a Saltzman colorimetric method using a spectrometer calibrated at 545 nm (Kumie et al., 2009). The exposure process sustained 5 h/day for 7 days. During the exposure process, treated groups and the control group were not allowed to eat or drink anything. Lung tissue samples from the rats were collected 24 h after the last time exposure. All experiments were conducted following the guidelines and under the approval of the university.

#### 2.2. Hematoxylin–eosin (H&E) staining

The tissue was rapidly removed, washed for several times with 0.1 M phosphate buffer saline (PBS, pH 7.4), fixed in 10% formalin for 24 h at RT, dehydrated by graded ethanol and embedded in paraffin. For HE staining, sections (5–6 μm-thick) were deparaffinized with xylene, stained with hematoxylin and eosin, after that, observed by light microscopy.

#### 2.3. Transmission electron microscopy (TEM) observation

About 1 mm × 1 mm × 1 mm pieces were rapidly cut from lungs, fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M Phosphate Buffer (PB) (pH = 7.4) for 2 h at room temperature and then post-fixed in 2.5% osmium tetroxide in 0.1 M PB. After that, En bloc stain with 2% aqueous uranyl acetate for 1 h at room temperature in dark, dehydrated by graded ethanol and embedded in beam capsules. Sections, 70–80 nm-thick, were cut from the embedded tissue and collected onto grids to air dry overnight. Stained grids with uranyl acetate for 15–30 min and lead citrate for 3–15 min, and then observe under transmission electron microscope (JEOL, JEM-1011, Japan) operating at 80 kV.

#### 2.4. RNA isolation and real-time PCR

Real-time RT-PCR analysis assay was performed as described previously (Li et al., 2011). Briefly, total RNA was isolated, quantified and then synthesized to complementary DNA (cDNA) by using TRIzol Reagent (Invitrogen Life Technologies) and reverse transcription kit (TaKaRa Biotechnology Co., Ltd., Dalian) according to the manufacturers’ protocol. RT-PCR with specific primers was run on a qTOWER 2.2 Real-Time PCR (Analytik Jena AG, Jena, Germany). Each 20 μl PCR reaction contained 1 μl cDNA (3-fold dilution of original cDNA product), 10 μl SYBR Premix Ex Taq II (TaKaRa Biotechnology Co., Ltd., Dalian), 8 μl RNase Free H2O, 0.5 μl each primer. Each treatment had six samples, and each PCR reaction carried out in duplicate. MUC5AC, IFN-γ, T-bet, GATA-3, JAK1, JAK3, STAT6, IL-4Rα, GAPDH gene used two-step method, their reaction conditions were as follows: 95 °C for 30 s, 95 °C for 5 s, and respectively annealing temperature (60 °C (GAPDH, IL-4Rα), 61 °C (MUC5AC, IFN-γ), 62 °C (T-bet, GATA-3, JAK1, JAK3, STAT6)) for 20 s, 95 °C for 5 s, and respectively annealing temperature (55 °C (IL-1β), 58 °C (ICAM-1)), 20 s at 72 °C. At the end of the PCR cycle, a dissociation curve was performed to confirm amplification of a single product. Quantification of the samples was calculated by the qPCRsoft 2.1 software from threshold cycle (Ct) by interpolation from the standard curve to yield copy numbers for the target samples. The relative quantification of the expression of the target genes was measured using GAPDH mRNA as an internal control. Primer sequence information is provided in Table 1.

#### 2.5. ELISA assay

The concentrations of IL-4 of lung tissues were measured according to the instruction of the enzyme-linked immunosorbent assay (ELISA) kits (XiTang Biotechnology Co., Ltd., Shanghai).

#### 2.6. Statistical analysis

Data were presented as mean ± SE. unless stated otherwise, ANOVA was applied for between-group statistical comparison using SPSS 17.0 software. Differences were considered significantly when P < 0.05, P < 0.01, P < 0.001.

### 3. Results

#### 3.1. Effects of NO2 exposure on body weight and lung-to-body weight ratio

The rat growth was recorded once a day for seven days. During the experimental period, the mean body weight gain was not different in rats exposed to NO2 compared with negative control...
3.3. Effects of NO2 exposure on the MUC5AC mRNA expression

To determine whether NO2 stimulated MUC5AC production, we detected MUC5AC mRNA expression in the lungs. As shown in Fig. 3, MUC5AC mRNA level statistically elevated after 5 mg/m3 NO2 exposure, and reached 3.14-fold of control ($P < 0.001$ versus control group).

3.4. Effects of NO2 exposure on the level of pro-inflammatory cytokines

Pro-inflammatory cytokines IL-1β, IL-6 and ICAM-1 occurred in the sputum and BAL fluid in individuals with asthma and amplified inflammation, and contributes to the increased release of multiple inflammatory genes (Barnes, 2008a). Also, these pro-inflammatory cytokines play a critical role on the human MUC5AC promoter activity. Therefore, we examined the levels of pro-inflammatory cytokines in the lungs after NO2 exposure. As shown in Fig. 4, IL-1β mRNA level tended to increase after NO2 inhalation, but no statistical difference was observed; whereas, IL-6 and ICAM-1 expression statistically elevated, and reached 1.36- and 1.34-fold of control ($P < 0.05$ versus control group).

3.5. Alteration of the imbalance in Th1/Th2 differentiation after NO2 exposure

IFN-γ is considered to be the product of Th1 cells, and down-regulates the Th2 cell differentiation; while, IL-4 is the product of Th2 cells, and down-regulates the Th1 cell differentiation (Heaton et al., 2005; Lama et al., 2011). Therefore, we assessed the IL-4 level and the IFN-γ expression in the lungs. After 5 mg/m3 NO2 exposure for 7 days (Fig. 5), the IL-4 content increased to 1.46-fold of control ($P < 0.05$), which was confirmed by the up-regulated expression of IL-4Rα ($P < 0.05$). In contrast to the change of IL-4, the IFN-γ expression statistically decreased to 0.53-fold of control ($P < 0.001$).

Since T-bet and GATA-3 reflect the changes in the Th1-specific cytokine IFN-γ and Th2-specific cytokine IL-4 (Chakir et al., 2003), we further detected their mRNA expression following above results. As presented in Fig. 5d and e, GATA-3 mRNA level...
significantly augmented after NO2 exposure (1.53-fold of control, \( P < 0.001 \)); whereas, T-bet expression statistically decreased (0.70-fold of control, \( P < 0.05 \)).

3.6. Activation of the bio-markers in JAK-STAT pathway after NO2 exposure

JAK-STAT pathway is the generally accepted mechanism for Th2 differentiation predominantly and Th1/Th2 differentiation imbalance (Ashino et al., 2014). To test the hypothesis, we examined the expression of STAT6 and JAK3. After NO2 exposure treatment, the levels of STAT6 and JAK3 in rat lung significantly increased and reached 1.30- (\( P < 0.05 \)) and 2.19-fold (\( P < 0.001 \)) of control (Fig. 6a and b), respectively. However, no statistical difference was observed on the JAK1 expression (Fig. 6c).

4. Discussion

NO2 is a toxic byproduct of combustion, a component of air pollution, and an endogenously-generated mediator of inflammation (Poynter, 2012). NO2 exposure correlates with asthma severity, disease exacerbation, risk of adverse outcomes in asthma, and development of asthma in adolescence (Sunyer et al., 2002; Delamater et al., 2012; Takenoue et al., 2012). Although evidence that NO2 poses inflammatory properties is steadily growing, the precise mechanisms underlying the acute inflammatory response are not well understood. The purpose of this study is to study the mechanism of airway inflammation induced by NO2 exposure. Previous study have showed that peak levels of NO2 can reach up to 0.4 ppm are encountered in the outdoors, particularly along kerbsides in downtown areas with heavy motor vehicular traffic, peak levels in the indoor environment can reach up to 4 ppm in garages, ferries, skating ice rinks, and kitchens with gas cookers (Pathmanathan et al., 2003), and peak levels about occupational exposure to NO2 can reach up to 7.6 mg/m\(^3\) (Chen et al., 2013). Studies by other investigators have confirmed that 5 mg/m\(^3\) are the peak level encountered outdoor and indoor, which is similar with the lowest effect value in humans (Hesterberg et al., 2009). In this study, NO2 at 5 mg/m\(^3\) (2.5 ppm) levels, some 5-fold greater than the typical urban concentration (0.5 ppm), was used to examine the responses of rat lungs. Although the experiments may be viewed as beyond the normal atmosphere encountered in the human environment, two important points must be taken into account. First, the animals were subjected to regular periods of extended exposure, with relief periods between protocols (i.e., 5 h/day, for 7 days, with 19 h between exposures). This may provide a corollary to individuals exposed to the gas in an occupa-
Fig. 4. Effects of NO$_2$ inhalation on the expression of IL-1$eta$(a), IL-6 (b) and ICAM-1 (c) in rat lungs. Male Wistar rats were exposed to 5 mg/m$^3$ NO$_2$ for 5 h/d for 7 d, and control group was exposed to filtered air using the same schedule. 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 ($n=6$); *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control group.

Fig. 5. Effects of NO$_2$ inhalation on the expression of IL-4(a), IL-4R$\alpha$ (b), IFN-γ (c), GATA-3 (d) and T-bet (e) in rat lungs. Male Wistar rats were exposed to 5 mg/m$^3$ NO$_2$ or filtered air for 5 h/day for 7 days. Then, the IL-4 level was assessed by ELISA assay, and the mRNA expressions of IFN-γ, IL-4R$\alpha$, GATA-3, T-bet mRNA were determined by RT-PCR. 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 are expressed as means ± SE ($n=6$); *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control group.

Fig. 6. Effects of NO$_2$ inhalation on the expression of STAT6 (a), JAK3 (b), JAK1 (c) in rat lungs. Male Wistar rats were exposed to 5 mg/m$^3$ NO$_2$ or filtered air for 5 h/day for 7 days. Then, the expressions of STAT6, JAK3, and JAK1 in the lungs were assessed by RT-PCR. 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 are expressed as means ± SE ($n=6$); *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control group.
for 7 days induced acute damages asso-
exposure, and provide scientific evidence for
caused the imbalance in the ratio of Th1/Th2 differentia-
and NO2 exposure up-regulated
promoted the
tional setting. Second, the rat is an obligate nose breather and the
release of pro-inflammatory mediators from airway epithelial cells
way smooth muscle, were considered as a result of hyperplasia
with collagen deposition under the epithelium and increased air-
thelial cells, and increased number and thickness of the collagen
bundles. It was reported that characteristic structural changes,
collagen deposition under the epithelium and increased air-
smooth muscle, were considered as a result of hyperplasia and hypertrophy (Barnes, 2008b). Therefore, previous investiga-
tions combined with our study give strong evidence that NO2 inha-
lration contributed to mucus hypersecretion and development of
asthma.
Chronic mucus hypersecretion develops in asthma, at least in
part, due to the pro-inflammatory such as IL-1β, IL-6 and ICAM-1 effects on airway epithelial glands (Chen et al., 2003; Lai and
Rogers, 2010). Following this, we further investigated the expres-
sion of several pro-inflammatory cytokines and effectors such as
IL-1β, IL-6 and ICAM-1, and found that NO2 exposure up-regulated their expression. The results were consistent with previous litera-
tures that O3 and NO2 might modulate asthma by increasing the release of pro-inflammatory mediators from airway epithelial cells (Bayram et al., 2001).
Asthma is considered as a complex disease, and the generally accepted view of its mechanism is that the subtypes of helper T lymphocyte Th2 differentiation predominantly, resulting in the imbalance in the ratio of Th1/Th2 differentiation (Zhang et al., 1999). IL-4, as a cytokine of Th2, plays a critical role in the differen-
tiation of Th2 cells from uncommitted Th0 cells. Expression of
IL-4 in the lung of IL-4 transgenic mice elicits an inflammatory response characterized by epithelial cell hypertrophy and accumu-
lation of macrophages, lymphocytes, eosinophils and neutrophils (Rankin et al., 1996). In addition, IL-4Rα as the type I receptor con-ers specificity for IL-4 binding and signal transduction (Miloux et al., 1997). Conversely, IFN-γ, a predominant cytokine produced
by Th1 and Tc1 cells, plays an important role in suppressing Th2-
mediated responses, and has been shown to be a major regulatory cytokine that inhibits the proliferation of Th2 cells in vitro and antagonizes the in vivo Th2-type responses such as IL-4-dependent IgE antibody production (Heaton et al., 2005; Lama et al., 2011). Thus, the IL-4/IFN-γ ratio can reflect the ratio of Th1/Th2 differen-
tiation. Our present study indicated that NO2 exposure up-regulated the IL-4 level and decreased the expression of IFN-γ.
Except the ratio of expression of IL-4 and IFN-γ, several factors such as GATA-3, JAK1, JAK3, STAT6 could promote the differen-
tiation of naïve T cells into Th2 cells and the secretion of Th2 cyto-
kines (Li et al., 2005; Kiwamoto et al., 2006; Ho and Pai, 2007; Malaviya and Laskin, 2010; Walford and Doherty, 2013). GATA-3, a member of the GATA family of zinc-finger transcription factors, has been identified as a key regulator of Th2 development (Kiwamoto et al., 2006). On the other hand, T-bet as a Th1 specific T-box transcription factor controls the expression of the hallmark Th1 cytokine, IFN-γ (Szabo et al., 2000). Therefore, the balance between T-bet and GATA-3 expression is also representative of
the balance between Th1 and Th2 cytokines (Chakir et al., 2003).
Similarly to the change of IL-4 and IFN-γ, NO2 promoted the
GATA-3 expression, and inhibited the T-bet level. The results imply that
NO2 caused the imbalance in the ratio of Th1/Th2 differentia-
tion and stimulated Th2 differentiation predominantly, which might be the major mechanism underlying the increased risk for mucus hypersecretion and development of asthma after environ-
mental exposure.

The analysis of STAT6-deficient mice has demonstrated that the vast majority of IL-4-induced responses are indeed mediated through the JAK-STAT pathway (Chitnis and Khoury, 2003; Kiu and Nicholson, 2012), and JAK/STAT pathway plays a critical role in the differen-
tiation of Th2 cells (Ashino et al., 2014). JAKs are regu-
ulators of signaling through cytokine receptors, among which JAK1 and JAK3 play critical roles in the initiation of inflammation and have been investigated as potent therapeutic targets in a variety of inflammatory diseases. A selective JAK1/3 inhibitor inhibited differen-
tiation of Th2 cells, down-regulated STAT6 and STAT5 phos-
phorylation and prevented the development of AHR, airway eosinophilia, mucus hypersecretion, and Th2 cytokine production (Ashino et al., 2014). STAT6 has been demonstrated to regulate many pathologic features of lung inflammatory responses in animal models including airway eosinophilia, epithelial mucus produc-
tion, smooth muscle changes, Th2 cell differentiation, and IgE production from B cells (Walford and Doherty, 2013). Also, a signif-
ificant increase of STAT6 phosphorylation was observed in mice with allergic bronchial asthma (Chiba et al., 2010). Especially,
Th2-dependent JAK/STAT activation pathway plays a critical role in the exacerbation of asthma. Thus, we analyzed the expression of STAT6, JAK3 and JAK1, and showed that STAT6, JAK3 mRNA expression were up-regulated significantly after one-week acute exposure to 5 mg/m3 NO2, with no significant increase being observed for JAK1 only, suggesting that JAK-STAT pathway was partly associated with NO2-induced mucus hypersecretion and development of asthma, and targeting the Th2-dependent JAK/STAT activation pathway might be help in therapeutic approach for the injuries in polluted area.

In conclusion, although the level of NO2 to which rats were exposed in the current study were significantly greater than those found in most indoor environments, it is important to realize that our data reflected a relatively acute (7 days) exposure to NO2 induced the pulmonary pathological injuries and increased MUC5AC expression and pro-inflammation mediator release (IL-1β, IL-6 and ICAM-1). Also, the biomarkers of Th1/Th2 differen-
tiation (IL-4/IFN-γ and GATA-3/T-bet) and JAK-STAT pathway (JAK1, JAK3 and STAT6) were influenced. The findings will clarify the mechanisms for mucus hypersecretion and development of asthma after NO2 exposure, and provide scientific evidence for assessing its biological safety and health effects.

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