

Effect of Nitrogen Dioxide Exposure on Allergic Asthma in a Murine Model*

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Study objectives: The purpose of this study was to examine the effects of NO₂, a major component of air pollution, on airway eosinophilic inflammation and bronchial hyperreactivity, using a mouse model of asthma.

Setting and subjects: BALB/c mice (eight mice per experimental group) were studied in a basic research laboratory at the University of Iowa.

Interventions: Using a standard murine model of asthma, BALB/c mice were sensitized to ovalbumin (OVA) by intraperitoneal (IP) injections (days 1 and 7) and were challenged with aerosolized OVA (days 13 and 14). Some mice were exposed to NO₂ (2 ppm) in an exposure chamber for 24 h before undergoing OVA aerosol challenge. A control group was exposed to OVA alone.

Measurements and results: The outcomes assessed included airway inflammation, bronchial hyperreactivity to inhaled methacholine, and goblet cell hyperplasia. We found that NO₂ exposure modestly increased airway neutrophilia but not airway eosinophilia in OVA-exposed mice. These mice exhibited epithelial damage and loss of epithelial mucin. Surprisingly, nonspecific bronchial hyperreactivity (*ie*, enhanced pause index) was not increased, although baseline smooth muscle tone was increased ($p < 0.05$) in the mice exposed to NO₂.

Conclusions: These data indicate that relatively short-term (24 h) exposure to NO₂ causes epithelial damage, reduced mucin expression, and increased tone of respiratory smooth muscle. Reduced mucin production may be a mechanism of injury following long-term exposure to inhaled NO₂. Despite enhancing epithelial damage in OVA-exposed mice, NO₂ exposure does not otherwise alter the expression of allergen-induced airway responses.

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Key words: air pollution; environmental tobacco smoke; indoor air quality; mucin

Abbreviations: AB-PAS = alcian blue periodic acid-Schiff stain; IP = intraperitoneal; OVA = ovalbumin; Penh = enhanced pause; Penh50 = enhanced pause recorded after inhalation of 50 mg/mL methacholine; PMN = polymorphonuclear

Epidemiologic studies have demonstrated a strong link between increased concentrations of NO₂ in polluted environments and respiratory symptoms,

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including rhinorrhea, cough, and infections of the lower respiratory tract.^{1,2} More recent evidence suggests that exposure to pollutants may also contribute to the development of the sensitization of atopic individuals to aeroallergens.³ Unlike ozone, NO₂ is a primary pollutant that is found both indoors and in the outdoor atmosphere. During high-temperature combustion, oxygen reacts with nitrogen to generate oxides of nitrogen, which mainly include nitrogen oxide and NO₂. In the outdoors, motor vehicular emissions represent the major source of NO₂. While indoor levels can reach up to 4 ppm in power plants, refineries, and ice-skating rinks, outdoor levels usually do not exceed 0.5 ppm.⁴ Gas stove cooking and environmental tobacco smoke are other sources of indoor household exposure.⁵

The effects of NO₂ exposure on airway disease are beginning to be better appreciated. Animal studies

have demonstrated that the terminal bronchiolar epithelium is particularly sensitive to NO₂-induced injury after brief exposures (*ie*, 1 to 6 h), the effects of which include epithelial flattening, loss of cilia and ciliated cells, epithelial cell hyperplasia, damage to surface membranes, and disruption of epithelial tight junctions.^{6,7} NO₂ also leads to an increased inflammatory cell influx^{8,9} and may affect lung defense mechanisms through reduced mucociliary clearance and changes in alveolar macrophages and other immune cells.¹⁰ NO₂ also may induce an inflammatory cell influx and eosinophil activation in humans.^{11–13}

NO₂ can potentiate responses to aeroallergens in mildly sensitive asthmatic persons.¹⁴ It has been shown that loratadine,^{14,15} an antihistamine, and fluticasone propionate,¹⁶ an inhaled steroid, can each block the effects of NO₂, suggesting that atopic mechanisms (*ie*, eosinophil-mediated or mast cell-mediated mechanisms) may be altered by NO₂. On the other hand, *in vitro* studies have not shown that NO₂ significantly influences bronchial smooth muscles hyperresponsiveness in wild-type guinea pigs¹⁷ or ovalbumin (OVA)-sensitized guinea pigs.^{17,18} The interactions between inhaled aeroallergens and NO₂ are not well understood. Thus, in the present study we decided to investigate the effect of relatively short-term exposure (24 h) to NO₂ on antigen-induced hyperresponsiveness, mucus production by epithelial cells, and airway inflammation *in vivo* in a murine model of allergic asthma.

MATERIALS AND METHODS

Animals

Six to eight-week-old female BALB/c mice (Jackson Laboratory; Bar Harbor, ME) were used for all studies. All animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed, and all protocols were reviewed and approved by the University of Iowa Animal Care and Use Committee. Eight mice were used for each experimental group.

Exposure Model

Mice were sensitized on days 0 and 7 with 10 µg OVA [Sigma; St. Louis, MO] adsorbed to alum by IP injection. Other mice received saline solution and served as negative controls. Prior to OVA inhalation, some mice were exposed to 2 ppm NO₂ for 24 h (day 13). The NO₂ concentration in the chamber was maintained at constant levels by the use of an analyzer (NO_x analyzer; TSI Incorporated; St Paul, MN). In preliminary studies (data not shown), we examined the effect of a range of concentrations of NO₂, and chose 2 ppm as a concentration that reliably induces airway inflammation and is within the range of exposure resulting from indoor air pollution. All mice other than negative controls were challenged with aerosolized OVA (1% solution, nebulized) for 30 min on days 14 and 15, then were killed 48 h later on day 16 (Table 1).

Table 1—Summary of the Mice Sensitization and Challenge Protocols

Variables	IP Sensitization, Boost (Days 0, 7)	Inhalation Exposure (Day 13)	Challenge by Aerosol (Days 14, 15)
Control	Saline solution	Ambient air	Saline solution
OVA	OVA/alum	Ambient air	OVA (1%)
NO ₂	Sham	NO ₂ 2 ppm 24 h	Saline solution
OVA + NO ₂	OVA/alum	NO ₂ 2 ppm 24 h	OVA (1%)

Whole-Lung Lavage

Following euthanasia, the trachea was cannulated and 3 mL saline solution was administered. The washing fluid was collected by gravitational flow. The lavage fluid samples were processed for total and differential cell counts (using Diff-Quik staining of cytopsin preparations).

Physiology

Airway hyperactivity was measured on day 16 (immediately prior to death) using a whole-body plethysmograph (Buxco Electronics; Troy, NY) and methacholine-induced airflow obstruction, as previously described.¹⁹ Airway resistance is approximated by the enhanced pause (Penh), which is normalized to the Penh following saline solution inhalation for that individual mouse, so that a Penh ratio of 1 implies a Penh value equivalent to that of the baseline, before the methacholine challenge.

Light Microscopy and Morphometry

At the time of death, the lungs of the mice were excised, fixed, and embedded in paraffin. Tissue pathology was studied in 5-µm-thick tissue sections, which were stained with hematoxylin-eosin, while other sections were stained with alcian-blue (pH 2.6) periodic acid-Schiff (AB-PAS) and hematoxylin. The percentage of the area of mucus on the epithelial surface stained with AB-PAS was determined by an image analyzer (SP 500; Olympus; Tokyo, Japan). The area of the respiratory epithelium was outlined, and the image analyzer quantified the area of AB-PAS-stained mucus within this reference area. The percentage of the area of the epithelial surface occupied by stored mucus was calculated over 2 mm of the basal lamina.

Statistical Analysis

Analysis was performed using a one-way analysis of variance or nonparametric Mann-Whitney tests using appropriate software (SPSS; SPSS Inc; Chicago, IL). Values for all measurements were expressed as the mean ± SEM. A *p* value of < 0.05 was considered to be significant.

RESULTS

Effects of NO₂ Exposure on Airway Inflammation

To examine the effects of NO₂ on airway inflammation, we exposed naive mice (*ie*, controls) and OVA-sensitized BALB/c mice to NO₂ (2 ppm for 24 h) or filtered air prior to the inhalation of

aerosolized OVA. The inhalation of NO₂ significantly induced an influx of neutrophils into the airways of naive mice (control mice, $2.0 \pm 0.8 \times 10^3$ polymorphonuclear (PMN) cells/mL [2.2 ± 0.4%]; NO₂ inhalation mice, $36.4 \pm 7.9 \times 10^3$ PMN cells/mL [12.5 ± 3.0%]; $p < 0.05$ [eight mice per group]) [Fig 1]. In contrast, among OVA-sensitized/exposed mice, NO₂ exposure did not significantly alter BAL neutrophilia (OVA mice, $14.9 \pm 4.1 \times 10^3$ PMN cells/mL [3.0 ± 0.7%]; OVA + NO₂-exposed mice, $9.6 \pm 4.2 \times 10^3$ PMN cells/mL [2.0 ± 0.7%]; difference was not significant [eight mice per group]) [Fig 1] or eosinophilia (OVA mice, $389.8 \pm 57.4 \times 10^3$ eosinophils/mL [80.3 ± 6.3%]; OVA + NO₂-exposed mice, $309.5 \pm 64.2 \times 10^3$ eosinophils/mL [64.5 ± 4.8%]; difference not significant [eight mice per group]).

Effects of NO₂ Exposure on Bronchial Hyperreactivity

We also evaluated the effects of inhaled NO₂ on the development of methacholine-induced bronchial hyperresponsiveness, as measured by whole-body plethysmography. In accordance with our previous findings, OVA-sensitized/OVA-challenged mice demonstrate marked increases in airway hyperresponsiveness compared to control mice (control mice with Penh recorded after inhalation of 50 mg/mL methacholine [Penh50]/Penh recorded after inhalation of saline solution, 4.84 ± 0.47 ; OVA Penh50, 10.29 ± 1.5 ; $p < 0.05$ [eight mice per group]). In contrast with the response to antigen exposure, the inhalation of NO₂ did not

significantly increase bronchial hyperreactivity (NO₂ Penh50, 2.97 ± 0.45 ; difference not significant [vs controls]; OVA + NO₂ Penh50, 3.28 ± 1.01 ; difference not significant [vs OVA]). However, NO₂ exposure increased the absolute (pre-methacholine challenge) Penh among the OVA-exposed mice (OVA Penh, 0.43 ± 0.09 ; OVA + NO₂ Penh, 1.03 ± 0.33 ; $p < 0.05$) but not the naive mice (control Penh, 0.4 ± 0.10 ; NO₂ Penh, 0.55 ± 0.16 ; difference not significant), perhaps reflecting structural changes of the airways (Fig 2).

Effects of NO₂ Exposure on Airway Epithelia

We next examined the histopathologic changes induced in the airways by NO₂ inhalation. One important alteration seen during asthma exacerbations is increased mucus production by the epithelial cells, which was associated with goblet cell hyperplasia/metaplasia.²⁰ As anticipated, OVA-sensitized/challenged mice exhibited significant increases in mucin-positive cells in comparison with control mice (controls, $8.17 \pm 0.17\%$; OVA-exposed mice, $18.35 \pm 1.91\%$; $p < 0.01$ [eight mice per group]). Surprisingly, mucin expression was actually reduced following the inhalation of NO₂ (NO₂-exposed mice, $1.98 \pm 0.24\%$; $p < 0.01$ [vs controls]; OVA + NO₂-exposed mice, $3.52 \pm 1.16\%$; $p < 0.01$ [vs OVA-exposed mice]). In contrast, epithelial disruption and denudement were seen to a significantly greater degree in mice exposed to NO₂ than in those not exposed (Fig 3).

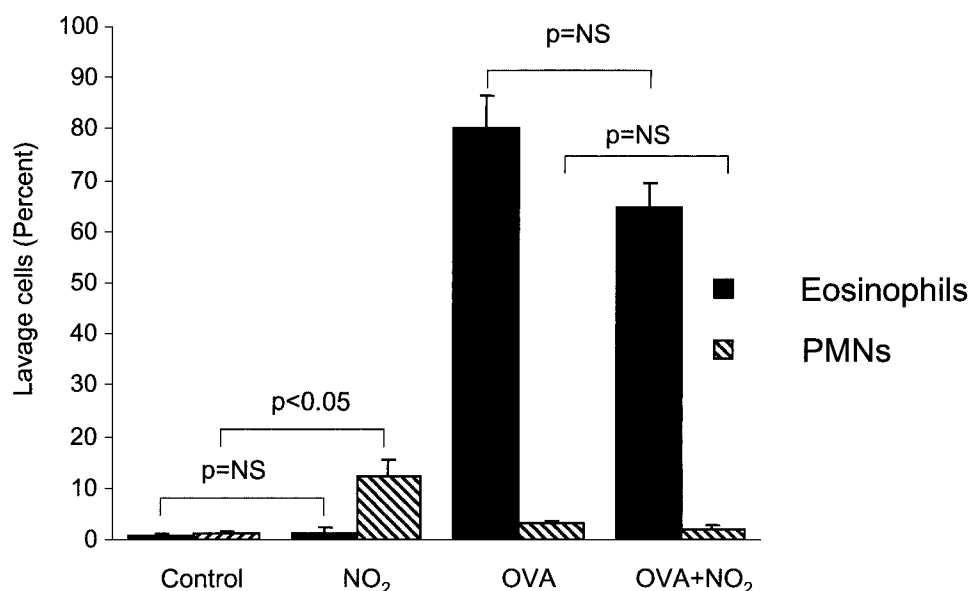


FIGURE 1. Exposure to 2.0 ppm NO₂ for 24 h induced significant airway neutrophilia. The exposure of OVA-sensitized/OVA-challenged mice to these levels of NO₂ did not significantly alter the number of airway eosinophils or PMN cells. NS = not significant.

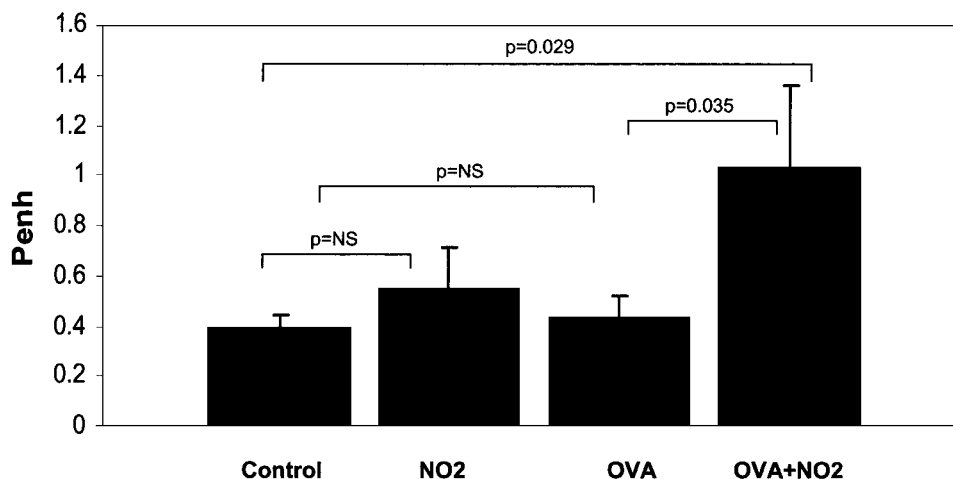


FIGURE 2. The effect of 2.0 ppm NO₂ exposure for 24 h on Penh, a correlate of airway resistance and thus smooth muscle tone. OVA-sensitized/OVA-challenged mice, when exposed to NO₂, have significantly increased Penh compared to control mice as well as mice exposed to OVA in the absence of NO₂ ($p < 0.05$).

DISCUSSION

These studies demonstrated, using a murine model, that exposure to 2 ppm NO₂ for 24 h induces airway neutrophilia in naive mice but did not alter the cellular inflammatory response to inhaled allergen in OVA-sensitized animals. The inhalation of NO₂, in this model, increased the Penh (a measure of airway resistance) of OVA-sensitized/challenged mice but did not increase bronchial hyperresponsiveness to inhaled methacholine. Most notably, a morphometric evaluation of the airways demonstrated that epithelial disruption was enhanced by the inhalation of NO₂, both in naive and allergic mice. In addition, mucus expression was reduced in the NO₂-exposed animals. These findings are consistent with the known toxic effects of NO₂ on the airway. Epithelial disruption and denudement are common in inflamed airways, and air pollution can symptomatically exacerbate atopic asthma.²¹ These epithelial changes, often seen before the onset of airway repair and remodeling, are acute responses to airway injury.

We speculate that the increased Penh values seen in NO₂-exposed mice were linked to the structural changes in their airway epithelia. Although some investigators^{22,23} have criticized the use of whole-body plethysmography to assess airway hyperresponsiveness, we have found that this measure correlates well with airway inflammation.^{19,24,25} In the current study, we think that the Penh measure reflects alterations that may occur throughout the air passages, integrating changes that extend from the nasal passageway through the lower airways.

NO₂, like sulfur dioxide and ozone, is a major component of air pollution. The threshold value for

both the primary and secondary national Ambient Air Quality Standard for NO₂ is 0.053 ppm (measured as an annual arithmetic mean concentration). Six-day integrated indoor and outdoor concentrations of NO₂ were measured in two communities in Southern California using passive samplers. The average indoor and outdoor NO₂ concentrations were 0.028 and 0.020 ppm, respectively.²⁶ Other studies of indoor air pollution have demonstrated average levels as high as 0.05 to 0.08 ppm, with higher levels associated with gas cooking and environmental tobacco smoke.^{5,27,28} Although the level of NO₂ to which mice were exposed in the current study were significantly greater than those found in most indoor environments, it is important to realize that our data reflect a relatively short-term (*ie*, 24 h) exposure to NO₂ and may substantially underestimate the effect of long-term or life-long exposures to polluted air.

It has been shown that exposures of 2 to 5 ppm NO₂ in healthy subjects increases the number of inflammatory cells found in BAL fluid.¹¹ Wang and coworkers^{16,29} studied the effects of NO₂ inhalation in subjects with seasonal allergic rhinitis and found that NO₂ exposure increased allergen-induced eosinophilic cationic protein, mast cell tryptase, myeloperoxidase, and interleukin-8. Another study showed that, in asthmatic subjects, short-term exposure to NO₂ from single episodes of gas cooking was associated with immediate airflow limitation. Continued exposure from repeated episodes of gas cooking by asthmatic women was associated with a greater use of rescue bronchodilators.³⁰ Blomberg et al³¹ showed that the inhalation of 2 ppm NO₂ for 6 h caused

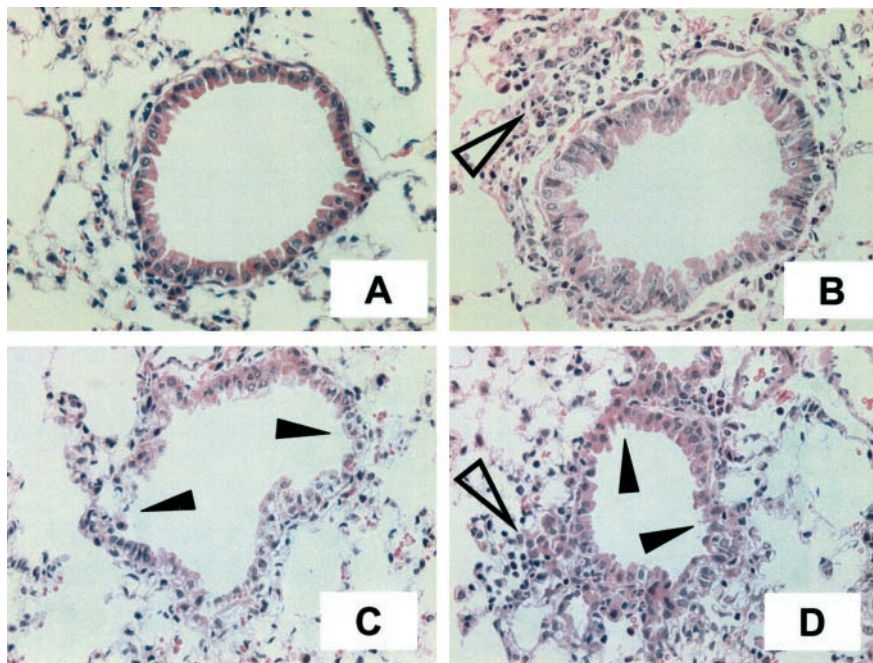


FIGURE 3. Lung samples from mice that were exposed to NO_2 (2 ppm for 24 h) showed signs of epithelial injury, including epithelial denudation (closed arrows), compared with mice not exposed to NO_2 . Sensitization to and challenge with OVA induced mucosal hyperplasia and increased mucosal thickness, regardless of NO_2 exposure. *Top left, A:* control mice; *top right, B:* OVA-sensitized/OVA-challenged mice; *bottom left, C:* NO_2 -exposed mice; *bottom right, D:* NO_2 exposure followed by OVA challenge (hematoxylin-eosin, original $\times 40$). Open arrows point to peribronchial cellular infiltrate after OVA challenge (*top right, B,* and *bottom right, D*).

significant decrements in FEV_1 and FVC after the first exposure, but these effects were attenuated following repeated exposures.

In contrast with clinical studies, *in vitro* studies on smooth muscles have not supported the existence of a direct role of NO_2 in inducing bronchospasm. Neither human nor murine bronchial smooth muscle demonstrates altered contractility following exposure to NO_2 .^{17,18} In this current study, we have presented data supporting those *in vitro* studies by demonstrating that short-term exposure to NO_2 does not significantly alter airway hyperreactivity. Blomberg et al³¹ showed that repeated exposure to NO_2 results in the attenuation of the changes in FEV_1 and FVC that develop after an initial exposure in healthy, adult nonsmokers. This attenuation of lung function response after repeated or prolonged exposure to NO_2 is consistent with the response patterns seen after repeated daily exposures to other environmental pollutants, such as ozone and carbon monoxide.^{32,33} The inability to induce hyperreactivity may be attributed to counterregulatory mechanisms, which might include replenishment of lost antioxidants in the epithelial lining fluid or the up-regulation of other defense elements. Like carbon monoxide, NO_2 can reduce bronchial hyperreactivity by the generation

of bronchodilating substances such as cyclic guanosine monophosphate.^{33,34} One important determinant of bronchial reactivity is the resting state of the airways.³⁵ An increase in resting bronchomotor tone, either by the direct action of spasmogens or by the autonomic nervous system, may potentiate a subsequent constrictor stimulus. In this study, we found that NO_2 significantly increased baseline airway smooth muscle time, as measured by baseline Penh.

Mucins are polydispersed and highly glycosylated molecules, and are the principal determinant of the viscoelastic properties of mucus.³⁶ Excessive mucus production by hyperplastic goblet cells has been reported in patients with acute and chronic asthma.³⁷ Allergic asthma is characterized by airway hyperresponsiveness to a variety of specific and nonspecific stimuli, chronic pulmonary eosinophilia, elevated serum IgE levels, and excessive airway mucus production.³⁸ Mucus hypersecretion is also an important part of the sequelae induced by a number of toxic insults to the lung such as inhaled irritants, neutrophil products, or viral and bacterial infections.^{39–41} The production of mucus, although at times used as a proxy for airway injury and “remodeling,” may actually serve as a defense against inhaled harmful

agents. Reduced levels of mucus expression may thus be a mechanism of, as well as a marker for, airway injury. The reduced amounts of airway mucin observed in this study after the inhalation of NO₂ might be due to a loss of production secondary to the noxious effects of this common environmental pollutant.

We conclude that short-term exposure to NO₂ induces neutrophilic airway inflammation, epithelial damage, and increased baseline airway smooth muscle tone in naive mice. Although enhancing epithelial disruption, this exposure did not significantly alter the influx of inflammatory cells or bronchial hyperresponsiveness in a murine model of atopic asthma.

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