

Does Nitrogen Dioxide Affect Inflammatory Markers after Nasal Allergen Challenge?

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ABSTRACT

Background: Exposure to high ambient levels of nitrogen dioxide (NO₂) enhances the bronchial inflammatory reaction to allergen in humans. We tested whether this NO₂ effect occurs also in the upper airways.

Methods: Sixteen allergic subjects with rhinitis and mild asthma were exposed at rest to either purified air or 500 µg/m³ NO₂ for 30 minutes, followed 4 hours later by a nasal allergen challenge. Nasal lavage was performed before air/NO₂ exposure, before allergen challenge, and 1, 4 and 18 hours after allergen challenge. Symptoms were recorded.

Results: The percentage of eosinophils and neutrophils, eosinophil cationic protein, and myeloperoxidase were similar after exposure to air + allergen and to NO₂ + allergen. We noticed

a tendency to increased sneezing the day after exposure to NO₂ + allergen.

Conclusion: The priming effect of an ambient brief NO₂ exposure on subsequent allergic response was not noticeable in activation of inflammatory cells and mediators in the upper airways. (American Journal of Rhinology 19, 560–566, 2005)

Nitrogen dioxide (NO₂) is a major air pollutant originating from outdoor traffic exhaust and indoors from gas appliances. Short-term exposure can affect pulmonary function and increase bronchial sensitivity, especially in asthmatic subjects.¹ In controlled human studies NO₂ enhanced the asthmatic response to inhaled allergen measured as decreased pulmonary function both after single and after repeated exposures.^{2–5} In a series of studies we used a design with preexposure of subjects with asthma to a high ambient level of NO₂ (500 µg/m³) before a bronchial allergen challenge 3–4 hours later.^{4–6} With this exposure model we showed that NO₂ increases the bronchial response to allergen measured as decreased pulmonary function. Preexposure to NO₂ also augments the allergic bronchial inflammation after allergen challenge, as reflected in raised levels of eosinophil cationic protein (ECP) and neutrophil cell counts in bronchoalveolar lavage fluid.⁶

There is a close link between the upper and lower airways,^{7–9} and nasal inflammatory changes in normal subjects after exposure to air pollutants such as ozone mimic the changes that occur in the lower airways.¹⁰ Whether NO₂ has this effect is unknown.

We wondered whether NO₂'s enhancing effect on the allergic response in the lower airways would be mirrored by a corresponding response in the upper airways. To address this hypothesis, we tested whether preexposure to NO₂ before nasal allergen challenge enhanced the aller-

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gic inflammatory response in the upper airways. We recorded symptoms and measured inflammatory cells and mediators in nasal lavage (NAL) fluid repeatedly after exposure to NO₂ followed by allergen in patients with allergic rhinitis.

MATERIALS AND METHODS

Subjects

Sixteen asymptomatic subjects with a history of seasonal allergic rhinitis and mild asthma participated in the study (Table I). One-half of the subjects were recruited from the allergy outpatient clinic and one-half were recruited by advertising in a newspaper. There were nine men and seven women. Their mean age was 31 years (range, 22–48 years). The diagnosis of seasonal allergy to either birch or timothy pollen was confirmed by a positive skin-prick test (>3 mm) and a positive nasal challenge with the relevant allergen. All subjects had an airway hyperresponsiveness according to histamine inhalation test. No subjects had any steroid, antihistamine, or other anti-inflammatory medication during the study period, which was out of pollen season.

Study Design

Subjects acted as their own controls (Fig.1). They were exposed for 30 minutes at rest in an exposure chamber to filtered air or to 500 µg/m³ (260 ppb) of NO₂ on two separate occasions at least 4 weeks apart. The order of exposure was randomized (8 subjects first NO₂, 8 first air).

Four hours after exposure a nasal challenge with an individually fixed dose of birch or timothy pollen was given. The time interval of 4 hours was the same as in our previous studies.^{4–6}

Nasal allergen challenge and lavage of the upper respiratory tract are minimally invasive and easily tolerated. These techniques have been applied to explore if environmental pollutants alter allergic responses in humans.^{11,12} NAL was performed five times: before NO₂/air exposure (NAL 1); before allergen challenge (NAL 2); and 1 (NAL 3), 4 (NAL 4), and 18 hours (NAL 5) after allergen challenge. Venous blood was obtained in the morning of day 1 as baseline and in the morning of day 2 to assess the effect of an exposure to NO₂ followed by a nasal allergen challenge. Symptoms were recorded during the exposures; before allergen challenge; and 1, 4 and 18 hours after allergen challenge. All subjects gave their written informed consent and the study was approved by the Medical Ethics Committee of the Karolinska University Hospital Huddinge.

Exposure Data

NO₂ concentrations in the exposure chamber were measured with a chemiluminescence instrument.¹³ The concentration mean was 498 and range was 488–510 µg/m³. During exposure to filtered air the NO₂ concentration was below 10 µg/m³. The temperature in the chamber was 23.2 ± 0.5°C (mean ± SD) during air exposure and 23.1 ± 0.6°C during NO₂ exposure. The corresponding values for

TABLE I

Clinical Characteristics of Subjects (n = 16)

Subject	Age (yr)	Sex	Smoking	Histamine PDS (Raw 100%, µg)	Allergen Inhaled	Skin-Prick Test (mm)	Allergen Dose (SQ Units)
1	35	M	Ex	74	B	9.8	1000
2	37	M	Ex	223	B	8.5	1000
3	40	M	Ex	124	B	6.5	1000
4	27	F	No	737	B	9.5	1000
5	26	M	No	32	B	10	1000
6	31	F	No	124	B	6.5	1000
7	29	F	No	115	B	7.5	1000
8	48	F	No	803	T	9	1000
9	22	F	No	440	B	9	1000
10	36	M	Ex	66	T	15.5	500
11	31	M	No	402	B	10.5	1000
12	24	F	No	803	T	12	100
13	25	M	Ex	37	B	8.5	1000
14	31	F	No	220	B	9.5	1000
15	31	M	Ex	530	T	4.5	1000
16	28	M	No	110	B	12	1000
Range	22–48			32–803		4.5–15.5	100–1000

Nasal allergen dose given after air/NO₂.

M = male; F = female; B = birch; T = timothy; skin-prick test >3 mm = positive reaction.

Study design

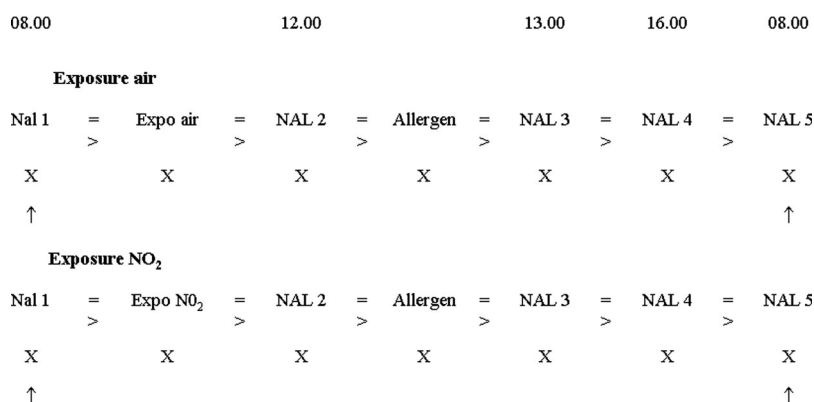


Figure 1. Study design. Exposure to NO₂ and control exposure to filtered air for 30 minutes followed by a nasal allergen challenge 4 hours later. NO₂ exposure and exposure to filtered air were separated for at least 4 weeks. Nasal lavage, recording of symptoms (X), and blood samples (↑) are shown in the figure.

relative humidity were $21 \pm 8\%$ and $22 \pm 5\%$. The concentration of NO₂ in ambient air outside the chamber, measured with a personal sampler carried by each subject,¹⁴ was $18 \pm 9 \mu\text{g}/\text{m}^3$ during the air + allergen and $18 \pm 8 \mu\text{g}/\text{m}^3$ during the NO₂ + allergen exposure days.

Nasal Allergen Challenge

Nasal allergen challenge was performed with standardized and freeze-dried birch or timothy allergen extracts (Aquagen; ALK-ABELLÓ, Copenhagen, Denmark) diluted in saline and used at a maximum of three concentrations: 100, 1000, and 10,000 standardized quality (SQ) allergen units per milliliter.¹⁵ Saline and allergen solutions were kept at room temperature for 1 hour before provocation. (0.9% saline.) After placebo challenge with diluents, the extracts were delivered starting with a dose of 10 SQ units, sprayed as 50 μL of the 100 SQ/mL solution into each nostril, using a mechanical pump spray.¹⁶ Then, the dose was increased by 10-fold dose increments (to 100 and 1000 SQ, respectively) until a clinical response was effectuated. The response was evaluated within 15 minutes after allergen administration and was regarded as positive when nasal blockage, itching, sneezing, and secretion occurred. The given allergen dose during the study period was set to the dose giving a positive reaction at inclusion (Table I).

Before the challenge session and 15 minutes after each dose of allergen the patient recorded blockage, secretion, and itching on a 4-point scale (0 = no symptoms, 1 = mild, 2 = moderate, and 3 = severe symptoms) and the number of sneezes was counted (0 = no sneeze, 1 = 1–4 sneezes, 2 = 5–9 sneezes, and 3 = >10 sneezes). A total nasal symptom score was calculated and a positive reaction was defined as symptom scores of at least 5.

NAL

Each nasal cavity was lavaged with 10 mL of warm (37°C) sterile isotonic saline with a syringe to which a tight-fitting nasal olive was adapted. The subject was sitting with the head leaned forward $\sim 60^\circ$, and breathing through the mouth. This position prevented the fluid from reaching the lower respiratory tract. The lavage fluid was passed slowly into the nasal cavity, kept there for 10 seconds, and withdrawn into the syringe five times.¹⁷ The lavage fluid from both nostrils was pooled into a sterile plastic container and put on ice for further analysis. The recovery from each five NALs in both nostrils was $16 \pm 0.96 \text{ mL}$ (mean $\pm$ SD) during air exposure and $16 \pm 0.74 \text{ mL}$ during NO₂ exposure.

Processing of NAL Fluid

The NAL fluid was filtered through a Dacron net (Millipore, Cork, Ireland). Cells were pelleted by centrifugation at 4°C $400 \times g$ for 10 minutes, and the supernatants were stored at -70°C after an additional centrifugation at 4°C $600 \times g$ for 10 minutes.

The cell pellets were resuspended in Hepes buffer. The total number of cells was counted in a Bürker chamber, and their viability was tested by the exclusion of trypan blue. Slides for differential counts were prepared by cytocentrifugation at 500 rpm for 3 minutes (Shandon Cytospin 3 cytocentrifuge, Southern Products, Ltd., Runcorn, UK). Slides were stained with May-Grünwald Giemsa, and 300 cells were counted. Staining for mast cells were done with acid toluidine blue and counterstained with Mayer's acid hematoxylin.

Soluble Components in NAL Fluid and Blood

Analyses of soluble mediators were performed in cell-free NAL fluid. The fluid was distributed in portions and stored at -70°C until analyzed. ECP levels were mea-

sured with a fluoroimmunoassay, Pharmacia ECP Cap System (Pharmacia & Upjohn, Uppsala, Sweden), with a detection limit of >2 ng/mL. Myeloperoxidase (MPO) was measured with a competitive radioimmunoassay (Pharmacia & Upjohn), with a detection limit of >8 μ g/mL.

Symptoms

After 4 and 28 minutes of exposure in the chamber, the subjects were asked 16 questions concerning respiratory symptoms and discomfort, reporting symptoms on an eight-step scale from none to maximal. Questions concerning specific nasal symptoms such as sneezing, itching, blockage, and secretion were posed at five times during the study days: before exposure to NO_2 /air; before allergen challenge; and 1, 4, and 18 hours after allergen. The answers were evaluated on a scale from 0 to 3 (0 = no symptoms; 3 = severe symptoms).

Statistical Analysis

Nonparametric statistics were used for description. The Wilcoxon signed-rank test was used for pair-wise comparisons. For analysis of variance (ANOVA) used to compare the effects of nasal allergen challenge after NO_2 and air, for cells and mediators, SAS "Mixed model" repeated measures ANOVA procedure was used. For symptoms, the Friedmans ANOVA was used for testing changes across time. Values of $p \leq 0.05$ were considered significant and values of $p > 0.05$ were considered nonsignificant (NS).

RESULTS

Exposure to NO_2 tended to increase the percentage of eosinophils and ECP levels in NAL fluid (NS). Neutrophils, MPO, and nasal symptoms were not affected by NO_2 exposure (Tables II and III; Figs. 2 and 3).

Exposure to filtered air followed by allergen challenge increased the percentage of eosinophils in NAL fluid 1 ($p \leq 0.001$), 4 ($p \leq 0.01$), and 18 hours ($p \leq 0.05$) after allergen challenge. ECP values in NAL fluid were not affected by allergen challenge (Tables II and III; Fig. 2 and 3). The percentage of neutrophils decreased in NAL fluid both 1

and 4 hours after allergen challenge ($p \leq 0.05$) but not at 18 hours. MPO values tended to decrease at the same time points as neutrophils (NS; Tables II and III). ECP and MPO in blood were not affected by air + allergen (Table IV). Total symptom score was increased 1 hour ($p \leq 0.01$) and 18 hours ($p \leq 0.05$) after air + allergen.

Exposure to NO_2 followed by allergen challenge increased the eosinophils 1 hour after allergen ($p \leq 0.05$) but not at later time points. Exposure to NO_2 followed by allergen decreased ECP in NAL fluid 1 hour after allergen ($p \leq 0.05$) but did not affect ECP levels at other time points (Tables II and III; Figs. 2 and 3). Neutrophils and MPO in NAL fluid were not affected by NO_2 + allergen at any time point (Tables II and III). ECP and MPO in blood were not affected by NO_2 + allergen (Table IV).

Total symptom score was increased 1 hour ($p \leq 0.001$) and 18 hours ($p \leq 0.01$) after NO_2 + allergen. At 18 hours after allergen challenge one subject reported severe symptoms, two subjects reported moderate symptoms, and four subjects reported mild symptoms of sneezing after NO_2 + allergen compared with one subject with moderate and one with mild symptoms of sneezing after air + allergen (NS; Fig. 4). There was no difference between air + allergen and NO_2 + allergen (NS) from preallergen to 18 hours after allergen for eosinophils, neutrophils, ECP, MPO in NAL fluid, and symptoms when we performed ANOVA of the whole data set.

DISCUSSION

The purpose of this study was to assess whether short-term exposure to NO_2 at an ambient level has an enhancing effect on the response to allergen in the upper airways. This study did not show any augmenting effect of NO_2 exposure on subsequent allergic response. There were no differences in the percentage of eosinophils and neutrophils or ECP and MPO levels after to air + allergen exposure compared with NO_2 + allergen exposure.

In this study, we did not find any statistically significant effect on ECP or eosinophils after exposure to NO_2 alone. This is in line with the findings by Wang who noticed that NO_2 exposure did not alter the levels of ECP in NAL fluid

TABLE II

Cells in NAL Fluid after Exposure to Air/ NO_2 and Allergen ($n = 16$)

	Eosinophils Air (%)	Eosinophils NO_2 (%)	Neutrophils Air (%)	Neutrophils NO_2 (%)
NAL 1	0.0 (0.0–1.8)	0.0 (0.0–4.8)	99 (98–100)	99 (95–100)
NAL 2	0.5 (0.0–7.0)	0.5 (0.0–8.3)	99 (93–100)	99 (91–100)
NAL 3	3.0 (1.0–8.0)	1.5 (0.0–17.8)	97 (90–98)	98 (82–100)
NAL 4	3.0 (1.3–9.0)	2.0 (0.0–6.8)	96 (91–98)	97 (93–99)
NAL 5	1.5 (0.0–11.0)	2.0 (0.0–9.0)	98 (88–99)	97 (90–99)

Data are given as medians and interquartile ranges. There was no difference between exposures to air and NO_2 (NS).

TABLE III

Soluble Mediators in NAL Fluid after Exposure to Air/NO₂ and Allergen (*n* = 16)

	ECP/Air ($\mu\text{g/L}$)	ECP/NO ₂ ($\mu\text{g/L}$)	MPO/Air ($\mu\text{g/L}$)	MPO/NO ₂ ($\mu\text{g/L}$)
NAL 1	2.5 (0–4.6)	2.8 (0–5.7)	190 (103–488)	170 (70–318)
NAL 2	2.9 (0–7.0)	3.5 (0.6–8.0)	165 (91–420)	215 (110–355)
NAL 3	2.6 (0–6.4)	1.1 (0–2.9)	70 (11.3–425)	130 (65–243)
NAL 4	2.5 (0–8.3)	3.4 (0–4.4)	110 (43–433)	133 (80–241)
NAL 5	3.5 (0–8.8)	3.6 (2.7–7.6)	185 (110–330)	160 (64–330)

Data are given as medians and interquartile ranges. There was no difference between exposures to air and NO₂ (NS).

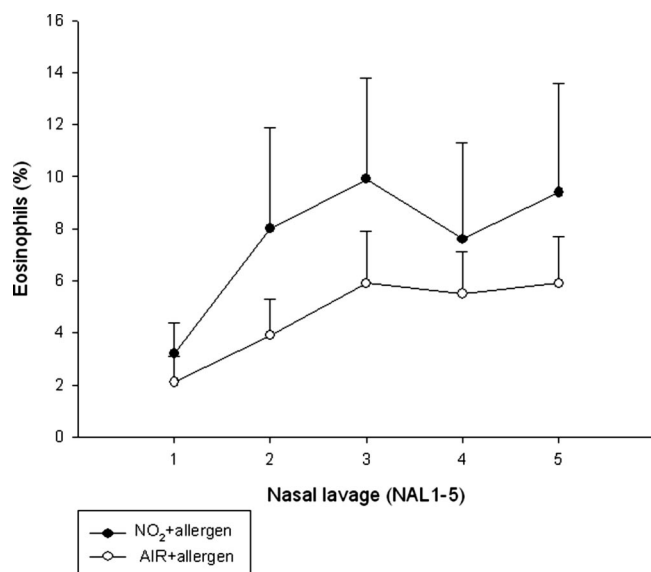


Figure 2. Eosinophils in NAL fluid: 1 = before exposure to air/NO₂, 2 = before allergen, 3 = 1 hour after allergen, 4 = 4 hours after allergen, and 5 = 18 hours after allergen. Data are reported by the means and standard error of the mean.

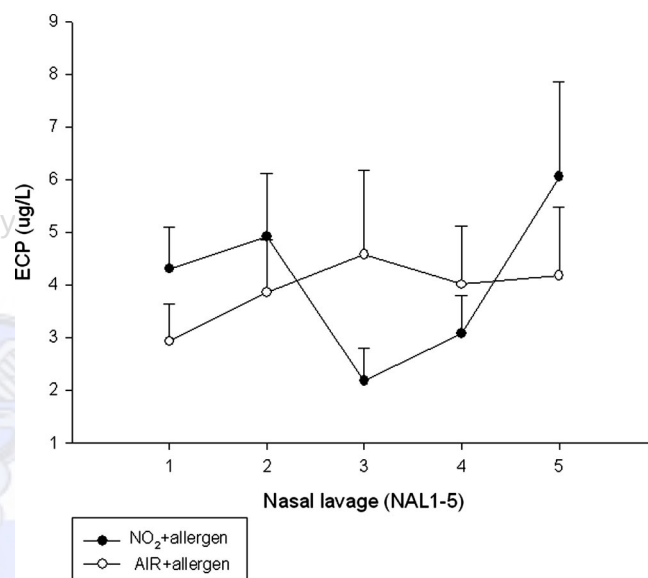


Figure 3. ECP in NAL fluid: 1 = before exposure to air/NO₂, 2 = before allergen, 3 = 1 hour after allergen, 4 = 4 hours after allergen, and 5 = 18 hours after allergen. Data are reported by the means and standard error of the mean.

in subjects with allergic rhinitis.¹⁸ Our data also is supported by the study by Vagaggini who studied the effects of exposure to peak outdoor concentrations of NO₂ on the upper and lower airways of mild atopic asthmatic subjects. No significant alterations were seen in differential cell counts in the NAL fluid after NO₂ compared with air. The NO₂ dose, exposure time, and interval from exposure to NAL were similar to the design of this study.¹⁹ These studies and the present study suggest that short-term exposure to NO₂ alone at a high ambient concentration does not induce an eosinophilic inflammatory reaction in the nasal mucosa. Our findings showed that preexposure to NO₂ did not alter the neutrophils or MPO at these ambient levels.

We found no effect of preexposure to NO₂ on ECP levels in NAL fluid after allergen challenge. This is opposed to the findings by Wang who noticed that exposure to NO₂ increased the ECP levels in NAL fluid 0.5 hours after nasal allergen challenge.¹⁸ A possible explanation of these incon-

gruent results is differences in study design. In the study by Wang, the NO₂ concentration was almost twofold higher and the exposure time was 12 times longer as in our study. This suggests that the priming effect of NO₂ at ambient levels on the eosinophils in the nasal mucosa for subsequent activation by allergen is dose dependent.

The total symptom score increased 1 hour and 18 hours after allergen challenge both after exposure to air + allergen and after exposure to NO₂ + allergen. This indicates that the given allergen dose was sufficient. There was a nonsignificant tendency at 18 hours after allergen, to more sneezing after NO₂ + allergen than after air + allergen, but there was no difference in itching, blockage, and secretion. Whether there is a true cause relationship between NO₂ exposure and increased sneezing is doubtful. The possible effect comes late and is seen only for one single symptom.

We hypothesized that NO₂'s enhancing effect on the

TABLE IV

Soluble Mediators in Blood after Exposure to Air/NO₂ and Allergen (*n* = 16)

	ECP/Air ($\mu\text{g/L}$)	ECP/NO ₂ ($\mu\text{g/L}$)	MPO/Air ($\mu\text{g/L}$)	MPO/NO ₂ ($\mu\text{g/L}$)
Day 1	7.7 (4.3–13.1)	7.3 (4.2–11.2)	160 (148–221)	165 (160–193)
Day 2	6.8 (4.8–10.2)	7.6 (4.8–12.0)	175 (134–185)	160 (133–189)

Data are given as medians and interquartile ranges. Wilcoxon signed-rank test showed no difference between exposures to air and NO₂ (NS).

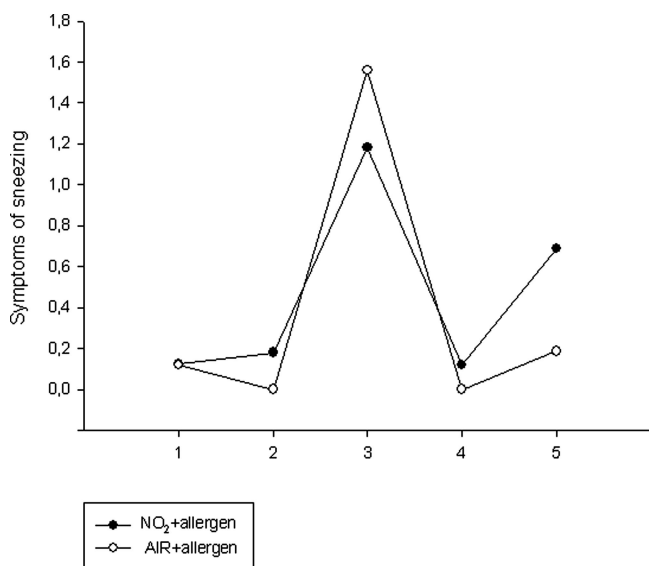


Figure 4. Symptoms of sneezing: 1 = before exposure to air/NO₂, 2 = before allergen, 3 = 1 hour after allergen, 4 = 4 hours after allergen, and 5 = 18 hours after allergen. Data are expressed as mean scores.

allergic response in the lower airways would be mirrored by a similar response in the upper airways. Therefore, we used an established exposure model with exposure to NO₂ followed by an allergen challenge 3–4 hours later. As in the previous studies, we used subjects with mild asthma and rhinitis and allergy to birch or timothy pollen. The level and duration of NO₂ exposure and interval between exposure and allergen challenge were similar to those that induced neutrophil influx and raised levels of ECP in bronchoalveolar lavage fluid.⁶

The only differences between the present and our previous study were the allergen challenge method and the allergen dose. First, here, we used nasal allergen challenge instead of bronchial challenge. These two methods are difficult to compare, because the response to allergen is assessed in two different ways. The response to the nasal allergen challenge was based on a symptom score, whereas the method we used for bronchial allergen challenge was based on changes in airway resistance. Second, the allergen dose given in the nose was higher (almost 10-fold) than the dose given in the bronchi in our previous study.

Moreover, NO₂ is not easily soluble in water and thereby not resorbed in the nasal mucosa, which might explain that higher exposure doses are needed for an effect in the nose. The poor nasal resorption of NO₂ also might explain that more is deposited in the lower airways where effects also are more visible. The nose is a smaller target organ than the lungs, and systemic effects, such as changes in mediators in blood, therefore are not as easy to detect after nasal provocation compared with pulmonary provocation.

To conclude, the priming effect of an ambient short NO₂ exposure on subsequent allergic response was not noticeable in activation of inflammatory cells and mediators in the upper airways. The nose is not as sensitive as the bronchi when studying effects of short-term exposure to ambient levels of NO₂ on the allergic inflammatory reaction in the respiratory tract.

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