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Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse

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Poynter, Matthew E., Rebecca L. Persinger, Charles G. Irvin, Kelly J. Butnor, Hans van Hirtum, Wendy Blay, Nicholas H. Heintz, Justin Robbins, David Hemenway, Douglas J. Taatjes, and Yvonne Janssen-Heininger. Nitrogen dioxide enhances allergic airway inflammation and hyperresponsiveness in the mouse. *Am J Physiol Lung Cell Mol Physiol* 290: L144–L152, 2006. First published August 5, 2005; doi:10.1152/ajplung.00131.2005.—In addition to being an air pollutant, NO₂ is a potent inflammatory oxidant generated endogenously by myeloperoxidase and eosinophil peroxidase. In these studies, we sought to determine the effects of NO₂ exposure on mice with ongoing allergic airway disease pathology. Mice were sensitized and challenged with the antigen ovalbumin (OVA) to generate airway inflammation and subsequently exposed to 5 or 25 ppm NO₂ for 3 days or 5 days followed by a 20-day recovery period. Whereas 5 ppm NO₂ elicited no pathological changes, inhalation of 25 ppm NO₂ alone induced acute lung injury, which peaked after 3 days and was characterized by increases in protein, LDH, and neutrophils recovered by BAL, as well as lesions within terminal bronchioles. Importantly, 25 ppm NO₂ was also sufficient to cause AHR in mice, a cardinal feature of asthma. The inflammatory changes were ameliorated after 5 days of inhalation and completely resolved after 20 days of recovery after the 5-day inhalation. In contrast, in mice immunized and challenged with OVA, inhalation of 25 ppm NO₂ caused a marked augmentation of eosinophilic inflammation and terminal bronchiolar lesions, which extended significantly into the alveoli. Moreover, 20 days postcessation of the 5-day 25 ppm NO₂ inhalation regimen, eosinophilic and neutrophilic inflammation, pulmonary lesions, and AHR were still present in mice immunized and challenged with OVA. Collectively, these observations suggest an important role for NO₂ in airway pathologies associated with asthma, both in modulation of degree and duration of inflammatory response, as well as in induction of AHR.

asthma; eosinophil; neutrophil; lung

THE TOXIC OXIDANT GAS, NO₂, is formed by the combustion process and is a pollutant found in both indoor and outdoor air (3, 7, 19, 30, 35). Not well recognized are recent reports demonstrating that NO₂ can also be formed within the lung during inflammation, as a result of the catalytic activity of eosinophil peroxidase and myeloperoxidase, or decomposition of peroxynitrite (ONOO[−]) (5, 41, 55, 57). Consequently, nitrotyrosine formation, a stable end product of NO₂ reactivity, is present in lungs from patients with asthma, chronic obstructive pulmonary disease, and many other inflammatory disorders (1, 9, 24, 26, 43, 44, 56) and may contribute to the pathophysiology of these diseases.

Epidemiological, clinical, and rodent studies have documented the adverse effects of NO₂ on the lung. Exposure to NO₂ is associated with increases in emergency room visits, hospital admissions, mortality, and acute respiratory complaints that include breathing difficulties, apnea, and exacerbation of asthma symptoms (3, 7), suggesting its role in augmenting respiratory illness. Epidemiologic studies performed in children have clearly documented an increased relative risk for adverse respiratory effects, including asthma exacerbations, which were associated with exposure to indoor or outdoor NO₂ (7, 32). The inhalation of NO₂ causes a number of biochemical, morphological, and functional changes in lungs of rodents (6, 12–15, 20, 25, 29, 47, 48). NO₂ is absorbed all along the respiratory tract and, depending on the concentration and dose, injury can occur in the trachea, bronchi, bronchioles, alveolar ducts, and the proximal airways (17, 34). The main site of NO₂ deposition and injury is at the distal conducting airways at the level of the terminal bronchioles (7).

Studies that address the contribution of NO₂ to pulmonary inflammation in mice are relevant because of the availability of genetically altered strains, which are instrumental to identify signaling pathways applicable to the pathophysiology of pulmonary diseases associated with the formation of reactive nitrogen species (RNS). To date, a limited number of studies using mice have demonstrated contradictory effects (22, 23, 40), possibly due to the use of different mouse strains, different doses of NO₂, and different timing of the NO₂ administration. The goal of the present study was to document the effects of NO₂ exposure on the mouse lung and to determine whether 5 or 25 ppm NO₂ exposure exacerbated allergic airways disease or promoted chronic alterations in the allergic inflamed lung.

MATERIALS AND METHODS

Ovalbumin model of allergic airway inflammation and hyperresponsiveness. Mice were subjected to ovalbumin (OVA) sensitization by administration of 100 μ l of OVA (20 μ g, grade V; Sigma, St. Louis, MO) with Alum (2.25 mg, Imject Alum; Pierce) via intraperitoneal injection on days 0 and 7. Mice were challenged using three doses of aerosolized 1% OVA in PBS for 30 min on days 14–16, as previously described (8, 38, 52).

Exposure to NO₂. C57BL/6 mice, obtained from The Jackson Laboratory (Bar Harbor, ME), were used for whole body inhalation studies conducted within our Association for Assessment and Accreditation of Laboratory Animal Care-accredited inhalation facility. All animal studies were reviewed and approved by the institutional animal care and use committee at the University of Vermont. The method-

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ology has been completely described elsewhere (25). In brief, a 50-l Teflon bag was filled with 1 part pure NO₂ to 3 parts dry N₂ gas, thus diluting the NO₂. This mixture was then pumped through a regulating valve using a stainless steel/Teflon diaphragm pump into a mixing chamber and then into the inhalation chamber where the animals were exposed. A nitric oxide analyzer equipped with an NO₂ thermal converter was used to continuously measure chamber NO₂ in the gas phase according to the manufacturer's instructions (Eco Physics, Ann Arbor, MI). The instrument was routinely calibrated through the use of NO₂ permeation tubes and was shown to adequately monitor concentrations from 0.05 to 50 ppm. The exhaust from the exposure chamber was passed through a stainless steel duct to release the gases through a negative pressure line to the top of the building. Mice were exposed to 5 or 25 ppm NO₂ for 1, 3, or 5 days, using daily exposure regimens of 6 h in duration. Selected groups of mice exposed to a 5- or 25-ppm NO₂ dose for 6 h/day for 5 days were allowed to recover for 20 days.

Pulmonary function assessment. After anesthesia and tracheostomy, mice were mechanically ventilated for the assessment of pulmonary function using the forced oscillation technique, as previously described (54). Mice were ventilated at a rate of 2.5 Hz, with a tidal volume of 0.2 ml and 3 cmH₂O positive end-expiratory pressure (flexiVent; SCIREQ, Montreal, PQ, Canada). Data from regular ventilation were collected to establish the baseline for each animal, and inhaled doses of aerosolized methacholine (Sigma-Aldrich) in saline were then administered in successive increasing concentrations (0, 3.125, 12.5, and 50 mg/ml) as an aerosol. Multiple linear regression was used to fit impedance spectra derived from measured pressure and volume to the constant phase model of the lung (18)

$$Z(f) = R_n + i\omega I + (G + iH_{ii})/\omega^\alpha$$

where Z is input impedance of the respiratory system, R_n is a Newtonian resistance composed mostly of the flow resistance of the conducting pulmonary airways, I is the inertance of the gas in the central airways, G reflects viscous dissipation of energy in the respiratory tissues, H_{ii} reflects elastic energy storage in the tissues, f is frequency, $i = \sqrt{-1}$, ω is $2\pi f$, and α couples G and H_{ii} . Using this model, the peak response for the physiological property of elastance (H_{ii} , a measure of parenchymal events) was determined, and the percentage change from baseline, as measured at the beginning of the protocol, was calculated.

Bronchoalveolar lavage. Mice were killed by a lethal dose of pentobarbital via intraperitoneal injection. Bronchoalveolar lavage fluid (BALF) was immediately collected from euthanized mice for the assessment of total and differential cell counts (39). Supernatants were collected for protein (Bio-Rad, Hercules, CA), and lactate dehydrogenase (LDH) was quantitated according to the manufacturer's instructions (Promega, Madison, WI).

Histopathology and transmission electron microscopy. After the mice were killed and bronchoalveolar lavage was performed, the left lung lobe was instilled with 4% paraformaldehyde in PBS (4% PFA) for 10 min at a pressure of 25 cmH₂O and placed into 4% PFA at 4°C overnight for further fixation of the tissue before being embedded in paraffin. Seven-micrometer sections were cut, affixed to glass microscope slides, deparaffinized with xylene, rehydrated through a series of ethanol (39), stained with hematoxylin and eosin or with periodic acid-Schiff stains, coverslipped, and examined by light microscopy. For transmission electron microscopy, the PFA-infused lungs were immersed in Karnovsky's fixative (2.5% glutaraldehyde, 1.0% PFA in Millonig's phosphate buffer) and fixed overnight at 4°C. After three 5-min rinses in buffer, the tissue pieces were postfixed for 45 min at 4°C in 1% osmium tetroxide. Finally, the tissue pieces were rinsed in buffer, dehydrated in a graded series of ethanol (finishing with propylene oxide), and embedded in Spurr's resin. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, retrieved onto copper grids, contrasted with uranyl acetate and lead citrate, and examined with a JEOL 1210 transmission electron microscope operating at 60 kV.

Quantitative RT-PCR. Lungs were removed from the mice immediately after bronchoalveolar lavage, snap frozen in liquid nitrogen, and pulverized using chilled mortars and pestles. RNA was extracted using TRIzol, DNase treated using RNeasy columns (Qiagen), and reverse transcribed into cDNA using Superscript II according to instructions by the manufacturer (GIBCO-BRL). Real-time semiquantitative RT-PCR was performed using the Taqman Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. The probes and primer sets used for mouse hypoxanthine guanine phosphoribosyltransferase (*HPRT*), forward: 5'-TTTCCCGCGA-GCCG-3', reverse: 5'-TAACCTGGTTCATCATCGCTAATC-3', probe: 5' FAM-(CGACCCGAGTCCCAGCGTC)-BHQ-1 3', and eotaxin-1, forward: 5'-AGAGCTCCACAGCTTCTATT-3', reverse: 5'-CTTACTGGTCATGATAAAGCAGCAG-3', probe: 5' FAM-(ACGGTCACTTCCTTCACCTCCCAGG)-BHQ-1 3', were purchased from Biosearch Technologies (Novato, CA), and the mouse Assay-On-Demand primers and probes *Gob-5*, Mm00489959_m1, *Muc5ac*, Mm0276725_g1, and *Ccl20* Mm00444228_m1 were purchased from Applied Biosystems (Foster City, CA). *Gob-5*, *eotaxin-1*, and *Ccl20* levels were normalized to *HPRT*, and the data are presented as average expression relative to the housekeeping gene.

Statistical analysis. Data were analyzed by two-way ANOVA, and Bonferroni correction was used for multiple comparisons.

RESULTS

Because elevated ambient levels of NO₂ are associated with bronchitic symptoms in asthmatics (32), and recent observations demonstrate that NO₂ can be generated endogenously through eosinophil peroxidase or myeloperoxidase catalyzed reactions (4), we sought to characterize the patterns of lung injury and repair following exposure to NO₂ in the mouse. Exposure to 5 ppm NO₂ for 6 h/day for 1, 3, or 5 days resulted in no remarkable lung inflammation or injury (data not shown). In contrast, exposure to 25 ppm NO₂ resulted in acute and reversible lung injury. Indicative of the reversible lung injury induced, mice exposed to 25 ppm NO₂ demonstrated increased LDH activity and protein levels in the BALF after 1 or 3 days of NO₂ exposure, which largely resolved after 2 additional days of exposure (Fig. 1). Interestingly, increased protein in the lavage was detected before LDH levels were elevated, suggesting that protein leak, possibly via plasma exudation, occurred before lung cellular damage.

We next examined the lung tissues of NO₂-exposed mice by histopathology (Fig. 2). Lung sections harvested from mice after 1 day of exposure to 25 ppm NO₂ showed rare foci of periterminal bronchiole inflammatory cells composed predominantly of neutrophils with rare mononuclear cells and reactive pneumocytes within small airways. After 3 days of exposure, diffuse alterations involving primarily the terminal bronchioles were apparent and consisted of increased numbers of periterminal bronchiole macrophages, with intermixed neutrophils, lymphocytes, and plasma cells, and markedly reactive pneumocytes lining alveoli and sloughed into alveolar spaces. The terminal bronchioles displayed respiratory epithelial cell injury characterized by flattening and focal loss of cilia with focal squamous metaplasia and hyperplastic changes extending into the first order of respiratory bronchioles. Collectively, these changes involved >75% of the terminal bronchioles. Additional assessment of the terminal bronchioles from mice exposed to 25 ppm NO₂ for 3 days by transmission electron microscopy confirmed hypertrophy and hyperplasia of type II cells, peribronchiolar hyperplasia, influx of macrophages and neutrophils, and occasional apoptotic bodies, compared with

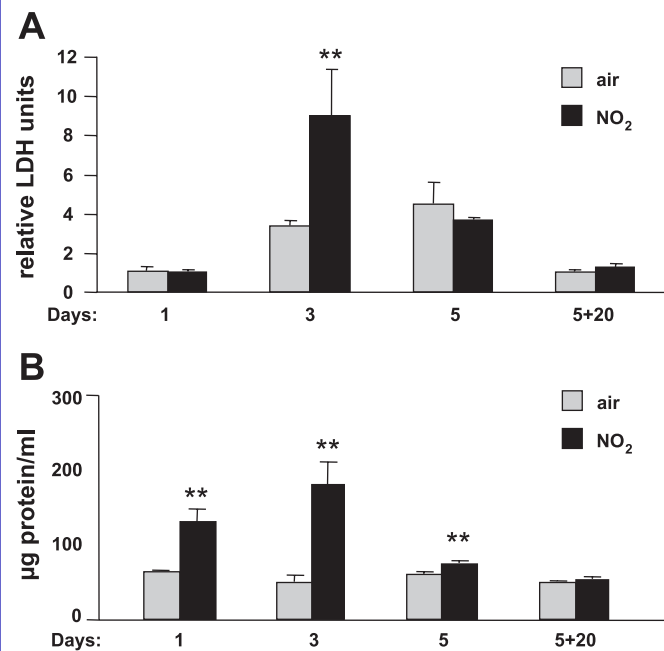


Fig. 1. Assessment of the injury markers lactate dehydrogenase (LDH; A) and protein (B) in bronchoalveolar lavage from C57BL/6 mice exposed to air or NO_2 . Mice were exposed to 25 ppm for 6 h/day for 1, 3, or 5 days. In addition, 1 group of animals was maintained for 20 days postcessation of the 5-day exposure period. Data are means $\pm$ SE of 5 mice/group. ** $P < 0.01$ for NO_2 vs. air.

sections from control mice (Fig. 3). Consistent with these inflammatory changes, elevated numbers of macrophages and neutrophils were observed in the BALF of mice exposed to 25 ppm NO_2 for 3 days (Fig. 4).

In parallel with the resolution of injury markers in the bronchoalveolar lavage (Fig. 1), lungs harvested from mice after 5 days of exposure to 25 ppm NO_2 displayed a dampened inflammatory infiltrate within terminal bronchioles (Fig. 2), consisting mainly of macrophages. After 20 days of recovery in room air following 5 days of exposure to 25 ppm NO_2 , the lung sections showed a near complete resolution of these changes (Fig. 2), demonstrating that this exposure regimen in the mouse causes a pattern of acute lung injury that resolves.

After having established that exposure to 25 ppm NO_2 is itself sufficient to induce lung inflammation and injury, whereas 5 ppm NO_2 is not, we next addressed what the consequences of exposure to these NO_2 doses are to mice with preexisting allergic airway inflammation. Mice were either sensitized via intraperitoneal injection with the antigen OVA plus the adjuvant aluminum hydroxide or mock sensitized with adjuvant alone (Alum) and then challenged with aerosolized OVA for 30 min/day for 3 days. This protocol induces airway inflammation and hyperresponsiveness with many of the cardinal features of asthma (38, 39). Forty-eight hours after the last aerosolized OVA challenge, mice were exposed to air, 5 ppm NO_2 , or 25 ppm NO_2 for 6 h/day for 3 days and analyzed the following day. Alternatively, 48 h after the last aerosolized OVA challenge, mice were exposed to air, 5 ppm NO_2 , or 25

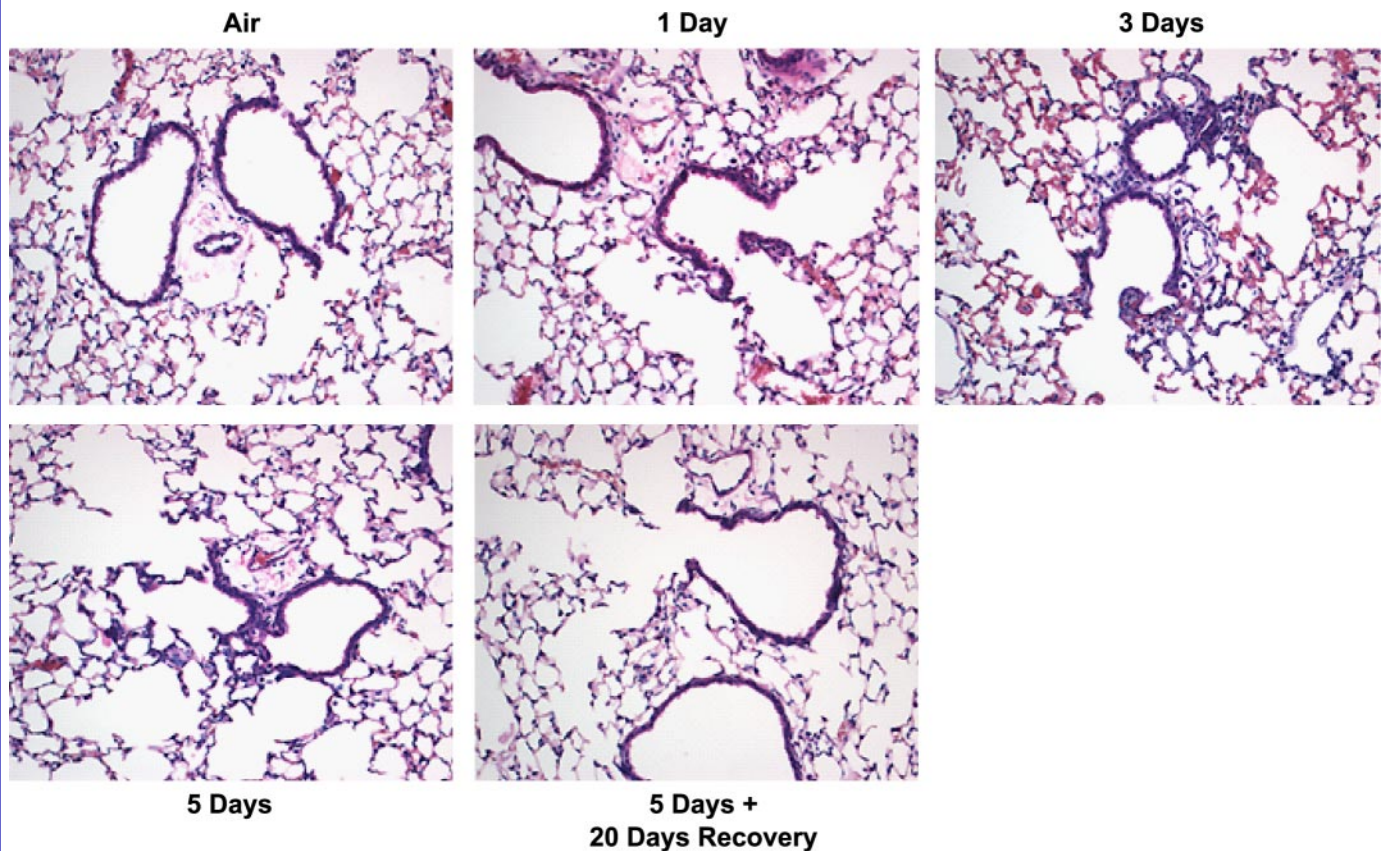


Fig. 2. Assessment of lung histopathology from mice exposed to 25 ppm NO_2 for 6 h/day for 1, 3, or 5 days. In addition, 1 group of 25 ppm NO_2 -exposed animals was kept 20 days postcessation of the 5-day exposure period. Control mice (air) were exposed to high-efficiency particulate air-filtered room air for 6 h/day for 5 days. Lung sections were stained with hematoxylin and eosin. Original magnification, $\times 200$. Data are representative of 5 mice/group.

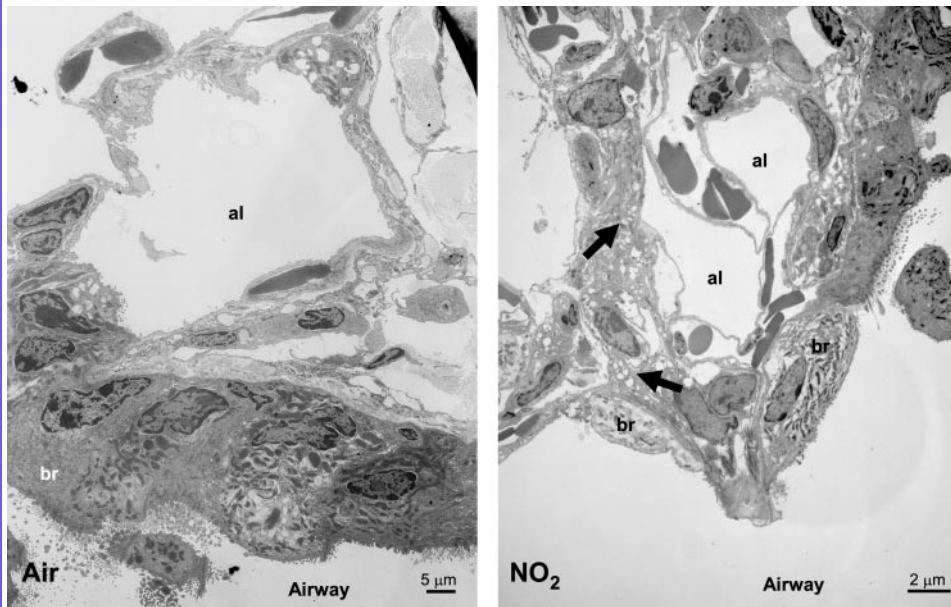


Fig. 3. Transmission electron micrographs of mouse lungs exposed to 25 ppm NO₂ for 6 h/day for 3 days or to air alone. Lung sections were prepared as described in MATERIALS AND METHODS. al, alveoli; br, bronchiolar epithelial cell; arrows, site of peribronchiolar hyperplasia and cytosolic alterations. Magnification, $\times 2,000$ (air) and $\times 1,200$ (NO₂). Data are representative of 3 mice/group.

ppm NO₂ for 6 h/day for 5 days and analyzed after a 20-day recovery period in room air. Whereas exposure to 25 ppm NO₂ alone led to an accumulation of macrophages and neutrophils in the BALF (Fig. 4), the BALF of mice exposed to the OVA sensitization and challenge regimen (OVA/OVA) demonstrated a predominance of inflammatory eosinophils in addition to resident alveolar macrophages (Fig. 5, A and B). In mice that were subjected to OVA/OVA, before exposure to 3 days of 5 ppm NO₂, no changes in the inflammatory response were observed relative to the OVA/OVA group exposed to room air (Fig. 5A). In contrast, mice subjected to OVA/OVA before exposure to 3 days of 25 ppm NO₂ demonstrated a markedly elevated inflammatory response, reflected by increased numbers of neutrophils and eosinophils in the bronchoalveolar lavage. In fact, the number of eosinophils recovered in these mice was nearly 10-fold the number recovered in the bronchoalveolar lavage from OVA/OVA mice that were exposed to air and analyzed at the same time point, reflecting a larger than additive effect (Fig. 5B). Histological examination of the lungs confirmed the enhanced response in mice exposed to the combined OVA/OVA and 3 days 25 ppm NO₂ exposure

regimens, over individual exposures (Fig. 5C). Although 25 ppm NO₂ itself was capable of inducing distal bronchiolar inflammatory lesions, described above, and the mice subjected to OVA/OVA regimen showed perivascular and peribronchiolar inflammation, the lungs from OVA/OVA plus 25 ppm NO₂-exposed mice showed a greater magnitude of the distal bronchiolar inflammatory lesions, which were composed primarily of eosinophils and lymphocytes and appeared to involve coalescing of alveoli into the inflammatory lesions as well (Fig. 5C). Lungs from mice exposed to 5 ppm NO₂ demonstrated no histological changes relative to the air-exposed controls or to the OVA/OVA groups (data not shown). These results demonstrate that exposure to 25 ppm NO₂ exacerbates the inflammatory response elicited by OVA/OVA.

Evaluation of the mRNA levels of the mucus cell-associated gene products *Gob5* (36) and *Muc5AC* (58), the Th2 and dendritic cell chemokine *CCL20* (42), and the eosinophil chemokine *eotaxin-1* (53) in lung tissue demonstrates marked increases in mice subjected to OVA/OVA (Fig. 5, D and E). No additional increases were apparent in mice subjected to OVA/OVA plus 25 ppm NO₂ exposure for 3 days compared with OVA/OVA exposure alone, despite the marked enhancement of the inflammatory response seen in mice subjected to combined exposures (Fig. 5, B and C). Furthermore, NO₂ exposure alone was not sufficient to increase expression of any of these genes.

We next determined whether inflammation was prolonged in mice subjected to the combined OVA/OVA plus NO₂ exposure regimens by examining lungs 20 days after recovery from 5 days of 5 or 25 ppm NO₂ exposure. Whereas the overt inflammatory lesions were resolved in mice subjected to OVA/OVA or NO₂ alone, or the combined exposure groups of OVA/OVA plus 5 ppm NO₂ (Fig. 6A) in mice exposed to OVA/OVA plus 25 ppm NO₂, residual inflammation was apparent based on the continued presence of neutrophils and eosinophils in the bronchoalveolar lavage (Fig. 6B) as well as the persistently elevated mRNA levels of *CCL20* and *eotaxin* (Fig. 5E). The apparent elevations in bronchoalveolar lavage

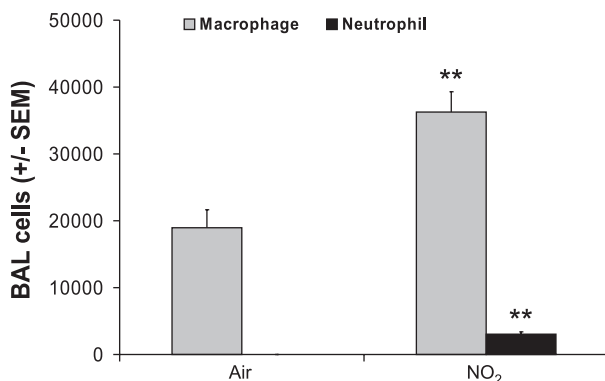
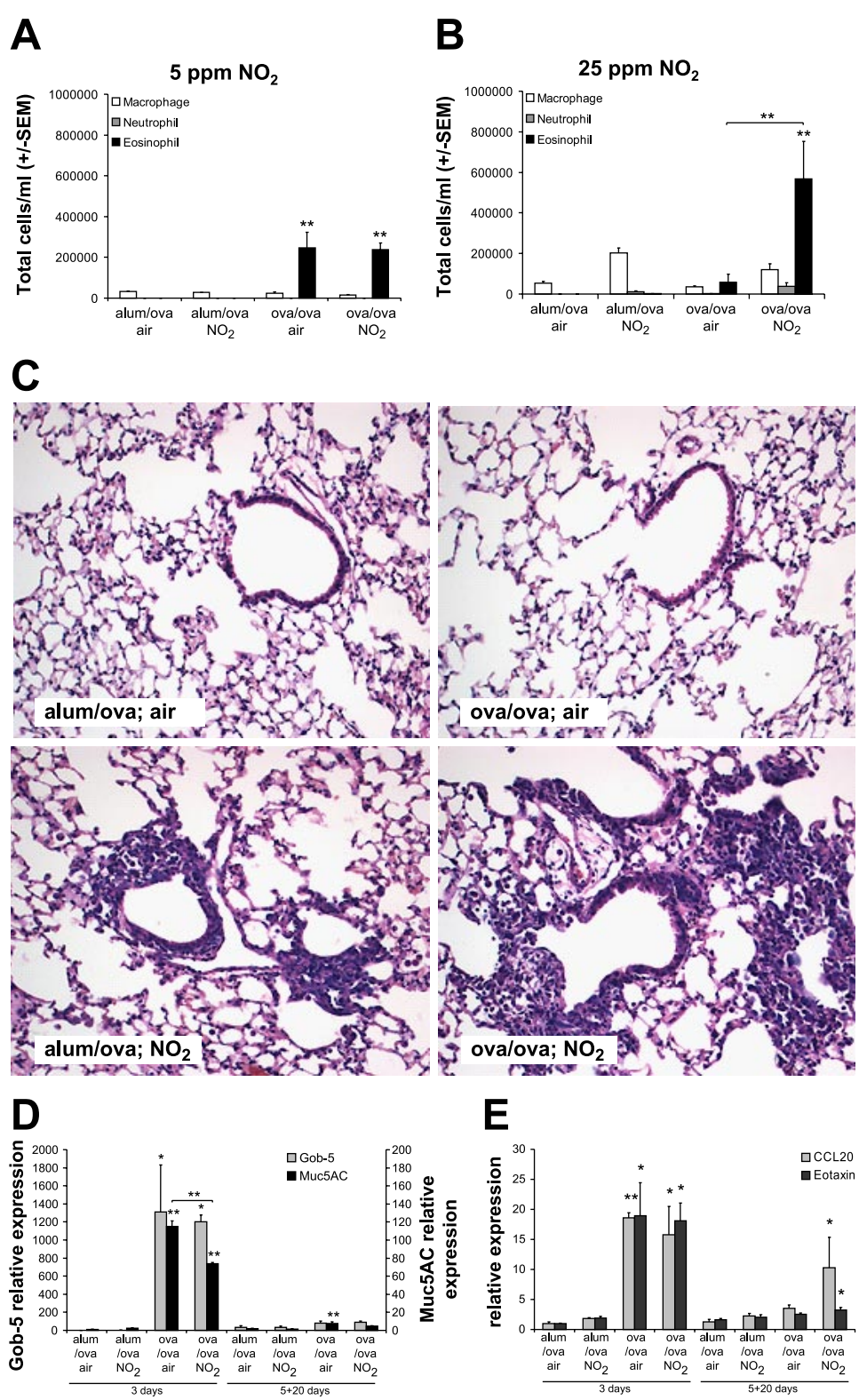


Fig. 4. Assessment of cells recovered by bronchoalveolar lavage from mice exposed to air or 25 ppm NO₂ for 6 h/day for 3 days. Data are means $\pm$ SE of 5 mice/group. *** $P < 0.01$ for NO₂ vs. air.

Fig. 5. Assessment of the inflammatory response in mice subjected to individual or combined ovalbumin (OVA)/OVA and NO₂ exposure regimens. Mice were either mock sensitized or OVA sensitized followed by challenge with OVA (Alum/OVA and OVA/OVA, respectively) on 3 sequential days (see MATERIALS AND METHODS). Forty-eight hours after the last OVA challenge, mice were either exposed to air or 5 or 25 ppm NO₂ for 6 h/day for 3 days. **A**: assessment of the inflammatory response via analysis of bronchoalveolar lavage fluid (BALF) from mice exposed to air or 5 ppm NO₂. Data are means $\pm$ SE of 5–6 mice/group. **B**: assessment of the inflammatory response via analysis of BALF from mice exposed to air or 25 ppm NO₂. Data are means $\pm$ SE of 5–6 mice/group and are representative of studies performed 3 times. **C**: assessment of lung histopathology from mice exposed to air or 25 ppm NO₂ by staining of lung sections with hematoxylin and eosin. Original magnification, $\times 200$. Data are representative of 5–6 mice/group of studies performed 3 times. **D** and **E**: assessment of mRNA expression of *Gob-5*, *Muc5AC*, *CCL20*, and *eotaxin-1* in lung tissues from mice exposed to air or 25 ppm NO₂. Lungs were harvested immediately after cessation of the 3-day NO₂ exposure regimen (*left*), whereas some mice were allowed to recover for 20 days after cessation of the 5-day NO₂ exposure regimen (*right*). Total lung cDNA was examined by quantitative PCR analysis, and data were normalized to the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase. All data are expressed as the level of expression relative to the group of mice that were mock sensitized, challenged, and exposed to air (Alum/OVA; air) for 3 days. Data are means $\pm$ SE of 5 mice/group. * $P < 0.05$, ** $P < 0.01$ vs. Alum/OVA + air exposure group; * or **over brackets indicate $P < 0.05$ or $P < 0.01$ between indicated groups, respectively.



eosinophils from the OVA/OVA plus 5 ppm NO₂ group were due to a single mouse and therefore did not reach statistical significance.

Finally, we determined respiratory mechanics in the mice using impedance measurements obtained through the forced oscillations technique, as previously described (38). We have

reported using this technique that the majority of pulmonary responsiveness to methacholine in the mouse model of allergic airway disease is manifest in parenchymal events, which are assessed as the tissue elastance parameter H_{ti} (38). Whereas 48 h after three challenges airway hyperresponsiveness (AHR) was maximal in mice subjected to OVA/OVA, 3 days later,

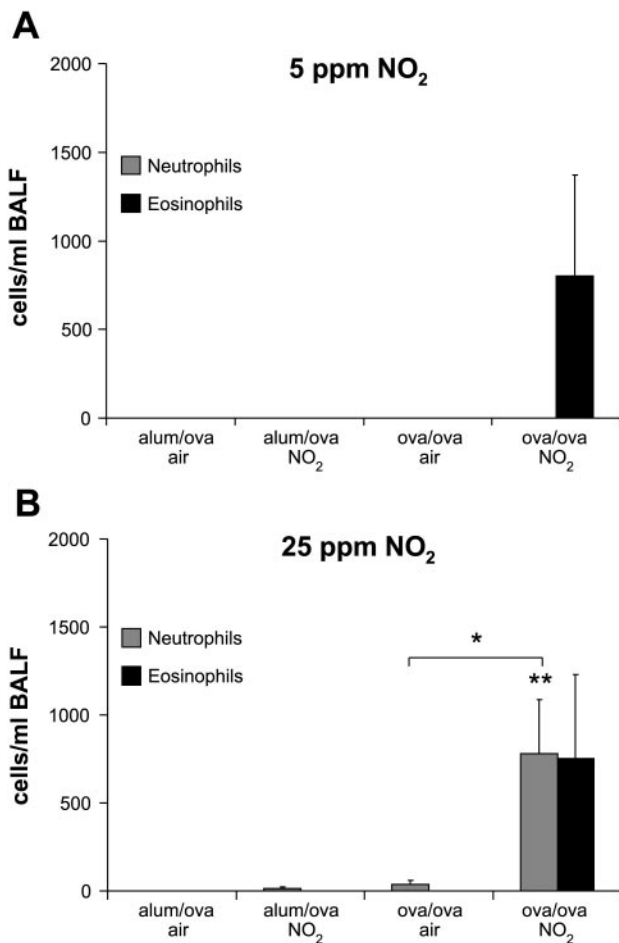


Fig. 6. Assessment of the persistence of the inflammatory response via BALF analysis from antigen-challenged mice that were either mock sensitized (Alum/OVA) or antigen sensitized (OVA/OVA) and subsequently exposed to air, 5 ppm NO₂ (A), or 25 ppm NO₂ (B) for 5 days and allowed to recover for an additional 20 days. ***P* < 0.01 for OVA/OVA + NO₂ exposure vs. Alum/OVA + air exposure; *over brackets indicates *P* < 0.05 between indicated groups.

AHR was still apparent (Fig. 7, A and C). Whereas 5 ppm NO₂ exposure did not cause AHR, 25 ppm NO₂ exposure alone was sufficient to elicit AHR in the mouse (Fig. 7, A and C). A 25-ppm NO₂ exposure did not further elevate OVA/OVA-induced AHR at this acute time point (Fig. 7C). However, 20 days after cessation of individual or combined exposures demonstrated that AHR in mice only exposed to NO₂ was resolved, AHR was retained in OVA/OVA plus 25 ppm NO₂-exposed mice (Fig. 7D). Mice exposed to OVA plus 5 ppm NO₂ demonstrated no long-term alterations in AHR (Fig. 5B). Collectively, these data demonstrate that, in the absence of eosinophilic inflammation, NO₂ inhalation is sufficient to acutely induce AHR to methacholine, and in the presence of preexisting allergic airway disease, NO₂ exposure augments and prolongs the inflammatory response and causes sustained increases in AHR.

DISCUSSION

Nitrogen dioxide represents a significant air pollutant for many populations, notably for individuals living in heavily polluted urban settings (32) and those exposed to indoor

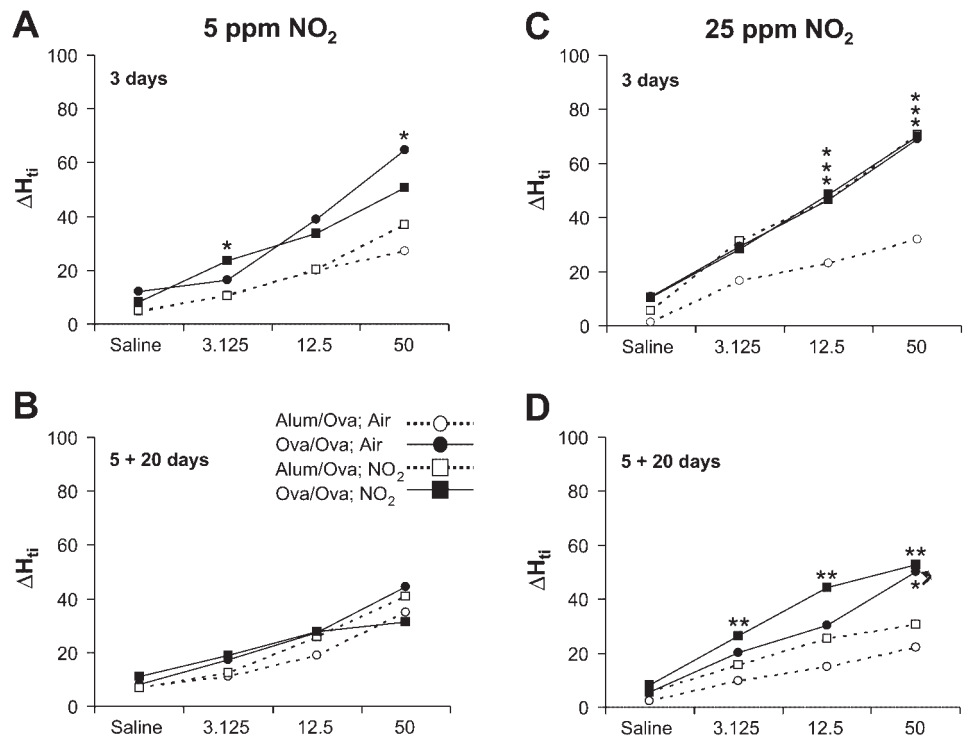
combustion byproducts generated during heating and cooking (7). In addition, its formation can also be triggered in the lung via peroxidase-catalyzed consumption of nitrite, providing an additional route of exposure (4). Recent studies also suggest that gaseous air pollutants can enhance the effects of allergens (10, 11), providing a putative mechanism whereby this reactive oxidant can exacerbate inflammatory lung disease. A number of various scenarios exist in which exposure to NO₂, either from exogenous or endogenous sources, could contribute to the causation or aggravation in inflammatory lung diseases including asthma (2, 49–51). A number of fundamental insights have previously been made in rats, including ultrastructural and morphological alterations (6, 13, 14, 47, 48), bronchoalveolar lavage protein, neutrophils (15), and LDH (20), as well as the reversibility of the phenotype (12). However, understanding the events elicited by NO₂ exposure in a genetically defined model organism, such as the inbred mouse, are important to further our understanding of the potential of NO₂ to cause lung pathophysiology.

Our findings described herein demonstrate that inhalation of 25 ppm NO₂ by C57BL/6 mice induces acute damage associated with inflammation and lesions in the alveolar duct region and an influx of macrophages and neutrophils into the lavageable air spaces. In addition, we also demonstrate that this exposure regimen of 25 ppm NO₂ augments allergic airway disease, evidenced from the enhanced and prolonged eosinophilic and neutrophilic inflammation, and prolonged AHR. The acute effect of 25 ppm NO₂ alone causing AHR could have been due, at least in part, to pulmonary edema. The molecular events responsible for the NO₂-induced aggravation of allergic airways disease were initially assessed by measuring *eotaxin-1* and *CCL20*, yet remain to be further elucidated. Evaluation of *eotaxin-1* mRNA, a chemokine important in eosinophil chemotaxis that is enhanced during allergic airways disease in the mouse, revealed no synergistic increases in mice subjected to combined antigen plus NO₂ exposures, which would have explained the markedly enhanced eosinophilia in the combined exposure groups. However, it is possible that at earlier time points, which were not examined in the present study, mRNA levels for this chemokine might have been elevated in the combined exposure groups. Alternatively, it is also possible that eosinophil chemokine gradients are not enhanced, but that eosinophil retention or survival are enhanced in NO₂-exposed mice via other mechanisms. Similarly, evaluation of *Gob5*, *Muc5AC*, and *CCL20* mRNAs also failed to elucidate clear increases in OVA/OVA plus 25 ppm NO₂ exposure groups compared with mice subjected to antigen alone after 3 days of NO₂ exposure. In addition, these chemotactic signals are unlikely to cause the sustained inflammation observed in the OVA/OVA plus NO₂ group, as they are at or near baseline levels 20 days after exposure, at a time of sustained inflammation and AHR.

Although the precise mechanism of action through which NO₂ injures lungs and promotes inflammation remains to be determined, we have recently described in vitro that alveolar type 2 epithelial cells undergo cell death with characteristics of both apoptosis and necrosis following exposure to NO₂ (37). RNS, NO₂, or ONOO[−] caused death of rat lung epithelial cells that were dividing or migrating, whereas confluent cultures were completely resistant. We also reported that RNS-induced death was induced via the activation of JNK, which occurred in



Fig. 7. Assessment of airway hyperresponsiveness in antigen-challenged mice that were either mock sensitized (Alum/OVA) or antigen sensitized (OVA/OVA) and exposed to air or 5 ppm NO₂ for 6 h/day for 3 days (A), or for 5 days and allowed to recover for an additional 20 days (B), or exposed to air or 25 ppm NO₂ for 6 h/day for 3 days (C), or for 5 days and allowed to recover for an additional 20 days (D). Data are means ($n = 4-6$ /group) of changes in tissue elastance (ΔH_{ti}) over baseline measurements taken before administration of increasing doses of methacholine. * $P < 0.05$ vs. Alum/OVA + air exposure, ** $P < 0.01$ for OVA/OVA + NO₂ exposure vs. Alum/OVA + air exposure. Stacked * are indicative of the significance of the respective overlapping groups below.



a Fas-dependent manner (46). It will be of great interest to determine whether Fas and JNK activation also play a role in the genesis of NO₂-induced acute lung injury and inflammation and whether these molecules are also important in the sustained inflammatory response observed in mice with allergic inflammation exposed to NO₂.

The phenotype of JNK-dependent cell death triggered by NO₂, which consists of mixed features of apoptosis and necrosis, may be important in the propagation of inflammation, based on recent studies demonstrating that necrotic cell constituents are potent activators of the transcription factor NF- κ B (31). NO₂-induced damage could result in the release of intracellular components, some of which are known to be sufficient to activate NF- κ B in neighboring healthy cells, which in turn could elicit an inflammatory response and perpetuate damage to the lung. A further elucidation of the constituents of the cell debris that is elaborated into the air spaces of NO₂-exposed mice, and their mechanism of activation of inflammatory signals, will be essential to address this hypothesis.

Toll-like receptors (TLRs) are a class of receptors that represents potential candidates to transduce NO₂-induced necrotic inflammatory signaling, given the recent studies demonstrating that the intracellular protein, heat shock protein 70, induces NF- κ B activation and inflammatory cytokine production through TLR2 (31). In addition, many other endogenous molecules may be capable of stimulating responses via TLR stimulation when released from necrotic cells or after their oxidative modification (45), the latter representing a plausible scenario after NO₂ exposure. Intriguingly, a critical role for TLR4 in the genesis of acute lung injury by NO₂ (21, 29) has emerged, based on observations that C3H/HeJ mice, which contain a mutation in the TLR4 gene that renders it nonfunctional, are particularly resistant to NO₂, although at least another candidate gene is likely to be involved. TLR4 signaling

has also been implicated in the pulmonary response to ozone exposure (27, 28). However, due to the lack of concordance of mouse strain responsiveness to NO₂ and ozone in nine tested strains, the genetic mechanisms controlling susceptibility to lung damage induced by these agents are unlikely to be the same (29). Whether TLRs and downstream intracellular signaling events are indeed important in NO₂-induced exacerbation of allergic airway disease remain to be formally tested.

Intriguingly, our present findings are not in complete agreement with recent reports on the effects of NO₂ exposure in a mouse model of allergic airways disease, which either describe a lack of effect of NO₂ on eosinophilia or a decrease in eosinophilic inflammation. These discrepancies can be explained by differences in the time frame of exposure to NO₂ relative to the OVA challenge, the concentration and/or the duration of NO₂ inhalation, and the genetic backgrounds of mice investigated. For example, using allergen-sensitized BALB/c mice exposed to 20 ppm NO₂ immediately before an intranasal challenge with allergen, Proust et al. (40) reported enhanced AHR and lung permeability 24 h after exposure, despite a lack of alterations in eosinophil numbers in the BALF at 24 or 72 h after challenge and a reduction of late mucosal metaplasia compared with mice sensitized and challenged with allergen (OVA) alone. This group also reported that 5 ppm NO₂ caused a reduction in many of the measured features of allergic airway disease (40). Using allergen-sensitized C57BL/6 mice exposed to 0.7 or 5 ppm NO₂, Hubbard et al. (22) also demonstrated reductions in eosinophil numbers in the BALF compared with air-exposed mice in response to subsequent challenge with allergen. Using BALB/c mice sensitized and challenged in a manner similar to our methodologies, Hussain et al. (23) reported that exposure to 2 ppm NO₂ 24 h before allergen challenge induced airway neutrophilia and epithelial damage but did not alter AHR (as assessed by

enhanced pause measurements) despite increasing baseline smooth muscle tone. Finally, using Brown Norway rats sensitized and challenged with house dust mite allergen, Gilmour et al. (16) reported that exposure to 5 ppm NO₂ for 3 h after both allergen sensitization and challenge induced higher antigen-specific immunoglobulin levels in the circulation, inflammatory cells in the lungs, and lymphocyte responsiveness to antigen. From these different reports, it is apparent that NO₂ exposure has distinct effects on pulmonary response to antigen in rodent models.

Although the dose of NO₂ administered to our mice was higher than those used in a number of previous studies (16, 22, 23, 40) and are higher than the concentrations seen in ambient air, the actual concentrations of NO₂ that reach the lung tissue are likely to be far lower than 25 ppm, given the high reactivity of NO₂ toward airway surface lining components. Consequently, the patterns of tyrosine nitration observed within the lung tissue in situ using the current NO₂ exposure regimen were not substantially different from air controls (data not shown). Additionally, in the allergic, inflamed lung, NO₂ concentrations in the tissue locally could be very high, due to the catalytic activity of eosinophil peroxidase, which can generate up to 5 ppm NO₂ (4) using concentrations of its substrates, H₂O₂ and nitrite, within the physiological range, leading to marked tyrosine nitration (4, 33). Therefore, although the levels of NO₂ present in the exposure chamber are higher than NO₂ concentrations found in association with air pollution, local concentrations of NO₂ generated endogenously during allergic inflammation may effectively be even higher than those in the present study.

It is generally appreciated that oxidative stress accompanies inflammatory diseases of the lung and that the extent of oxidative stress appears to correlate with disease severity. For example, the degree of nitrotyrosine reactivity correlates directly with functional abnormalities in patients with asthma (44). Because NO₂ is the ultimate oxidant responsible for tyrosine nitration, these findings suggest a role for NO₂ in the pathophysiology of asthma. Indeed, our present study demonstrates that NO₂ is sufficient to induce AHR, a hallmark feature of allergic airways disease. More significantly, we demonstrate that NO₂ augments and prolongs OVA-induced inflammation and AHR. These findings provide “proof of concept” toward the notion that NO₂ is an important mediator that can contribute to the pathological effects seen in the allergic inflamed lung.

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