Repeated exposure to an ambient level of NO₂ enhances asthmatic response to a nonsymptomatic allergen dose

V. Strand*,**, M. Svartengren**,+, S. Rak**, C. Barck*, G. Bylin*

Nitrogen dioxide (NO₂) is a major air pollutant produced by combustion, the main sources being traffic exhaust outdoors and gas appliances indoors. Evidence from several epidemiological studies suggests that ambient levels of nitrogen dioxide may increase the risk for exacerbations of asthma [1, 2]. Controlled human exposure studies on the other hand have not shown that lung function is affected by ambient levels of NO₂ [3, 4]. However, an increase in airway responsiveness to nonspecific stimuli like histamine and methacholine has been reported in asthmatics at NO₂ concentrations <1,000 µg·m⁻³ [5-7]. It is possible that NO₂ may exert its effects by an interaction with other air pollutants, such as particles, SO₂ and O₃. In a study by JÖRRES et al. [8], the lung function response was increased after NO₂ followed by SO₂ in asthmatics, and in healthy females when NO₂ was followed by O₃ exposure 3 h later [9], but not in asthmatics exposed to NO₂ and O₃ simultaneously [10]. Furthermore, in a recent study, bronchial response to birch and timothy pollen was enhanced by prior exposure to 500 µg·m⁻³ NO₂ for 30 min [11]. Similar results were shown for house dust mite after exposure to 800 µg·m⁻³ (0.4 ppm) NO₂ for 1 h [12]. These latter reports show that ambient levels of NO₂ can also enhance the airway responsiveness to allergens in humans. However, the allergen doses given in the experiments mentioned above considerably exceed those encountered in normal life. This makes it difficult to draw any firm conclusions on the clinical implications of the findings. As an increase in nonspecific bronchial responsiveness and the presence of late asthmatic reactions have also been reported after low doses of pollen allergen [13, 14], the use of a nonsymptomatic allergen dose would imitate seasonal exposure better.

In order to mimic real-life exposure, the aim of the present study was to investigate the effect of daily repeated exposure to a nonsymptomatic allergen dose preceded by a short exposure to 500 µg·m⁻³ NO₂. We wanted to determine whether the NO₂ effect on asthmatic response was reproducible with this significantly lower allergen dose and whether the magnitude of the response to NO₂ changed during repeated exposure.

**Methods**

Subjects

Subjects were recruited through the hospital outpatient clinic and by local advertising. All subjects gave informed
consent to participate in the study. The study was approved by the Ethics Committee at Huddinge University Hospital.

Sixteen subjects, 6 females and 10 males, age 21–52 yrs with mild-to-moderate seasonal asthma and allergy to pollen (12 to birch, 4 to timothy) participated in the study. Anthropometric and clinical data are given in table 1.

The subjects were included if they had a diagnosis of asthma based on reversible attacks of dyspnoea during the pollen season and airway hyperresponsiveness to histamine. Their allergy to either birch or timothy pollen was confirmed at an inclusion test by a positive skin-prick test and a positive bronchial challenge with the relevant allergen. The study was performed out of the pollen season. Lung function at inclusion expressed as forced expiratory volume in one second (FEV1) was 93±16% predicted (mean ±SD).

They all used inhaled β₂-agonist as needed, and nine used inhaled steroids during the pollen season. None received inhaled steroids or antihistamines during the study period, and the time span between the end of inhaled corticosteroid treatment and first visit in the study was at least 4 months.

All subjects were nonsmokers (13 had never smoked, three were exsmokers for at least 2 yrs). The subjects sensitive to animal dander did not have pets in their homes, and no subject had any domestic gas appliances.

**Study design**

The subjects were exposed for 30 min at rest in an exposure chamber to filtered air or to a concentration of 500 µg·m⁻³ (260 ppb) NO₂ on 4 consecutive days, at least 4 weeks apart. The exposures were performed single-blind according to DUBOIS et al. [15, 16].

Between 08:00 and 09:00 h and the order of exposure was randomized (9 subjects first to NO₂, 7 first to air).

The procedure on days 1–4 was as follows: after arrival at the laboratory, the subject rested for 15 min, and the specific airway resistance (sRaw) and thoracic gas volume (TGV) were measured with a whole-body plethysmograph and FEV₁ with a portable spirometer (Diary Card Spirometer®; Micromedical Ltd., Chatham, Kent, UK). The subject then rested for 10 min more before entering the exposure chamber. During the 30 min of exposure to air/NO₂, the lung function (sRaw, TGV) was measured at 4, 15, and 30 min, and an interview concerning symptoms after 3 and 26 min was conducted with the help of a questionnaire.

After exposure, FEV₁ was measured hourly with the portable spirometer. Four hours later, before the allergen inhalation, lung function was again measured by means of spirometry and plethysmography. After the allergen inhalation, the subject went home and continued to measure FEV₁ hourly. The subjects were instructed to use an inhaled bronchodilator if necessary and to keep a daily record on symptoms and medication.

On day 5, a histamine challenge was performed in the laboratory between 08:00 and 09:00 h preceded by sRaw, TGV and FEV₁ measurements.

The exposures were coded to the investigators analysing the results from the portable computerized spirometer.

**Whole-body plethysmography**

Airway resistance (Raw) and TGV were measured in a constant-volume body plethysmograph (Model 2000 TB; Cardio-Pulmonary Instruments, Houston, TX, USA) according to DeBOIS et al. [15, 16].

**Table 1. – Anthropometric and clinical data**

<table>
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<tr>
<th>Subject</th>
<th>Age yrs</th>
<th>Sex</th>
<th>Asthma duration yrs</th>
<th>Allergen inhaled</th>
<th>Positive SPT for allergens</th>
<th>Smoking</th>
<th>FEV₁ % pred</th>
<th>Allergen dose SQ units</th>
<th>Allergen PD₅₀Raw100% SQ units</th>
<th>Allergen PD₁₀₀Raw100% μg</th>
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F: female; M: male; N: nonsmoker; E: exsmoker; SPT: skin-prick test; T: timothy grass pollen; B: birch pollen; D: dog; C: cat; H: horse; FEV₁: forced expiratory volume in one second; PD₁₀₀Raw100%: provocative dose causing a 100% increase in specific airway resistance; SQ: standard quality. *: positive SPT defined as allergen induced weal of at least the size of the histamine skin prick (positive control); **: inhaled allergen dose studies days 1–4;
The gas flow-box pressure slopes were measured between gas flow +0.5 and -0.5 L·s⁻¹ (expiration - inspiration) as a mean of two to three slopes. The mouth-box pressure slopes were measured between the end-points, again as the mean of two to three curves. All panting manoeuvres were made at about 1 Hz, the subject being guided by a metronome.

**Spirometry**

We used a portable combined spirometer and electronic diary card (EDC) symptom registration developed by Micromedical Ltd. The equipment has been used by several groups, and it is well accepted by the patients and seems to give reliable data [11, 17]. The best FEV₁ out of two blows was used in the analysis. The average FEV₁ value 3–10 h after allergen inhalation was used as a quantitative estimate of the asthmatic reaction during the late phase of the bronchial allergic response, and the minimum single FEV₁ value was used as a qualitative assessment of a late asthmatic reaction (LAR) defined as a ≥15% decrease in FEV₁ from prechallenge values.

**Histamine bronchial challenge**

Histamine provocation tests were performed by using an automatic, inhalation synchronized dosimeter jet nebulizer (Spira Elektro 2; Respiratory Care Center, Hameenlinna, Finland) with an adjustable aerosol delivery time according to a method previously described [11, 18]. Briefly, doubling doses of histamine diphosphate at three concentrations, 1, 8, and 64 µg·m⁻³ were administered from an initial dose of 14 µg until a 100% increase in s_RAW was reached. s_RAW and TGV were measured 3 min after each dose. The provocative dose (PDs_RAW100%) was calculated by linear interpolation from a logarithmic scale.

**Allergen challenge and allergen inhalation**

The allergen challenge at study inclusion and the allergen inhalation during the study was performed with the same dosimeter jet nebulizer (Spira Elektro 2) as for the histamine inhalation. Standardized and freeze-dried birch or timothy allergen extracts (Aquagen, ALK, Copenhagen, Denmark) were used at a maximum of four concentrations, 1,000, 4,000, 16,000 and 64,000 standardized quality (SQ) allergen units·mL⁻¹. At each concentration, two and four breaths could be taken and, if needed, followed by eight and 16 breaths at the highest concentration. Fifteen minutes after each dose s_RAW and TGV were measured. After measuring baseline s_RAW, the subject inhaled doubling doses of allergen, starting from an initial dose of 14 SQ until a 100% increase in s_RAW was reached. PDs_RAW100% for the allergen was calculated by linear interpolation from a logarithmic scale.

The daily inhaled allergen dose during the study period was set to 10% of the PDs_RAW100% at study inclusion or, when this was impossible to administer for practical reasons, the dose above (table 1). FEV₁ and s_RAW were recorded immediately before and 15 min after the single dose of allergen was inhaled.

**Questionnaire**

After 3 and 26 min of exposure in the chamber, the subject was asked 16 questions concerning respiratory symptoms and perceptions of discomfort (i.e. tight chest, cough, headache, odour), estimated on a scale of 1–7. Throughout the exposure week, the subjects kept a self-administered daily record of airways symptoms, nose and eye symptoms, and bronchodilator medication. Medication was counted as the number of inhalations during the night and day, respectively. Nightly asthmatic bronchial symptoms were registered on a five-category scale: 1) no symptoms; 2) awaken by symptoms once or earlier than usual; 3) awaken more than once; 4) awake for greater part of the night; and 5) severe symptoms such that no sleep at all had been possible. Daytime symptoms were expressed on a six-category scale: 1) no symptoms; 2) symptoms once; 3) symptoms more than once; 4) symptoms during all day; 5) symptoms affecting normal activities; and 6) symptoms hindering normal duties. Irritability symptoms from bronchi, nose and eyes were noted for the last 24 h on a four-category scale: 1) no symptoms; 2) tolerable symptoms; 3) symptoms affecting normal activity; 4) no normal activity possible.

**Gas dilution and exposure system**

NO₂, kept in a gas bottle (Alfax, approximately 8,000 mg·m⁻³ NO₂) was diluted in two steps to a final concentration of about 500 µg·m⁻³ NO₂ and fed into the exposure chamber (volume 7 m³). The gas dilution and exposure system is presented in more detail elsewhere [19].

**Chemical analyses**

NO₂ concentrations in the exposure chamber were measured with a chemiluminescence instrument (Model 8440 Nitrogen Oxides Analyzer; Monitor Laboratories, Engelwood, CO, USA). For calibration, a NO₂ permeation tube and NO calibration gas (Model 8500 Calibrator; Monitor Laboratories, Engelwood, CO, USA; AGA Special Gas, 100 ppm NO) was used. A calibration procedure was run daily.

The subject’s individual exposure to NO₂ was measured with a personal, passive (filter badge) sampler (Toyo Roshi Kaisha Ltd, Tokyo, Japan). Sampling was made from the morning of day 1 to the morning of day 5 with the exception of the 30 min of NO₂/air exposure in the chamber. The analytical technique as well as the accuracy and reproducibility of the measurements with the sampler are presented in detail elsewhere [20].

**Exposure data**

NO₂ concentrations in the exposure chamber were measured in the breathing zone of the subject. The concentration was 499±8; 493–519 µg·m⁻³ (average of the mean concentrations during 4 days ±SD, range). During exposure to filtered air, the NO₂ concentration was <10 µg·m⁻³. The temperature in the exposure chamber was 25.1±1.1°C (mean±SD) during air and 25.2±1.0°C during NO₂ exposure. The corresponding values for relative humidity was 35.9±7.6 and 39.5±8%. 
The exposure to NO\textsubscript{2} in ambient air measured with the personal sampler was 18±7, 9–33 µg·m\textsuperscript{-3} (4 days mean± so, range) during NO\textsubscript{2} and 16±7, 4–32 µg·m\textsuperscript{-3} during air exposure.

The concentrations of outdoor air pollutants in the Stockholm area were low or moderate during the study period (NO\textsubscript{2}, 40–60 µg·m\textsuperscript{-3}; SO\textsubscript{2}, 3–6 µg·m\textsuperscript{-3}; O\textsubscript{3}, 25–62 µg·m\textsuperscript{-3}; monthly means, Stockholm Air Quality and Noise Analysis, The Environmental Administration of the City of Stockholm).

### Data analysis

The early-phase (EP) reaction after allergen was defined as the percentage change in FEV\textsubscript{1} from immediately before to 15 min after the allergen. The late-phase (LP) reaction was defined as the percentage change in FEV\textsubscript{1} from immediately before to 15 min after the allergen. The minimum FEV\textsubscript{1} value 3–10 h after the allergen inhalation was used as a qualitative assessment of a late asthmatic reaction (LAR) defined as §15% decrease in FEV\textsubscript{1} from preallergen values.

The effects on FEV\textsubscript{1} and sRaw after a single exposure and also histamine responsiveness and subjective symptoms were tested using Wilcoxon’s nonparametric sign rank test for two related samples for data not normally distributed. Differences between NO\textsubscript{2} and air in FEV\textsubscript{1} and sRaw after repeated exposure were tested by analysis of variance (ANOVA) for repeated measures. The statistical programme used was SPSS for Power Macintosh (SPSS Inc. Chicago, IL, USA). Probability values <5% were considered significant.

### Results

All 16 subjects completed both exposure series.

### Table 3 – Individual changes in forced expiratory volume in one second (FEV\textsubscript{1}) (%) after allergen preceded by air or 500 µg·m\textsuperscript{-3} NO\textsubscript{2}

<table>
<thead>
<tr>
<th>Subject</th>
<th>ΔFEV\textsubscript{1} % EP</th>
<th>Day 1 Air</th>
<th>Day 2 Air</th>
<th>Day 3 Air</th>
<th>Day 4 Air</th>
<th>Day 1 NO\textsubscript{2}</th>
<th>Day 2 NO\textsubscript{2}</th>
<th>Day 3 NO\textsubscript{2}</th>
<th>Day 4 NO\textsubscript{2}</th>
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</thead>
</table>

### ΔFEV\textsubscript{1} EP: difference during early phase (EP) in FEV\textsubscript{1} 15 min after allergen inhalation as a percentage change from immediately before inhalation. ΔFEV\textsubscript{1} LP: difference during late phase (LP) in FEV\textsubscript{1} 3–10 h after allergen inhalation as a mean percentage change from immediately before allergen inhalation. Difference between air and NO\textsubscript{2} significant for EP p<0.02 and LP p<0.01, analysis of variance.

### Effects of NO\textsubscript{2} exposure on lung function

Data are shown in table 2. After a single exposure, there was no immediate reaction either during (mean sRaw, data not shown) or after NO\textsubscript{2} when comparing baseline (morning) FEV\textsubscript{1} to FEV\textsubscript{1} 15 min after the end of NO\textsubscript{2} exposure on day 1. In addition, there was no delayed reaction to NO\textsubscript{2} after a single exposure when comparing FEV\textsubscript{1} baseline (morning) values to the values 4 h after exposure, before the allergen.

After repeated exposure, the mean sRaw during the exposure sessions to NO\textsubscript{2} did not, on any day, differ significantly from the baseline (morning) values before exposure or from the air exposure week (data not shown). There was a trend, albeit not statistically significant (p=0.07), towards lower FEV\textsubscript{1} values 4 h after exposure during the NO\textsubscript{2} week compared to the control.

### Effects of NO\textsubscript{2} on asthmatic reaction to allergen after single exposure

Spirometric results are shown in table 3. FEV\textsubscript{1} decreased significantly after allergen preceded by NO\textsubscript{2} at the...
early phase with an average decrement of -2.5% (p=0.01) versus -0.1% after air (p=0.94) and with the difference between air-allergen and NO$_2$-allergen responses reaching statistical significance (p=0.03). The corresponding change in FEV$_1$ after NO$_2$ at the LP response was -4.2% (p=0.005) versus -1.7% after air (p=0.29), although the difference between air and NO$_2$ was not statistically significant (p=0.21). Baseline (morning) FEV$_1$ and $\Delta$Raw did not change from day 1–2 either for air or for NO$_2$, which indicates that no effect of NO$_2$ or allergen remained from day 1.

**Effects of NO$_2$ on asthmatic reaction to allergen after repeated exposure**

Spirometric results are shown in table 3, and figures 1 and 2. The EP response was significantly increased by NO$_2$, and the allergen induced fall in FEV$_1$ for the 4 days was, on average, -2.5 versus -0.4% after air (p=0.018, analysis of variance). The LP response was also significantly greater after NO$_2$ after repeated exposure for 4 days, with an average decrement in FEV$_1$ of -4.4 versus -1.9% after air (p=0.009, analysis of variance).

The number of late asthmatic reactions 3–10 h after allergen with a fall in FEV$_1$ >15% was seven after air (five subjects) and 12 after NO$_2$ (6 subjects, NS). There was no difference in baseline (morning) FEV$_1$ or $\Delta$Raw values between the air and NO$_2$ weeks.

**Histamine responsiveness**

Histamine PD$_{250}$Raw$100\%$ was at inclusion 154 (126,372) µg and decreased after allergen preceded by air to 137 (58,261) µg and after allergen preceded by NO$_2$ to 100 (58,239) µg (median value (25th percentile, 75th percentile)). The decrease from inclusion in histamine PD$_{250}$Raw$100\%$ was significant after air plus allergen (-17%, p=0.01) as well as after NO$_2$ plus allergen (-35%, p=0.002). There was, however, no statistically significant difference between the decrease after air plus allergen and NO$_2$ plus allergen.

**Subjective complaints and medication**

Subjective symptoms and medication did not differ significantly between NO$_2$-allergen and air plus allergen, although there was a trend towards increased night-time symptoms of asthma after NO$_2$ plus allergen (score 18) versus after air plus allergen (score 9), p=0.07. During the 4 days, four doses of inhaled $\beta_2$-agonist were used after NO$_2$ plus allergen (one subject, days 2, 3 and 4) versus 6 after air plus allergen (3 subjects, days 2, 3 and 4).

**Discussion**

The major aim of the present study was to investigate the effects of 500 µg·m$^{-3}$ NO$_2$ and a nonsymptomatic dose of pollen allergen on both EP and LP asthmatic response after 1) single and 2) repeated exposure to allergen. An attempt to imitate real life exposure was made in three ways, using: 1) a NO$_2$ concentration that may occur in areas with heavy traffic or in homes with unvented gas appliances; 2) a repeated and short exposure to NO$_2$ for several days resembling the exposure pattern during morning rush hours; and 3) a repeated inhalation of a small dose of an outdoor allergen. In this group of subjects with mild asthma, we found a small, but statistically significant, enhancement by NO$_2$ on both the EP and the LP asthmatic response to the nonsymptomatic allergen dose. These effects remained after repeated exposure.

Exposure to 500 µg·m$^{-3}$ NO$_2$ alone did not induce any acute lung function changes either during or immediately after the exposure session, which is in accordance with earlier reports [3, 8, 19]. After repeated exposure, there was a trend to a delayed effect 4 h after NO$_2$ exposure, although the decrease in FEV$_1$ did not reach statistical significance.

We found an increase in EP response to allergen when preceded by a single exposure to NO$_2$. It should be noted that the allergen dose was so low that it did not per se cause any change in FEV$_1$ at the control exposure. Our
present results not only confirm that the EP asthmatic response can be potentiated by NO$_2$, as shown by TUNNICLIFFE et al. [12] but also indicate that this effect can arise at a significantly lower dose of allergen and also a lower dose of NO$_2$. In our former study [11], a statistically significant effect was seen only on LP; but our method for assessing EP in that study (change in allergen provocative dose) might not have been sensitive enough.

In contrast to these short-exposure studies (30 min and 1 h, respectively), the response to allergen after a six-hour exposure to NO$_2$ alone was not altered [21]. The reason for this inconsistency is unclear but could be caused by differences in the subjects' sensitivity to allergen or the fact that the allergen was inhaled immediately after the end of NO$_2$ exposure in the six-hour study.

It was shown in this study for the first time that an enhancing effect of NO$_2$ on the asthmatic response is seen over 4 days of repeated exposure. This contrasts with the adaptation of lung function response known from repeated O$_3$ exposure [22, 23] and also with the inflammatory response to NO$_2$ in bronchoalveolar lavage (BAL) studies in healthy subjects where the response changes from an increase after single to a decrease after repeated exposure to NO$_2$ [24].

There is no clear time pattern of the EP and the LP response. The LP response after NO$_2$ does not show any obvious signs of adaptation, and the difference in FEV$_1$ between air and NO$_2$ on day 4 (-3.1%) is not less than the difference on day 1 (-2.5%). Nevertheless, the duration of the effect had vanished after 18 h (on the basis of morning difference on day 1 (-2.5%)). Therefore, the small effects found in this study could be more emphasized and clinically manifested in allergic asthmatics with a more severe disease or when exposed to a combination of pollutants. Further studies are necessary in order to assess the clinical implications of NO$_2$ exposure on the population of people with allergic asthma.

In our exposure model, we intended to mimic natural exposure to NO$_2$ and allergen, but there are obvious limitations related to a study in a laboratory setting. One is the brief exposure period compared to the natural allergen exposure persisting for a whole pollen season or even throughout the whole year as for indoor allergens. Another limitation is the very low level of other pollutants and a favourable climate with a temperature of 25°C and relative humidity around 37% in the laboratory.

Epidemiological studies have suggested an association between ambient levels of NO$_2$ and increased risk of exacerbations of asthma [1, 2], first-time asthma in children [26] and a decrease in lung function [27]. However, in these studies, data on the exposure to allergens are rarely available.

A wide range of mechanisms may be involved in the NO$_2$-induced potentiation of the allergen response. Although the allergic reaction is relatively well known and there are data on cell and mediator changes after exposure to NO$_2$, there are few reports on the combined effect of NO$_2$ and allergen in the airways. Eosinophils and eosi-nophil cationic protein (ECP) in peripheral blood did not increase after airway exposure to 500 µg·m$^{-3}$ NO$_2$ and allergen [11], indicating either that the enhancing effect of NO$_2$ on the late allergic reaction was not mediated by eosinophils or that a recruitment of eosinophils from the blood to the lung had already taken place. In a nasal lavage study, ECP was found to be increased after NO$_2$ and allergen, supporting the hypothesis that activated eosinophils are involved in the adjuvant effect of NO$_2$ [25]. Studies of the local allergic reaction in the lung after NO$_2$ by means of bronchial lavage and other techniques are needed.

This study shows that an ambient concentration of NO$_2$ enhances the asthmatic response to allergen at an allergen dose that does not cause any asthmatic reaction by itself, and that the effect is reproducible after repeated exposure. The results indicate that NO$_2$ may affect the health of individuals with allergic asthma in real life. However, it is difficult to assess how substantial this NO$_2$ effect could be based on data from this laboratory experiment. On the one hand, the mean FEV$_1$ change is small and close to the individual normal variability in asthmatics, and the subjects did not report any statistically significant increase in symptoms of asthma or use of bronchodilators. On the other hand, our group of subjects all had mild asthmatic disease, they were all nonsmokers with a normal lung function, with few exceptions. They did not require any inhaled steroids outside of the pollen season and had few subjective symptoms of asthma according to the diary card 24 h before the start of the study. In this group of mild asthmatics, a LAR was, nevertheless, experienced on 12 occasions after NO$_2$ compared to seven after air, and the tendency to develop asthmatic symptoms at night during the NO$_2$ week could harmonize with the spirometric results. Therefore, the small effects found in this study could be more emphasized and clinically manifested in allergic asthmatics with a more severe disease or when exposed to a combination of pollutants. Further studies are necessary in order to assess the clinical implications of NO$_2$ exposure on the population of people with allergic asthma.

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References


