

Effects of Nitrogen Dioxide on Allergic Airway Responses in Subjects With Asthma

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Objective: We sought to determine whether nitrogen dioxide (NO_2) can enhance airway inflammation after allergen challenge in asthmatic subjects. **Methods:** Fifteen house-dust-mite (HDM)-sensitive asthmatic subjects were exposed for 3 hours to filtered air or 0.4 ppm NO_2 , followed by inhalational challenge with HDM allergen. Markers of inflammation were measured in sputum at 6 hours and 26 hours after allergen challenge. **Results:** After exposure to NO_2 , eosinophil concentration decreased significantly in the 6-hour postallergen sputum. No significant NO_2 -related difference was observed for other variables. **Conclusions:** Our results suggest that, in most asthmatic individuals, multihour exposure to a high ambient concentration of NO_2 does not enhance the inflammatory response to subsequent inhaled allergen as assessed by cell distribution in induced sputum. Because the decrease in airway eosinophils has been reported in previous animal studies, future research should be directed toward the mechanism of this effect. (J Occup Environ Med. 2005;47:1250–1259)

Nitrogen dioxide (NO_2) is a common environmental air pollutant that is primarily generated by the combustion of fossil fuels. Outdoor levels predominantly are associated with traffic density and can reach peak concentrations of 0.2 to 0.6 ppm,^{1,2} although indoor levels often exceed outdoor levels. In households with unvented combustion sources, NO_2 levels can peak at greater than 2 ppm³ and levels as high as 4 to 5 ppm have been measured at indoor ice arenas.⁴

Inhaled nitrogen dioxide is absorbed along the entire respiratory tract, with the greatest dose to epithelial tissue delivered to the peripheral airways at the junction between conducting and respiratory airways.⁵ NO_2 interacts with airway lining fluid and epithelial cell membranes before passing through the airway epithelium into tissue. Although the exact mechanism by which NO_2 reacts with tissue is not known, it is suspected to induce damage to cell membranes by generating reactive oxygen intermediates and inducing cytokine and other mediator release from inflammatory cells in the airways.⁶

Epidemiological studies indicate asthmatic individuals or individuals with airway hyperresponsiveness are more susceptible to the respiratory effects of exposure to ambient NO_2 .^{7–12} For people with asthma, exposure to NO_2 is associated with increased risk of respiratory symptoms and lower respiratory illness^{7–9,13–21} and emergency department visits or hospitalizations for asthma.^{8,10,11,22–32}

Several studies have demonstrated that NO_2 exposure may increase bronchoconstrictor responses to inhaled allergen in sensitized subjects.^{33–38} In one of the first of such

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studies, mild asthmatic subjects exposed to 0.4 ppm NO₂ for 1 hour had increased early-phase (EPR) and late-phase (LPR) bronchoconstrictor responses to a fixed dose of allergen.³⁷ A later set of experiments demonstrated that exposure to NO₂ resulted in concentration and time-dependent effects on the dose of allergen required to cause a 20% decrease in forced expiratory volume in one second (FEV₁; allergen PD₂₀-FEV₁).³³ Another group of investigators has observed enhancement of late bronchoconstrictor responses with a concentration of NO₂ as low as 0.26 ppm and a duration of exposure as short as 30 minutes.³⁵

LPR bronchoconstriction occurs 4 to 8 hours after allergen inhalation and is thought to be attributable to inflammatory cell influx, mucosal edema, and smooth muscle contraction.^{39,40} Late-phase inflammation is characterized by increased eosinophils, neutrophils, their degranulation products, and other mediators in the airways^{39,40} and has been measured in induced sputum and bronchoalveolar lavage (BAL) samples.

Given that NO₂ exposure has been shown to enhance the LPR to allergen, we hypothesized that NO₂ exposure also would increase the

airway inflammatory response to allergen in specifically sensitized asthmatic individuals. To test this hypothesis, we designed a controlled exposure study that used sputum induction to sample airway lining fluid after exposure to NO₂ and allergen challenge.

Materials and Methods

Subjects

Fifteen mild-to-moderate atopic asthmatics were recruited for the study. Subject characteristics are listed in Table 1. Inclusion criteria were age 18 to 50 years; nonsmoking status (defined as cumulative history <1 pack-yr, >3 months abstinence); physician diagnosis of asthma confirmed by methacholine challenge; and house dust mite (HDM) allergy confirmed by skin prick testing to *D. pteronyssinus* (*D. pter*) allergen. Subjects refrained from inhaled steroids for 2 weeks; long-acting bronchodilators for 2 days; short-acting bronchodilators for 8 hours; nonsteroidal anti-inflammatory medications (including leukotriene antagonists, antihistamines and other allergy medications) for 5 days, and caffeine for 4 hours before each visit. Subjects had no asthma exacerbations,

respiratory tract infections, or more than usual exposure to allergy provoking agents for 3 weeks before each visit.

Procedures used in this human research were in accordance with the recommendations found in the Helsinki Declaration of 1975. All subjects gave informed consent. The study protocol was approved by the Committee on Human Research at the University of California, San Francisco.

Study Design

Subjects were exposed to either filtered air (FA) or 0.4 ppm NO₂ for 3 hours with intermittent exercise in a randomized, single-blind, counter-balanced design. Spirometry was performed, and symptom questionnaires completed immediately before and after each exposure. Each exposure was immediately followed by an inhalational allergen challenge with doubling doses of *D. pter* until a 20% decrease in FEV₁ was achieved. Induced sputum was obtained at 6 (S-6) and 26 (S-26) hr after each allergen challenge. Spirometry and symptom questionnaires were performed for 6 hours after each allergen challenge, with additional spirometry before each sputum in-

TABLE 1
Subject Characteristics

Subject No.	Age (yr)	Sex	Ht (cm)	Wt (kg)	FEV ₁ (L)	FEV ₁ % pred	MPC ₂₀ (mg/mL)	Meds
1	30	M	175	75	3.71	85.5	4.13	None
2	24	F	163	64	2.38	70.6	0.88	IS; SB; LA; AH
3	21	F	173	84	3.53	93.0	0.47	SB
4	34	M	170	80	3.29	81.7	0.75	IS; SB
5	23	F	173	52	3.01	80.4	0.91	None
6	30	M	183	80	4.05	87.1	4.62	SB
7	37	F	175	67	3.34	96.1	5.60	AH
8	30	F	163	61	1.87	58.1	0.24	IS; LB; SB; LA; NS; AH
9	48	F	170	98	2.07	68.5	0.81	IS; SB
10	43	M	175	68	3.90	97.0	0.70	SB; AH; DE
11	34	F	163	56	3.10	99.5	4.22	LB; SB; LA; NS
12	26	M	183	99	3.78	79.6	0.58	None
13	45	M	185	84	3.45	78.6	4.39	None
14	35	F	165	136	2.04	64.2	1.63	IS; LA; SB
15	21	F	168	61	3.20	88.4	2.44	None
Mean	32		172	78	3.11	81.9	2.16	
(SD)	(8.6)		(7.5)	(22)	(0.71)	(12.4)	(1.88)	

MPC₂₀ indicates methacholine provocative concentration causing 20% reduction in FEV₁; IS, inhaled steroid; SB, short-acting bronchodilator; LB, long-acting bronchodilator; LA, leukotriene antagonist; AH, antihistamines; NS, nasal steroid; DE, decongestant/expectorant.

duction and at intervals overnight. The exposures were separated by at least 3 weeks.

Spirometry

Spirometry was performed using previously described standardized procedures.⁴¹ At the inclusion visit, spirometry was performed using a dry rolling seal spirometer (Collins Medical, Inc.). Spirometry during the exposure days was performed using a hand-held spirometer (EasyOne, ndd Medical Technologies, Zurich, Switzerland) to allow subject monitoring at a clinical research center.⁴² Each subject used the same hand-held spirometer for both exposure visits. The best of at least two consistent efforts based on FEV₁ was recorded for each time point.

Methacholine Challenge

Methacholine (Provacholine, Metapharm, Inc., Ontario, Canada) inhalation challenge was performed following a protocol modified from the American Thoracic Society guidelines,⁴³ using a nebulizer (DeVilbiss) and dosimeter (Rosen-thal) set to deliver 9 μ L per breath. Subjects inhaled aerosol from the nebulizer in five breaths (one breath every 12 seconds over the course of a 1-minute period), and spirometry was measured 3 minutes after each dose. The next dose was administered within 30 seconds of completing the spirometry. Increasing doses of methacholine (0.0625, 0.25, 1, 2, 4, 8 mg/mL) were given, until a 20% fall in FEV₁ from saline FEV₁ was achieved. Methacholine PC₂₀ (MPC₂₀) was calculated from linear interpolation of the last two values on the log dose-response curve. A positive methacholine test was defined as a MPC₂₀ < 8 mg/mL.

Skin Prick Testing

Allergy to *D. pter* (Hollister-Stier Laboratories, LLC, Spokane, WA) was confirmed by skin-prick test, with a positive response defined by a skin weal >2 mm after 15 minutes. Dilutions of allergen were prepared

by diluting stock solution in sterile normal saline. Skin sensitivity (SS) to *D. pter* was determined by a dilutional skin-prick test using dilutions ranging from 0.057 to 30,000 AU/mL, and was defined as the dilution that produced a >2 mm weal after 15 minutes.

Exposure with Exercise

The exposures were performed in a custom-built stainless-steel and glass exposure chamber (Model No. W003273R; Nor-Lake Inc., Hudson, WI), which is 2.5 $\times$ 2.5 $\times$ 2.4 m in dimension. Temperature- and humidity-controlled air was supplied to the chamber at an average airflow rate of 9.3 L/min as previously described.⁴⁴

NO₂ from a gas cylinder (Airgas, certified 5000 ppm, balance air) was supplied to the inlet duct of the chamber using a dual regulator/flow meter system. During exposure, NO₂ concentration inside the chamber was continuously monitored with a chemiluminescence NO_x analyzer (Model No 8840; Monitor Labs, Los Angeles, CA), with sampling via Teflon tubing in the breathing zone of the subject and was recorded every 10 minutes.

Exposures were performed with intermittent exercise (first 30 minutes of each hour). Breathing frequency and tidal volume were measured at 10 and 20 minutes during each exercise period using a pneumotachograph (Fleish, Model No 3) and minute ventilation was calculated. Exercise was adjusted to approximate the calculated target ventilation (body surface area m² $\times$ 25 L/min).

Allergen Challenge

Predicted Allergen PC₂₀ (APC₂₀) was calculated according to Cockcroft⁴⁵ using the formula:

$$\log_{10} (\text{APC}_{20}) \\ = 0.68 \log_{10} (\text{MPC}_{20} \times \text{SS}).$$

The starting dose for allergen challenge was four doubling doses less than the predicted APC₂₀ dose. The range of allergen doses was 0.057 to

30,000 AU/mL, and doses were thawed to room temperature before inhalation. Using the same nebulizer and dosimeter from the methacholine challenge, subjects inhaled normal saline with five breaths (one every 12 seconds over the course of 1 minute), performing spirometry 10 minutes after inhalation. Increasing doses of allergen were inhaled until a 20% decrease in FEV₁ from saline baseline was observed or all doses were given. Spirometry was performed at 10, 20, 30, 45, and 60 minutes and hourly until 6 hours after the challenge.

Cumulative allergen PD₂₀ was calculated by linear interpolation of the last two values on the log dose-response curve. The maximum EPR was recorded as the greatest drop in FEV₁ in the first hour after allergen challenge. A LPR was characterized by a fall of >15% in FEV₁ from baseline 4 to 6 hours after allergen challenge.

Symptom Questionnaire

Subjects completed a symptom questionnaire before and after each exposure, and hourly during the 6 hours monitoring period after allergen challenge. Symptoms included six pulmonary (chest tightness, chest pain, cough, phlegm, shortness of breath, and wheezing) and six general (headache, nausea, anxiety, eye irritation, nasal irritation, and throat irritation) symptoms and were rated from 0 (none) to 4 (severe).

Sputum Induction

Sputum induction (SI) was performed in an isolation booth (Biosafety, Aerostar Systems, San Diego, CA). Subjects were pretreated 15 minutes before SI with 400 μ g of albuterol. Subjects inhaled nebulized (DeVilbiss Ultra-Neb 99) 3% sterile saline for 20 minutes; at 2-minute intervals, saliva was cleared from the mouth before the subject coughed and sputum was collected. The sample was diluted in an equal volume of 0.1% dithiothreitol (Sputalysin; Behring Diagnostics, Inc., Somerville,

NJ) and incubated in a 37°C shaking water bath for 15 minutes with sample manipulation to ensure complete homogenization. An aliquot for total cell count and cytospin preparation was separated, and then the sample was centrifuged at 1000 rpm for 15 minutes at 4°C. The supernatant was recentrifuged at 3000 rpm for 15 minutes at 4°C and stored in aliquots at -80°C until analysis.

Total cell counts were performed using a hemocytometer (Fisher Scientific, Hampton, NH) and were expressed as number of cells per milliliter of sputum. Cytospin slides were prepared using a cytocentrifuge (Thermo Shandon) at 500 rpm for 5 minutes. Slides were stained with Diff-Quik (Dade Behring). Differential cell counts were obtained by enumeration of 400 nonsquamous cells per slide, performed by two counters. Macrophages, lymphocytes, neutrophils, and eosinophils were expressed as a percent of leukocytes. Cell concentrations were calculated from the differential percent and total leukocyte cell count and were expressed as number of cells per milliliter of sputum.

Biochemical Analyses

Analyses of soluble mediators in sputum were performed on samples after additional centrifugation at 10,000 rpm for 3 minutes at 4°C. Eosinophilic cationic protein (ECP) was measured using the UniCAP fluoroenzymeimmunoassay (Pharmacia & Upjohn Diagnostics AB, Portage, MI) with a detection limit of 2 µg/L. Levels of interleukin (IL)-8 were measured using a Quantiglo ELISA (R&D Systems, Minneapolis, MN), with a detection limit of 0.8 pg/mL. Interleukin-5 (IL-5) was measured with a Quantikine enzyme-linked immunosorbent assay (ELISA; R&D Systems) with a detection limit of 3 pg/mL. Granulocyte/macrophage-colony stimulating factor (GM-CSF) was measured using a Quantikine HS ELISA (R&D Systems) with a detection limit of 0.26 pg/mL. Quantitation

of total protein was determined using the BCA Protein Assay kit (Pierce).

Statistical Analyses

Most of the sputum and spirometric data were not normally distributed. Therefore, statistical analysis of the data comparing FA and NO₂ conditions was performed using the nonparametric Wilcoxon signed rank test. The reproducibility of allergen challenge is considered to be ± 1 doubling dose of allergen.⁴⁶ Subjects who had a difference within ± 1 doubling dose of allergen between the FA and NO₂ allergen challenges were designated “nonresponders” to the NO₂-allergen exposure sequence according to this criterion, whereas subjects who had a ≥ 2 doubling-dose difference between allergen challenges were designated “responders” because they required less allergen to reach a PD₂₀ after NO₂ exposure compared with FA. Comparisons of responders to nonresponders were performed as follows. First, the differences in all measured variables between the two exposure conditions (FA-allergen vs. NO₂-allergen) were calculated for each subject for both the S-6 and S-26 time points. Second, these differences for the two groups (responders vs. nonresponders) were compared using the Mann-Whitney *U* test. All analyses were conducted using the statistical program SYSTAT (SPSS Inc., Chicago, IL). Statistical significance was set at $P < 0.05$.

Results

Exposure Conditions

The concentration for the NO₂ exposures was (mean $\pm$ SD) 0.40 $\pm$ 0.01 ppm. The temperature and humidity inside the chamber for FA were 19.8 $\pm$ 0.3°C and 54.4 $\pm$ 3.9%, and for NO₂ exposure were 19.9 $\pm$ 0.6°C and 51.8 $\pm$ 4.5%, respectively. Minute ventilation for FA and NO₂ was 41.7 $\pm$ 6.7 L and 40.5 $\pm$ 6.8 L, respectively. There was no statistical

significant difference in these variables between the two conditions.

Spirometry

Table 2 displays the FEV₁ responses to the two experimental conditions: FA/allergen challenge and NO₂/allergen challenge. There was no statistically significant difference in FEV₁ before the FA and NO₂ exposures or change in FEV₁ for either exposure. After FA exposure, 13 of the 15 subjects had an EPR during the allergen challenge and seven had a LPR. After NO₂ exposure and allergen challenge, 15 had an EPR and five had a LPR. However, there was no statistically significant difference in FEV₁ or %drop in FEV₁ in either phase, comparing NO₂ to FA exposure arms.

Allergen PD₂₀-FEV₁

There was no statistically significant difference in allergen PD₂₀-FEV₁ comparing NO₂ to FA for the 15 subjects as a whole. Of the 15 subjects, 12 had a difference within $\pm$ one doubling dose of allergen between the FA and NO₂ allergen challenges (nonresponders). The remaining three subjects (responders) had a $\geq$ two doubling-dose difference between FA and NO₂ allergen challenges (Fig. 1).

Subject Symptoms

There were no statistically significant differences respiratory symptoms comparing NO₂ and FA exposure arms.

Sputum Volume

Sputum volumes for FA and NO₂ S-6 sputum samples were (median [IQR]) 5.9 (3.8–7.4) mL and 5.0 (4.7–7.2) mL, and for S-26 were 5.1 (3.6–6.3) mL and 4.8 (3.9–6.3) mL, respectively. There was no statistical significant difference between the two conditions.

Sputum Cell Distribution

Cell distribution for the sputum samples at 6 and 26 hours after allergen challenge are shown in Table 3. When comparing NO₂ to FA,

TABLE 2

FEV₁ Responses to Filtered Air/HDM Allergen and Nitrogen Dioxide/HDM Allergen

Subject	Pre Exp FEV ₁		Pre AC FEV ₁		EPR FEV ₁		EPR % Change		LPR FEV ₁		LPR % Change		PD ₂₀ -FEV ₁	
	FA	NO ₂	FA	NO ₂	FA	NO ₂	FA	NO ₂	FA	NO ₂	FA	NO ₂	FA	NO ₂
1	3.99	3.89	4.01	3.99	3.11	2.86	-22	-28	3.93	3.64	-2	-9	327	598
2	2.56	2.46	2.71	2.31	1.61	1.80	-41	-22	1.98	2.04	-27	-12	7.9	18
3	3.52	3.40	3.62	3.89	3.15	2.89	-13	-26	3.26	3.45	-10	-11	>2710	42 R
4	3.61	3.64	3.71	3.58	2.76	1.72	-25	-52	2.24	2.75	-40	-23	4.2	8.4
5	3.86	3.31	3.61	3.47	2.34	2.38	-35	-31	3.73	3.57	+3	+3	14	7.2
6	4.04	3.99	4.11	3.99	3.66	1.98	-11	-50	3.56	3.26	-13	-18	>2697	480 R
7	3.24	3.34	3.40	3.41	2.11	2.24	-38	-34	2.05	2.25	-40	-34	24	18
8	2.31	1.82	2.10	1.97	1.65	1.53	-21	-22	1.86	1.76	-11	-11	8.9	22
9	1.97	1.99	1.99	2.22	1.54	1.49	-23	-33	1.76	1.85	-12	-17	1112	402
10	4.01	4.23	3.98	4.19	3.00	2.56	-25	-39	3.01	3.27	-25	-22	2.4	1.9
11	2.97	3.07	3.28	3.17	2.14	2.05	-35	-35	2.79	2.81	-15	-11	401	23 R
12	3.66	3.75	3.80	3.97	2.88	3.08	-24	-22	3.68	3.67	-3	-8	9.0	9.3
13	3.71	3.91	3.67	3.78	2.58	2.66	-30	-30	3.08	3.31	-16	-12	12	27
14	2.27	2.27	2.32	2.13	1.57	1.52	-32	-29	1.63	2.00	-30	-6	56	139
15	3.11	2.98	3.16	3.07	1.79	2.43	-44	-21	3.00	2.93	-5	-5	866	674
Median	3.52	3.34	3.61	3.47	2.34	2.24	-25	-30	3.00	2.93	-13	-11	24	23
IQR	2.77-3.79	2.72-3.82	2.94-3.76	2.69-3.93	1.72-2.94	1.76-2.61	-22.5, -35	-24, -34.5	2.02-3.41	2.15-3.38	-7.5, -26	-8.5, -17.5	9-634	14-271

FEV₁ (L); HDM, house dust mite; AC, allergen challenge; EPR, early-phase response (0-1 hour); LPR, late-phase response (4-6 hours); PD₂₀-FEV₁, provocative dose of HDM allergen causing 20% decrease in FEV₁; AU, allergen units; FA, filtered air; NO₂, nitrogen dioxide; R, responder (difference $\geq$ 2 doubling dose).

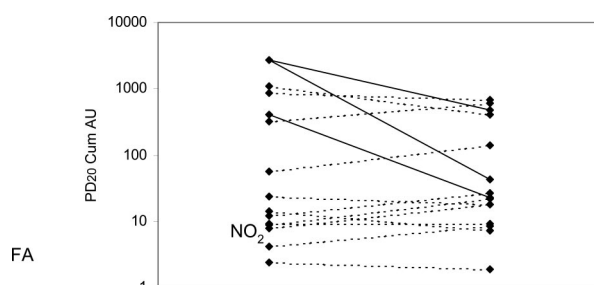
we noted a statistically significant decrease in eosinophil concentration in the S-6 sample ($P = 0.012$). However, there was no statistically significant difference in total leukocyte concentration, percent, or concentration of macrophages, lymphocytes, and neutrophils for S-6 or S-26 samples comparing NO₂ to FA. Analysis of cell concentrations corrected for cumulative allergen dose inhaled during allergen challenge showed no statistically significant difference for any cell type comparing NO₂ to FA (data not shown).

Sputum Biochemical Analyses

There was no statistical difference in IL-5, IL-8, GM-CSF, ECP, or total protein, comparing NO₂ to FA for either S-6 or S-26 samples. Analysis of data corrected for total protein showed a small but significant increase in IL-5/TP ratio ($P = 0.031$) after NO₂ exposure. Correction of concentrations and total protein ratios for cumulative allergen dose inhaled during allergen challenge resulted in no statistically significant difference for any concentration or ratio, comparing NO₂ to FA (data not shown).

Subset of Responders

Table 5 shows the study data stratified by whether the subjects are responders or nonresponders to NO₂. Comparison of the three responders to the 12 nonresponders showed the following statistically significant differences between the two groups: (1) the responders had higher allergen PD₂₀-FEV₁ and macrophage concentration in both S-6 and S-26 sputum samples after FA exposure and (2) they had higher IL-8 and IL-8/TP in both S-6 and S-26 samples after NO₂ exposure. Although the small number of subjects in the responder group limits the statistical power of this analysis, inspection of the values in Table 5 suggests a difference in the inflammatory responses of the two groups. At 6 hours after allergen challenge, there are nonsignificant increased NO₂-related changes in



PD20 expressed as cumulative allergen units of house dust mite (*D. pteronyssinus*) causing 20% reduction in FEV1. FA: filtered air, NO2: nitrogen dioxide.

Fig. 1. Effect of 0.4 ppm NO₂ on house dust mite allergen PD₂₀ in asthmatics.

TABLE 3

Leukocytes in Induced Sputum at 6 and 26 Hours After HDM Allergen Challenge With Prior Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26	
	FA	NO ₂	FA	NO ₂
Total leukocyte cells × 10 ³ /mL	152	140	132	122
	93–230	78–220	77–231	67–266
% Macrophage	26.1	24.5	23.3	29.1
	15.2–36.3	17.3–31.7	19.5–34.5	21.3–50.3
% Neutrophil	57.1	66.9	66.5	55.0
	50.5–68.6	50.7–72.4	52.7–75.5	39.7–68.8
% Eosinophil	7.2	4.5	3.5	2.1
	2.5–12.6	1.6–7.7	0.9–7.4	0.1–5.9
% Lymphocyte	3.4	3.3	4.5	4.8
	2.9–5.3	2.7–4.8	2.9–5.8	2.4–7.6
Macrophage cells × 10 ³ /mL	34.6	26.7	42.2	33.3
	23.3–55.7	16.9–48.9	16.1–62.9	18.6–74.2
Neutrophil cells × 10 ³ /mL	82.6	76.3	75.7	53.2
	55.7–156.6	35.8–161.3	50.7–146.4	37.0–127.0
Eosinophil cells × 10 ³ /mL	6.2	3.1*	5.3	4.52
	3.0–15.2	1.0–8.9	1.3–15.8	0.05–5.7
Lymphocyte cells × 10 ³ /mL	6.0	3.7	6.7	4.3
	2.4–7.7	1.5–11.6	2.8–13.1	2.2–11.7

HDM, house dust mite; FA, filtered air; NO₂, nitrogen dioxide; S-6 and S-26, sputum induction 6 and 26 hrs after allergen challenge. Values expressed as median; interquartile range.

**P* = 0.012.

neutrophils, eosinophils, IL-5, GM-CSF, and ECP in the responders compared to the non-responders. Most of these NO₂-related increased responses persisted at 26 hours after allergen challenge. Of note, the baseline lung function was non-significantly higher and both the non-specific and specific allergen airway reactivity of the three responders was non-significantly lower than the 12 nonresponders.

Discussion

This study was designed to test the hypothesis that exposure to a high

ambient concentration of NO₂ would enhance the airway inflammatory response to inhaled allergen in mild allergic asthmatic subjects. Using an exposure regimen previously reported to have enhanced the early-phase bronchoconstrictor response to inhaled allergen in similar groups of subjects,^{33,34} we failed to confirm our hypothesis. In fact, we also failed to observe enhancement of the EPR in most subjects. Our results, placed in the context of previous reports,^{33–38,47} indicate that it is only a subset of asthmatic subjects who respond to ambient NO₂

exposures with increased bronchoconstriction upon inhalational challenge with an allergen to which they are specifically sensitized.

Although we did not find that NO₂ exposure led to enhanced allergen-induced neutrophils or eosinophils in induced sputum obtained 6 hours after inhalational challenge, there were not significant suggested increases in any of the four inflammatory mediators measured (IL-5, IL-8, GM-CSF, and ECP) after NO₂ exposure as compared with filtered air. We also found a statistically significant decrease in eosinophils at 6 hours after NO₂. There may be decreased transit of eosinophils across the bronchial mucosa associated with increased activation and cytotoxicity^{48,49} after mild NO₂-induced oxidant injury (hence explaining the apparent inconsistency between decreased sputum eosinophils and increased ECP after NO₂). Bronchial tissue sampling is required to determine whether this occurs. Two recent toxicological studies using ovalbumin (OVA)-sensitized animal models support the concept that NO₂ exposure might actually cause reduced instead of increased allergen-induced airway eosinophilia. One study using BALB/c mice showed that a 3-hour exposure to 5 ppm NO₂ caused a marked reduction in BAL eosinophils with a subsequent OVA challenge compared with air-OVA controls,⁵⁰ and a second study using C57B1/6 mice showed that 2-hour exposures to NO₂ at either 0.7 or 5 ppm on 3 consecutive days reduced BAL eosinophil levels compared to air-OVA controls.⁵¹

When we designed and began data collection for this study, there were no published reports addressing the airway inflammatory response to inhaled allergen after exposure to NO₂. Recently, however, the results of such a study have been reported by Barck and coworkers.⁵² Using BAL 19 hours after allergen challenge, these investigators found NO₂-related increased neutrophils but not eosinophils. Because a different NO₂

TABLE 4

Biochemical Analysis of Induced Sputum at 6 and 26 Hours After HDM Allergen Challenge With Prior Exposure to Filtered Air or Nitrogen Dioxide

	S-6		S-26	
	FA	NO ₂	FA	NO ₂
IL-5, pg/mL	13.4 9.9–16.5	18.6 11.4–32.1	11.6 6.3–14.6	9.0 6.0–19.4
ECP, pg/mL	270 127–566	330 191–647	446 159–735	362 81–860
GM-CSF, pg/mL	1.04 0.74–2.85	1.46 0.52–3.79	0.54 0.52–1.56	0.75 0.52–0.95
IL-8, pg/mL	2580 1503–4055	3640 1224–4966	2783 1958–7390	4250 1028–8106
Total protein, mg/mL	3.19 2.89–3.51	2.93 2.59–3.50	3.22 2.81–3.89	3.07 2.53–3.42
IL-5/TP, pg/mg	4.2 3.2–5.9	6.6* 4.7–8.6	3.3 2.1–5.8	3.2 2.0–7.0
ECP/TP, pg/mg	90 36–160	102 67–217	146 44–244	126 27–294
GM-CSF/TP, pg/mg	0.37 0.22–0.59	0.44 0.22–1.44	0.19 0.16–0.48	0.23 0.21–0.34
IL-8/TP, pg/mg	809 470–1129	1180 437–1707	1158 605–2103	1481 356–3930

HDM, house dust mite; FA, filtered air; NO₂, nitrogen dioxide; S-6 and S-26, sputum induction 6 and 26 hours after allergen challenge; IL-5, interleukin-5; ECP, eosinophilic cationic protein; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-8, interleukin-8; TP, total protein. Values expressed as median and interquartile range.

exposure regimen and allergen challenge protocol were used by Barck and coworkers, their results and ours can not be directly compared. A methodological difference between the two studies is that we used a multidose allergen challenge protocol to achieve a 20% decrease in FEV₁ rather than a fixed-dose allergen challenge. In addition, we recruited subjects sensitized to *D. pteronyssinus*, whereas the Swedish investigators recruited subjects sensitized to either one of two pollens, birch and timothy. The timing of the allergen challenge after exposure to NO₂ may also be an issue; ours was immediately after a 3-hour exposure to NO₂ whereas Barck et al used 4 hours after a 30-minute exposure.

One interesting finding that both our study and the Barck et al study do share is a failure to observe an overall group enhancement of the EPR to allergen challenge by prechallenge exposure to NO₂. As noted previously, a number of studies by several groups, including members of the Barck study team, have

previously demonstrated such an NO₂-enhancing effect.^{33–38,47} A close examination of the individual data in these studies reveals that an enhanced sensitivity to inhaled allergen is not a universal result following NO₂ exposure. In fact, there are “responders” and “nonresponders” in every study. Understanding the basis of this differential response to NO₂ among asthmatic individuals should be a focus of future research. One possibility is that genetically determined differences in antioxidant defenses play a critical role in mediating the effects of an oxidant pollutant like NO₂. Previous work has suggested that individuals with the GSTM1 null genotype (involving the complete absence of this antioxidant enzyme) have increased risk of adverse effects from exposure to ambient ozone.⁵³ This genotype, as well as other polymorphisms in antioxidant enzyme genes, also may be a determinant of susceptibility to NO₂.

A potential limitation of the current study is the method used to sample airway lining fluid, sputum

induction. We chose this method because it less invasive and less costly in terms of both time and resources than fiberoptic bronchoscopy with BAL. We have experience with both techniques in assessing the inflammatory response to ozone and have directly compared them in one study.⁵⁴ There is considerably greater within-subject variability in sputum neutrophils than in BAL neutrophils leading to a greater signal-to-noise ratio with the former technique when used to assess a pollutant-induced inflammatory response. In other words, our sample size may have been too small to have detected a significant effect of NO₂ exposure on the airway inflammatory response to subsequent allergen challenge given the “noise” inherent in using induced sputum data. Moreover, if the primary site where NO₂ affects allergen-induced inflammation is the peripheral airways,⁵ then BAL may be a more appropriate method for sampling these airways than induced sputum.

Other potential limitations of our study relate to the experimental protocol. It is possible that the exposure to NO₂ (0.4 ppm for 3 hours with intermittent exercise) was too low to induce enhancement of the late airway inflammatory response to inhaled allergen. We selected this exposure protocol because it had been used in several studies by other investigators who reported NO₂ enhancement of the early phase response to the allergen we used, *D. pteronyssinus*, but a larger effective dose of NO₂ may be required to affect the late phase response. However, the NO₂ exposure studied by Barck et al was actually lower than what we used.⁵²

Another limitation is the allergen challenge dose regimen. Ideally the same dose of allergen would be used for both arms of the study. However, given the suspected enhancement of the bronchoconstrictor response to inhaled allergen by previous exposure to NO₂ and that previous studies had used a multiple

TABLE 5
Responders Versus Nonresponders

	Responders (n = 3)		Non-Responders (n = 12)	
FEV ₁ % pred. (baseline)		93		80
Methacholine PC ₂₀		4.22		0.90
	FA	NO ₂	FA	NO ₂
Pre-exposure FEV ₁	3.52	3.40	3.43	3.33
Allergen PD ₂₀	2697*	42	13*	20
EPR % change in FEV ₁	13	35	28	30
LPR % change in FEV ₁	13	11	14	12
S6				
Leukocytes cells × 10 ³ /mL	227	422	128	124
% Macrophage	34.3	19.9	25.3	27.4
% Neutrophil	55.7	71.4	61.6	58.6
% Eosinophil	2.7	5.1	8.2	4.0
Macrophage cells × 10 ³ /mL	86.8*	82.3	25.0*	23.4
Neutrophil cells × 10 ³ /mL	126.5	292.3	78.1	74.5
Eosinophil cells × 10 ³ /mL	6.2	3.8	7.3	2.7
IL-8, pg/mL	4921	5303*	2490	2835*
IL-8/TP, pg/mg	1727	2040*	745	812*
IL-5, pg/mL	9.50	18.64	14.37	18.27
IL-5/TP, pg/mg	2.98	6.62	4.74	6.69
GM-CSF, pg/mL	0.95	3.97	1.10	1.44
GM-CSF/TP, pg/mg	0.25	1.49	0.38	0.43
ECP	428	580	269	310
ECP/TP, pg/mg	137	218	79	101
TP, mg/mL	3.12	2.66	3.23	3.07
S26				
Leukocytes cells × 10 ³ /mL	261	375	121	109
% Macrophage	25.8	24.0	22.7	31.0
% Neutrophil	66.5	61.2	66.0	54.2
% Eosinophil	2.0	5.0	4.4	1.4
Macrophage cells × 10 ³ /mL	90.4*	89.7	20.7*	25.2
Neutrophil cells × 10 ³ /mL	153.6	229.2	55.7	47.9
Eosinophil cells × 10 ³ /mL	5.3	5.1	5.1	1.3
IL-8, pg/mL	8606	25587*	2781	2726*
IL-8/TP, pg/mg	2212	8612*	1081	770*
IL-5, pg/mL	6.69	9.86	12.63	6.99
IL-5/TP, pg/mg	1.94	3.77	4.03	2.65
GM-CSF, pg/mL	0.52	0.95	0.60	0.72
GM-CSF/TP, pg/mg	0.16	0.36	0.20	0.23
ECP	502	804	357	264
ECP/TP, pg/mg	146	245	125	82
TP, mg/mL	3.44	2.67	3.02	3.10

FA, filtered air; NO₂, nitrogen dioxide; S-6 and S-26, sputum induction 6 and 26 hours after allergen challenge; PC₂₀, provocative concentration causing 20% reduction in FEV₁; PD₂₀, provocative dose causing 20% reduction in FEV₁; EPR, early-phase response (0–1 hours); LPR, late-phase response (4–6 hours); IL-5, interleukin-5; ECP, eosinophilic cationic protein; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-8, interleukin-8; TP, total protein. Values expressed as median.

*P < 0.05 for these comparisons between responders and nonresponders.

dose regime,^{12–14} we decided against the use of a single dose on the basis of subject safety. In retrospect, the responder group inhaled less allergen after NO₂ exposure, which may have decreased the inflammatory response and made it difficult finding a statistically significant inflammatory ef-

fect of NO₂. In addition, allergen challenge performed immediately after NO₂ exposure may not be at the optimal time. Rusznak et al³⁴ has shown that the enhancement of airway responsiveness to inhaled allergen in asthmatic subjects may be maximal at 24 hours after combined

NO₂ and sulfur dioxide exposure. However, the maximal airway inflammatory response to allergen challenge may occur at a different time point than the maximal bronchoconstrictor response to allergen.

In conclusion, multihour exposure to a high ambient concentration of NO₂ did not enhance airway responses to a subsequent allergen challenge in most asthmatic subjects studied. The most notable finding related to NO₂ exposure was a decrease in allergen-induced eosinophils in sputum obtained 6 hours after inhalational challenge. A subset of subjects did experience marked enhancement of the early phase response. Future research in the area of NO₂ modulation of airway responses to inhaled allergen should include efforts to understand the mechanisms underlying the decrease in luminal eosinophils and the between-subject variability in responses after NO₂ exposure.

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