Airway Responses to NO₂ and Allergen in Asthmatics

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Sic transit gloria mundi!
ABSTRACT
Nitrogen dioxide (NO$_2$), a gas produced by combustion, is a common environmental air pollutant. Individuals with asthma are more sensitive to NO$_2$ exposure than healthy subjects, according to results from controlled human-exposure studies. NO$_2$ can enhance the asthmatic response to inhaled allergen. The mechanisms for NO$_2$’s enhancing effect on the asthmatic reaction to allergen appear to be related to an increased inflammatory reaction in the airways.

The general aim of the studies I-III was to examine whether an ambient level of an air pollutant, nitrogen dioxide, interacts with pollen allergens so as to enhance the allergic inflammation in the upper and lower airways. Efforts were made to mimic real exposure situations with ambient doses of air pollutants and allergen.

The aim of study IV was to evaluate a simplified method to study intra-individual changes in eosinophil cationic protein (ECP) in induced sputum.

I. Thirteen subjects with mild asthma and allergy were exposed at rest to 500 µg/m$^3$ NO$_2$ or purified air for 30 minutes, followed four hours later by an allergen inhalation challenge. Bronchoscopy was performed 19 hours after the allergen challenge. This single ½-hour exposure to NO$_2$ followed by allergen gave an increase in the number of neutrophilic cells both in the bronchial and the bronchoalveolar lavage fluid. The levels of ECP were higher in bronchial wash after NO$_2$+allergen. No alteration was seen in symptoms or pulmonary function between NO$_2$+allergen and purified air+allergen exposure.

II. We used the same exposure protocol as in study I but on sixteen subjects who had asthma and rhinitis. The aim was to investigate if similar changes occurred in the upper airways. Nasal lavage was made before exposure, before nasal allergen challenge, and one, four and 18 hours after allergen challenge. No such priming effect of ambient NO$_2$ exposure on subsequent allergic response in the upper airways (activation of inflammatory cells and mediators) was noticeable.

III. Eighteen subjects with mild asthma were exposed to 500 µg/m$^3$ NO$_2$ or purified air for 15 minutes on day 1 and 2x15 minutes on day 2. Four hours after the exposure (day 1), and the two exposures (day 2) an allergen dose was inhaled. Sputum was induced and daily blood samples were taken. ECP in both sputum and blood increased after the 3 repeated exposures but not after a single brief exposure. No alteration was seen in symptoms or pulmonary function between NO$_2$+allergen and purified air+allergen exposure.

IV. In thirteen mild asthmatics sputum was induced before and 24 hours after allergen challenge. The entire sputum sample was incubated and divided into 2 parts. One part was processed according to the conventional method, and released ECP levels in the supernatant were measured. The second part was treated with a lysing reagent, and total ECP (intracellular and extracellular) was measured. We found a good correlation between total ECP levels in the entire sputum sample and released ECP levels in the supernatant before and 24 hours after allergen challenge. The changes in total and released ECP after the allergen challenge also correlated. Total ECP seems to reflect the eosinophilic inflammatory changes in asthma, and might be a useful tool in monitoring asthma in clinical practise and in exposure studies.

These studies (I-III) show for the first time that there is an interactive effect of the air pollutant NO$_2$ and allergen on the inflammatory reaction in the bronchi of persons with asthma. NO$_2$ has an enhancing effect on the eosinophilic inflammation by increasing the release of ECP. This was found both in bronchial lavage fluid, induced sputum and peripheral blood. There was also an increase of the neutrophilic cells in the airways. These NO$_2$ effects on eosinophilic and neutrophilic leukocytes were found in the lower but not in the upper airways. The NO$_2$ concentrations and allergen doses were comparable to the levels you can encounter in the outdoor environment.

This data suggests that ambient NO$_2$ can enhance the allergic inflammatory reaction in the airways without causing symptoms or pulmonary dysfunction.
LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

   Ambient level of NO₂ augments the inflammatory response to inhaled allergen in asthmatics.
   *Respiratory Medicine* 2002; 96:907-917

II. Barck C, Lundahl J, Holmström M, Bylin G.
    Does nitrogen dioxide affect inflammatory markers after nasal allergen challenge?
    *Submitted*

III. Barck C, Lundahl J, Halldén G, Bylin G.
    Brief exposures to NO₂ augment the allergic inflammation in asthmatics.
    *Environmental Research* 2005; 97:58-66

IV. Barck C, Lundahl J, Halldén G, Bylin G.
    Total eosinophil cationic protein levels in induced sputum as a marker of changes in eosinophilic inflammation in patient with allergic asthma.
    *Annals of Allergy, Asthma & Immunology* 2005; 94(May)
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BW</td>
<td>Bronchial wash</td>
</tr>
<tr>
<td>EAR</td>
<td>Early asthmatic reaction, change in lung function immediately after allergen inhalation</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in one second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>LAR</td>
<td>Late asthmatic reaction, change in lung function 3-10 hours after allergen inhalation</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NAL</td>
<td>Nasal lavage</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>PDS&lt;sub&gt;Raw100%&lt;/sub&gt;</td>
<td>Provocative dose causing a doubling of the specific airway resistance</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophilic cells</td>
</tr>
<tr>
<td>Sraw</td>
<td>Specific airway resistance</td>
</tr>
<tr>
<td>SQ</td>
<td>Standardized quality of allergen, units/mL</td>
</tr>
<tr>
<td>TGV</td>
<td>Thoracic gas volume</td>
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INTRODUCTION

For many hundreds of years it has been a well known fact that people suffering from airway disease are sensitive to air pollution. Already in the year 79 A.D. Plinius the Younger described in his letters to Tacitus the fatal respiratory disorders caused by natural air pollution during the eruption of Vesuvius. He indicated for the first time the importance of the inflammation of the airways in describing the airways of Plinius the elder as being of weak nature (1).

After the natural air pollution from Vesuvius mankind has created many sources of air pollution. The major man-made sources of ambient air pollution include industries, automobiles and power generation. After the industrialization people changed their way of living by moving from the countryside to urban areas. To date, road traffic constitutes the major source of air pollution in the larger cities of industrialized countries.

Many people in Sweden have rhinitis and asthma and a large percentage of them are also allergic to airborne allergens (2-4). When they are outdoors they can be exposed to both air pollutants and airborne allergens such as pollen. The time for such combined outdoor exposure to both air pollutants and allergens is usually short, as people in industrialized countries spend most of their time indoors. People indoors also become exposed to pollutants such as nitrogen dioxide from gas appliances in combination with allergen from animal dander or mites.

Epidemiologic studies carried out in various geographical regions in the world show a significant and consistent association between ambient levels of pollutants and increased asthma and rhinitis symptoms (5). Other studies show that there is good evidence of a relationship between urban air pollution and morbidity and mortality (6).

It is recognized that exposure to pollutants also enhances the airway response to inhaled allergens in asthmatics (7-9). However, the mechanisms underlying these effects are not fully understood.

The aim of this thesis was therefore to study the inflammatory mechanisms in the upper and lower airways after combined exposure to a major air pollutant (NO₂) and an allergen. The purpose behind the experiments was to create situations which might possibly occur in the outdoor environment during the pollen season with both ambient levels of an air pollutant (NO₂) and an airborne allergen (pollen).

AIR POLLUTANTS

Air pollutants can be defined as atmospheric accumulations of irritants, indoors or outdoors, that can be harmful to humans, animals and plants. Industrialization is associated with
environmental air pollution and people living all over the world in industrialized urban areas are periodically exposed to levels of air-pollution that exceed health-based air quality guidelines (10).

Air pollution in Swedish urban areas emanates mainly from road traffic (11). The most common air pollutants are ozone ($O_3$), sulfur dioxide ($SO_2$), nitrogen dioxide ($NO_2$) and particulate matter (PM) (12). $SO_2$ levels have decreased during the last years, due to less heating with fossil fuels and less emission from industries. Ozone is found in high concentrations during spring/summer and is usually lower in cities with heavy traffic due to reaction with nitrogen oxide and formation of nitrogen dioxide.

Particulate matter and $NO_2$ are at the highest levels in winter due to greater emissions from cars and heating and less favorable dispersion conditions. This thesis concerns first of all the effects of $NO_2$.

**$NO_2$ and its chemical properties**

Nitrogen dioxide is a combustion-generated oxidant gas. Outdoors, it comes primarily from vehicle emission and power plants in urban areas. Indoors, $NO_2$ is generated by gas cooking and heating. The levels of $NO_2$ are fluctuating and are higher outdoors during wintertime, reaching the highest levels at rush-hour (13). Indoors the levels can be even higher than outdoors when unvented gas fuelled stoves are used and also in skating ice rinks (14, 15).

The international unit for nitrogen dioxide concentration is micrograms per cubic meter, $\mu g/m^3$. The unit is a mass per unit volume and is expressed at a specific temperature and pressure. 1880 $\mu g/m^3$ equals 1 ppm at 25°C and 1 atmosphere. The current WHO guideline values for $NO_2$ are a 1-hour level of 200 $\mu g/m^3$ and an annual average of 40 $\mu g/m^3$ mean level (12). Recommended levels for $NO_2$ in Sweden are a 1-hour level of 110 $\mu g/m^3$ and a 24-hour mean level of 75 $\mu g/m^3$ (11). Most nitrogen oxides are emitted as NO but are oxidized to $NO_2$ in the presence of ozone. $NO_2$ is an oxidant gas that is inadequately water soluble and less reactive than ozone. The following reaction is the most important in ambient air: $NO + O_3 \rightarrow NO_2 + O_2$
Pulmonary deposition of NO₂

Most part of the inhaled gas is retained in the lung and about 60% deposited in the peripheral airways, particularly in the terminal bronchiolar region (16). The deposit increases with exercise. NO₂ reacts either within the lung epithelial lining fluid or in the epithelial cell membrane and does not penetrate beyond the epithelium as an intact molecule (17). Respiratory toxicity is probably related to these effects of NO₂ and its reaction on alveolar macrophages and airway epithelial cells (18).

Health effects of air pollutants

Epidemiologic studies have demonstrated consistent associations between exacerbations of respiratory disease and air pollution (6, 19). Further studies carried out in various geographical regions in the world have also shown a significant and consistent association between ambient levels of pollutants and increased asthma and rhinitis symptoms (5). Air pollution is convincingly associated with many signs of asthma exacerbation, e.g. increased bronchial responsiveness, emergency treatment, hospital admissions, and increased use of medication (20, 21). Outdoor air pollution in childhood may lead to reduced lung function in adulthood (22).
The prevalence of allergic diseases has increased in the past decades in industrialized countries. Air pollution has been implicated as one of the factors that are responsible for this increase (23, 24). Experimental data supports that opinion. Diesel exhaust particles can adsorb aeroallergens from pollen grains and can prolong the retention of the allergens so as to provide for an enhanced IgE-mediated response (25). It has also been demonstrated that diesel exhaust particles increase the allergic inflammatory response in the nose after allergen challenge by an increased production of IgE (26, 27). Recently, it was demonstrated that nitrogen dioxide and ozone can make the airborne allergens more powerful by nitration (28). However, epidemiologic studies have not given a definite support for air pollutants being a risk factor for the induction of allergies in humans (29).

**Epidemiologic studies of health effects of NO₂**

Multiple studies from various different countries have demonstrated associations between NO₂ levels and emergency treatment or hospitalization for asthma. (30, 31). However, NO₂ might simply be an indicator of polluted air, and epidemiologic studies are often unable to distinguish the relative importance of NO₂ in causing adverse health effects (12). There is some evidence that NO₂ exposure may decrease lung-function and increase the risk of respiratory symptoms (12). In the Southern California Children’s Study the lung function levels were lower in communities with higher NO₂ concentrations (32). Lung function growth, evaluated in a longitudinal study, was also impaired among children (22). A Swiss cross-sectional study, SAPALDIA gives support to the association of NO₂ exposure and lung function decrement in adults (33, 34). In an Australian study of indoor NO₂ exposure and gas stove use, atopic children had greater risk for respiratory symptoms associated with gas stove use than non-atopic children (35). A number of epidemiologic studies of relatively large populations exposed indoors to peak levels of nitrogen dioxide from gas-combustion appliances have not provided consistent evidence of adverse pulmonary function effects (12).

**Controlled human exposure studies in healthy subjects**

Exposures to concentrations below 1880 µg/m³ do not have any significant effect on lung function in normal subjects (36-38). Overall, there is little convincing evidence that exposure of healthy volunteers to NO₂ at levels as high as 7520 µg/m³ (4 ppm) alters airway mechanics, as measured by spirometry or flow resistance (39, 40). A meta-analysis of all published reports on bronchial responsiveness after exposure to NO₂ has shown a statistically significant increase in bronchial responsiveness at concentrations of above 1880 µg/m³ (1 ppm) in healthy subjects (41).
NO2 appears to be much less potent than ozone in eliciting a neutrophilic inflammatory response. Unlike ozone exposure, NO2 exposure at < 3760 µg/m³ (<2 ppm) does not cause a significant influx of polymorphonuclear neutrophilic cells (PMNs) into the airways and alveoli (42). Mild airway inflammation is found expressed as mild increases of PMNs in the bronchial wash (BW) of bronchoalveolar lavage (BAL) after a single as well as after repeated prolonged exposure (4-6 hours) to NO2 at a concentration of 3760 µg/m³ (2 ppm) (43-45)

Controlled human exposure studies in asthmatics

There are several indications that asthmatics are more susceptible to NO2 than healthy subjects. Inhalation of 560 µg/m³ NO2 at rest for 20 min followed by 10 min exercise resulted in a reduction in FEV1 which returned to baseline values one hour later (46). Exposure to 560 µg/m³ NO2 for 3 hours, including light exercise, resulted in decreased FEV1 and an increase in SRaw after 60 min exposure that returned to baseline after 3 hours exposure (47). Other investigators have however been unable to confirm effects of 188-376 µg/m³ (0.1-0.3 ppm) NO2 on lung function in mildly asthmatic adults (37, 38, 48, 49). Overall, there is little convincing evidence that short-term exposures to NO2 at outdoor ambient concentrations significantly alter lung function in most people with mild asthma.

Orehek reported 1976 that relatively brief exposures (1 hour) of asthmatics to low-level 188 µg/m³ (0.1 ppm) NO2 might enhance subsequent airway responsiveness to a bronchoconstricting drug (50). Some subsequent studies have shown that exposure to NO2 increases airway responsiveness in asthmatics (38, 46) while others have failed to confirm this (51-53). In a meta-analysis on airway responsiveness after exposure to NO2 a concentration of above 188 µg/m³ (0.1 ppm) was found to give increased bronchial responsiveness in asthmatics (41). This was confirmed by Strand who made histamine challenge of asthmatic subjects and found increased airway responsiveness 5 hours after a 30 minute exposure to 500 µg/m³ NO2, with intermittent exercise. This effect had disappeared 27 hours after exposure (8).

In a study of NO2's effects on the upper airways by Wang, nasal lavage was made directly after 6 hours exposure to 752 µg/m³ (0.4 ppm) NO2 on subjects with allergic rhinitis. The results did not show any effect of NO2 on the inflammatory markers (54). Vagaggini studied the effects of exposure to 564 µg/m³ (0.3 ppm) NO2 for 3 hours on the upper airways in mild atopic asthmatics (49). NAL was performed 2 hours later. No effect of NO2 was seen in NAL differential cell counts. Vagaggini also made induced sputum 2 hours after exposure to 564 µg/m³ (0.3 ppm) NO2 for 3 hours without finding any alterations in sputum differential cell counts (49). Similar results were found by Jörres who did not find any influx of neutrophils in BAL-fluid after exposure to 1880 µg/m³ (1 ppm) NO2 during 3 hours (55).
ALLERGIC AIRWAY DISEASE

In Sweden the most common outdoor airborne allergens are pollen from trees and grass. About 1-1.5 million people in Sweden are allergic to pollen (56). The birch pollen season starts at the end of April in southern Sweden and the grass pollen season starts in the middle of June. Pollen circulating in the air varies annually as well as daily and also depends on the weather. The amount of pollen in the outdoor air is about 200 times greater than in the indoor air. The pollen grains are between 25-40 µm in size and contain a vast amount of proteins which can circulate for a long time in the air as small particles smaller than 1µm. It is the small proteins inside the pollen grains that can circulate in the air as free particles that are allergenic (57).

Rhinitis and asthma

Allergic airway diseases such as rhinitis and asthma are steadily increasing in the western world (3, 58). Allergic rhinitis and asthma are inflammatory disorders in the airways. In susceptible individuals genetic predisposition leads to the production of specific IgE antibodies against common environmental antigens, for example airborne pollens.

Rhinitis is defined as inflammation of the nasal membranes and is characterized by a symptom complex that consists of any combination of the following: sneezing, nasal congestion, nasal itching, and rhinorrhea. The eyes, ears, sinuses and throat can also be affected. Allergic rhinitis
is the most common type of rhinitis, and the most frequent allergens are pollen and animal dander. Many Swedish people, especially children and young adults, suffer from seasonal rhinitis during the pollen season. There is an increase in the morbidity of seasonal rhinitis, and more than 20% of Swedish adults are affected (59). People suffering from rhinitis have a two to three-fold increased risk of developing bronchial asthma.

Asthma is currently defined as a chronic inflammatory airway disease in which many cells, in particular mast cells, eosinophils, and T-lymphocytes, play a role. In susceptible individuals, the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough. The symptoms are usually associated with variable airflow limitation that is at least partly reversible, spontaneously or with treatment. Chronic inflammation is associated with increases in airway responsiveness to various stimuli (60, 61). Asthma is a common disease and the prevalence of asthma in Sweden is about 8%, and the morbidity is increasing (62).

**Combined airway disease**

A high percentage of persons with allergic rhinitis have concomitant asthma, and many patients with asthma have allergic rhinitis (63). In atopic children, e.g., the incidence of concomitant asthma and allergic rhinitis is estimated to about 50% (64). During recent years allergic rhinitis and asthma have often been considered part of an allergic syndrome. The nose and lungs are anatomically and physiologically closely related organs. The nose acts as a filter and air-conditioner protecting the lower airways. The contribution of several common cells and mediators in both allergic rhinitis and asthma supports the concept of a unified airway. Individuals with asthma can have increased nasal inflammation, indicated by eosinophil infiltration in the nasal mucosa regardless of the presence or absence of clinical rhinitis (65). In asthmatic patients, who have concomitant upper airway pathology, nasal therapy can reduce lower airway symptoms (66, 67).

**Allergic inflammation**

The allergen exposure gives rise to the development of allergic inflammation followed by allergy symptoms (68, 69). When the IgE antibodies bind to the basophils and mast cells they become activated and the allergic reaction starts with a release of a wide range of inflammatory mediators (70). The resulting inflammation can be divided into early (vascular) and late events (cellular). The early phase, which occurs within 10-30 min after allergen exposure follows with activation of histamine, prostaglandins and other rapidly synthesized molecules, which cause a rapid increase in the vascular permeability and the contraction of smooth muscles. The late phase of the inflammatory response, which some individuals develop, is characterized by accumulation and activation of eosinophils and neutrophils (71, 72). The degranulation of
Eosinophils can cause epithelial damage and submucosal edema, which in turn contributes to airflow obstruction and increased hyperresponsiveness (73, 74). Research has demonstrated a pathogenic and destructive role for eosinophils in asthma (75). Asthma severity or airway hyperresponsiveness has been correlated with the number of eosinophils in peripheral blood, sputum and BAL-fluid or eosinophil cationic protein (ECP) levels (76).

The eosinophil granulocyte is produced in the bone marrow. When the eosinophils migrate from the bone marrow to the bloodstream, they circulate with a half-life of about 18 hours before migrating into the tissues. The peripheral tissue contains the majority of mature eosinophils (77). A characteristic feature of eosinophils is their variability. The state of activity may change dramatically as a consequence of exposure to various cytokines produced mainly by the T-lymphocyte but also by the mast cells. IL-5 is one cytokine essential for the activation and survival of the eosinophil (78). It is likely that the cell when it is activated can cause damage to the asthmatic airway that may lead to increased bronchial reactivity (79). In allergic and some other diseases, eosinophil counts are elevated in both peripheral blood and affected tissue. Activated eosinophils are known to release ECP, and high levels have been found in BAL-fluid from patients with allergic asthma (80, 81).

The main function of the neutrophil granulocytes is to protect the body against bacteria. The cells are produced in the bone-marrow. Neutrophils are increased in the airways of patients with chronic and severe asthma especially during respiratory viral exacerbations or after exposure to air pollutants but their role in asthma tissue pathogenesis requires elucidation. The number of neutrophils increases during the first hours after an allergen challenge and later returns to normal levels (82-84). Activated neutrophils produce different interleukines, IL-3, IL-6 and IL-8. IL-6 and IL-8 activate the neutrophils to adhesion, migration and degranulation, and IL-8 is regarded as neutrophil specific. The neutrophil granulae contain proteins called proteases. One of the most important proteases is a peroxidase called myeloperoxidase (MPO). The role of MPO is debated. It has been proposed that MPO has an anti-inflammatory function and participates in the down-regulation of the inflammatory process. It is clear that MPO in blood mirrors the amount and activity of the neutrophils (85).

**Monitoring of inflammation in allergic airway disease**

There are different ways to monitor the different cells and their mediators in the allergic inflammatory reaction. Blood analysis is a rapid and easy method to study inflammatory cells and markers, but does not necessarily reflect the events in the airways. For the upper airway, nasal lavage (NAL) is a simple and repeatable method to examine the inflammation. Bronchoalveolar lavage, is a more invasive method that reflects both the bronchial (BW) and
the more distal parts (BAL) of the lower airways. The induced sputum method has become more and more used as a non-invasive method to monitor the allergic inflammation of the proximal lower airways. One draw back is the processing which is time-consuming and requires skilled laboratory personnel. In addition, if the sputum sample is not processed immediately, there is a risk that the cells degranulate ex vivo, which can bias the interpretation of data.

**EFFECTS OF NO**₂** AND ALLERGEN IN CONTROLLED HUMAN EXPOSURE STUDIES**

It has been shown that exposure to ambient levels of NO₂ enhance the response to specific allergen challenge in mild asthmatics (7, 86). An increased responsiveness to a fixed dose of allergen, both during the early and late phases of the response was found after exposure of subjects with asthma for one hour at rest to 752 µg/m³ (0.4 ppm) 400 ppb NO₂ (7). The same results have been reported with lower levels (500 µg/m³), shorter exposure time (30 min) and repeated exposures (86).

The only study of interactions between NO₂ and allergen, which so far is published, is by Wang who studied the effects of 6 hours exposure to 752 µg/m³ (0.4 ppm) NO₂ followed by a nasal allergen challenge with grass pollen (54). In a control group nasal lavage (NAL) was performed immediately after exposure. In the index group NAL was performed half an hour after allergen challenge. NO₂ alone had no effect on inflammatory markers (including IL-8, myeloperoxidase, tryptase and ECP). NO₂ followed by allergen challenge gave a significant increase in ECP in NAL-fluid. No cellular data was presented in this study.
AIMS

The general aim of study I-III was to evaluate if there was an interaction between an ambient level of the air pollutant nitrogen dioxide and pollen allergen, giving rise to an enhanced allergic inflammation. The evaluation concerned both the upper and lower airways. Special emphasis was laid to mimic real exposure situations with low doses of air pollutants and allergen.

The specific aims were as follows:

- To study if a single exposure to an ambient level of NO₂ prior to a bronchial allergen challenge modulated the inflammatory response in the lower airways.
- To study if a single exposure to an ambient level of NO₂ prior to a nasal allergen challenge modulated the inflammatory response in the upper airways.
- To study if brief exposures to an ambient level of NO₂ prior to a bronchial allergen challenge modulated the inflammatory response in the lower airways.
- To evaluate a simplified method to monitor allergic eosinophilic activity in induced sputum.
SUBJECTS AND METHODS

Subjects and study design

The subjects were recruited from the out-patient clinic at the Department of Respiratory Medicine and Allergy, Karolinska University Hospital Huddinge or from advertising in a local newspaper. All the subjects had mild asthma according to the GINA guidelines (87). The diagnosis of asthma was based on a history of reversible seasonal asthma symptoms and bronchial hyperresponsiveness to histamine, $\text{PD}_{\text{SRaw100\%}} \leq 660 \mu g$ histamine. Lung function expressed as $\text{FEV}_1$ was within the normal range. Allergy to birch or timothy pollen was confirmed by a positive skin prick-test and by a positive bronchial or nasal allergen challenge test. A late asthmatic reaction was based on a fall in $\text{FEV}_1 \geq 15\%$ 3-10 hours after allergen bronchial challenge. All of the subjects in the studies (except 1 in study IV) were non-smokers, 42 had never smoked and 17 were ex-smokers, who had not smoked for at least 2 years. They were not using corticosteroids, disodium chromoglycate or theophyllamines continuously, but only inhaled $\beta_2$-agonist occasionally, when needed. They were free of airway infection within at least 4 weeks prior to and during the study period. This was also confirmed by a CRP at a normal level (<10) in studies I, III and IV.

Verbal and written informed consent was obtained from all subjects and approval from the local Ethics Committee at the Karolinska University Hospital Huddinge was given for each study.

<table>
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<th>Study</th>
<th>Subject no</th>
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<th>Sex F/M</th>
<th>Asthma duration years mean±SD</th>
<th>Skin prick test median mm</th>
<th>Smoking no/ex</th>
<th>FEV$_1$ % predicted mean±SD</th>
<th>Histamine dose at inclusion $\mu g$</th>
<th>Allergen dose after NO$_2$/allergen medium IQ range SQ units</th>
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*1 subject was a smoker; $^\dagger$ nasal challenge; $^\S$ late asthmatic reaction

All investigations, including the inclusion of the subjects in the 4 studies were made outside the pollen season. In studies I, II and III, all the exposures to NO$_2$ and control exposure to purified
air were made in an exposure chamber at our department. The exposures were randomized and single-blinded and NO$_2$/air exposures of each subject were performed at the same time of the day.

![Figure 3. NO$_2$ exposure chamber.](image)

**Study I**

Thirteen adult non-smoking subjects with mild asthma and allergy to birch or timothy pollen participated in the study. They were exposed at rest in an exposure chamber to either purified air or 500 µg/m$^3$ NO$_2$ for 30 min. The exposures (NO$_2$/air) were performed in random order (7 first NO$_2$, 6 first air) and at least 4 weeks apart. Four hours after exposure, a bronchial challenge with an individually fixed dose of allergen was given. The dose was 40% of the dose estimated to give a 100% increase in specific airway resistance (PD$_{SRaw100\%}$) at a screening visit, based on a previous study with NO$_2$ and allergen (86). The time interval of 4 hours was based on an established protocol from previous studies (88). Lung function during NO$_2$/air exposure and allergen challenge was measured by plethysmography, and then hourly by portable spirometry after exposures. Symptoms were recorded during and after exposure. Bronchoscopy with bronchial wash (BW) and bronchoalveolar lavage (BAL) was performed 19 hours after allergen challenge.
**Study II**

Sixteen adult non-smoking subjects with rhinitis and mild asthma and allergy to birch or timothy pollen were exposed at rest to either purified air or 500 µg/m³ NO₂ for 30 min. The exposures (NO₂/air) were performed in random order and at least 4 weeks apart. The order of exposure was randomized (8 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 between NO₂/air and allergen was the same as in study I. Nasal lavage was performed 5 times, before NO₂/air exposure, before allergen challenge, and 1, 4 and 18 hours after. Symptoms were recorded during the exposure and in connection with nasal lavage. The morning of Day 1 and 2 blood samples were obtained.

**Study III**

Eighteen adult non-smoking subjects with mild asthma and allergy to birch or timothy pollen were exposed at rest to purified air or to 500 µg/m³ NO₂ for 15 min on Day 1 and for 2×15 min on Day 2. Four hours after the exposure (Day 1) and 3-4 hours after the 2 exposures (Day 2), an allergen dose was inhaled. The time interval between NO₂/air and allergen was the same as in study I and II. The daily allergen dose was set to 20% of the PD₅₀Raw¹₀₀% at the screening visit. This dose was repeated over 2 days and gave an accumulated dose of 40% of the PD₅₀Raw¹₀₀%, which equals the single dose given in study I. The exposures (NO₂/air) were performed in random order and at least 4 weeks apart. Sputum was induced and blood samples were obtained on the morning of Day 1, 2 and 3 in order to measure the inflammatory response. Lung function during NO₂/air exposure and allergen challenge was measured by plethysmography, and then hourly during the two days by portable spirometry. Symptoms were recorded during and after exposure.

**Study IV**

Thirteen adult subjects with mild asthma, and allergy were entered into the study. Each subject visited the laboratory on 2 consecutive days. The morning of Day 1 sputum was induced, and blood sample was obtained. Directly afterwards, an allergen bronchial provocation with birch or timothy allergen was performed. Specific airway resistance (SRaw), thoracic gas volume (TGV) and forced expiratory volume in 1 s (FEV₁) were measured before and immediately after allergen inhalation. The FEV₁ was then registered every hour during the day. Symptoms were also recorded. The morning of Day 2, the subject returned to the laboratory for FEV₁ measurement, blood sample collection, and sputum induction.

**Exposure chamber and exposure data**

The exposure chamber had a volume of 7 m³ and a body plethysmograph was placed in the chamber. A separate ventilation system with filters for particulates and charcoal filters for gases
provided purified air to the chamber. The NO₂ gas, kept in a gas bottle (Alfax, approx. 8000 mg/m³ NO₂), was diluted in 2 steps to a final concentration of about 500 µg/m³ NO₂ and fed into the exposure chamber. The gas dilution and exposure system has been presented in detail (38). NO₂ concentrations in the exposure chamber were measured with a chemiluminescence instrument (Model 8440 Nitrogen Oxides Analyzer; Monitor Laboratories, Englewood, Colorado, USA). For calibration, a NO₂ permeation tube and NO calibration gas (Model 8500 Calibrator; Monitor Laboratories, Englewood, Colorado, USA; AGA Special gas, 100 ppm/m³ NO) was used. A calibration procedure was run daily. NO₂ concentrations in the exposure chamber were measured in the breathing zone of the subject.

Individual ambient NO₂ measurements

The subject’s individual exposure to NO₂ during the exposure period was measured with a personal, passive (filter badge) sampler (Toyo Roshi Kaisha, Ltd., Japan). The analytical technique, the accuracy and reproducibility of the measurements with the sampler have been presented in detail elsewhere (89).

Bronchial allergen challenge

The same nebulizer system was used for histamine and bronchial allergen challenge. The provocation tests were performed by using an automatic, inhalation-synchronized dosimetric jet nebulizer (Spira Electro 2; respiratory Care Center, Hameenlinna, Finland) with an adjustable, aerosol delivery time, according to a method previously described (90). With the equipment, the start of the aerosolization was determined by a threshold volume of inspiration. The inhalation flow, the number of nebulizations and the volume of each inhalation were displayed. The nebulization time was set to 0.5 s and the aerosolization started after an inspired volume of 100 mL and ended with the tidal volume. The inspiratory flow rate was 0.5 L/s. Standardized and freeze-dried birch or timothy allergen extracts (Aquagen, Alk, Copenhagen, Denmark) were diluted and used at a maximum of 4 concentrations: 1000, 4000, 16000 and 64000 SQ
(Standardized Quality) allergen units/mL. In each concentration, 2 and 4 breaths could be taken, and if needed even followed by 8 and 16 breaths at the highest concentration. SRaw and TGV were measured 15 min after each dose of allergen. After measuring the baseline SRaw, the subject inhaled double doses of allergen, starting from an initial dose of 14 SQ units until a 100% increase in SRaw was reached. PD_{SRaw100%} for allergen was calculated by linear interpolation on a logarithmic scale.

In study I, the inhaled allergen dose was set to 40% of the PD_{SRaw100%} at inclusion or, when this was impossible to administer for practical reasons, the dose above. For safety reasons, the inhalation started with 20% of the inclusion dose. Another 20% was then inhaled, provided that the SRaw increase after 15 min was less than 50% compared with the baseline. If the SRaw increase after the first dose was 50-75% compared with baseline, the second allergen dose was reduced to 10% of the inclusion dose.

In study III the inhaled allergen dose during the study period was set to 20% of the PD_{SRaw100%} at the screening visit. This dose repeated during 2 days gave an accumulated dose that equals the single dose given in study I. When it was impossible to administer exactly this dose for practical reasons, the dose above was given.

**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 3 concentrations: 100, 1000 and 10 000 SQ allergen units/mL (91). Saline and allergen solutions were kept at room temperature for 1 hour before provocation. (0.9% saline.) 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 (92). The dose was then increased by 10-fold dose increments (to 100 and 1000 SQ, respectively) until a clinical response was effectuated. A possible response was evaluated within 15 min after allergen administration and was regarded as positive when nasal blockage, itching, sneezing and hypersecretion occurred. The allergen was administered during breath-hold, and the patients were carefully advised to keep their heads in a forward prone position to avoid swallowing or aspiring allergen or nasal secretions into the lower respiratory tract. Placebo challenge was performed in the same manner, using allergen diluent (ALK-ABELLÓ). The given allergen dose during the study period was set to the dose giving a positive reaction at inclusion.

Before the challenge session and 15 min after each dose of allergen the patient recorded their symptoms (nasal blockage, secretions, itching and sneezing) using a detailed symptom score sheet. Symptoms of blockage, secretion and itching were scored by using a 4-point scale
(0=no symptoms; 1=mild; 2=moderate; and 3=severe symptoms) and the number of sneezes were counted (0= no sneezing; 1=1-4; 2=5-9 and 3=≥10 sneezes). A total nasal symptom score was calculated and a positive reaction was defined as symptom scores of at least 5.

**Spirometry and body plethysmography**

In study I, III and IV we used a computerized spirometer to record forced expiratory volume in 1 second (FEV₁) hourly from 8 a.m. on the day of exposure and allergen challenge and on control days. The measurements in study I were made with a portable computerized spirometer (Diary Card spirometer, Micromedical Ltd. Chatham, Kent, UK) and in study III and IV with another portable spirometer (Spirobank, MIR Srl. Roma, Italy). As a qualitative assessment of the late asthmatic reaction (LAR), defined as ≥15% decrease from pre challenge values, the lowest single FEV₁ value 3-10 hours after allergen challenge was used (93, 94). Measurements of lung function during exposure to air or NO₂ in studies I and III were made with a body plethysmograph (Model 2000 TB; Cardio-pulmonary Instruments, Houston, Texas) in the exposure chamber. The same equipment was used for the bronchial challenge with histamine in all studies and with allergen in study I, III and IV. Airway resistance (Raw) and thoracic gas volume (TGV) were measured according to the method of Du Bois and colleagues (95, 96). The gas flow/box pressure slopes were measured at flows +0.5 and -0.5 L/s (expiration-inspiration) as a mean of 2 to 3 slopes. The mouth-/box pressure slopes were measured between the end points, again as the mean of 2 to 3 curves. All panting manoeuvres were made at a frequency of 1Hz, with the subject being guided by a metronome.

**Bronchoscopies and bronchoalveolar lavage**

All bronchoscopies were done between 8 and 9 a.m. FVC and FEV₁ were measured before the procedure. Fifteen min before the bronchoscopy all subjects inhaled 0.4 mg salbutamol. Fiberoptic bronchoscopy was performed after premedication with morphine hydrochloride and scopolamine hydro bromide (Morfin-scopolamin®, Pharmacia, Uppsala, Sweden). Local anaesthesia was administered before and during the procedure with lidocaine (Xylocain®, Astra, Södertälje, Sweden). The Olympus B1-IT 20 (Olympus Optical Company, Tokyo, Japan) bronchoscope was introduced through the nose or the mouth into the lung, and wedged in a sub segmental bronchus of the middle lobe. Lavage was performed using 1 aliquot of 20 mL, and 3 aliquots of 50 mL sterile warm 0.9% NaCl, instilled into the middle lobe sub segment. The fluid was gently aspirated after each aliquot and collected in propylene tubes. The first 20 mL that was instilled, BW, was collected separately from the remaining 150 mL, BAL.
**BW and BAL analyses**

The lavage fluid was filtered through a Dacron net (Millipore, Cork, Ireland). Cells were pelleted by centrifugation at 4°C 400 g for 10 min, and the supernatants were stored at -70°C after an additional centrifugation at 4°C 600 g for 10 min. The cell pellets were resuspended in Hepes buffer (RPMI). The total number of cells were counted in a Bürker chamber, and their viability was tested by the exclusion of trypane blue. Slides for differential counts were prepared by cytocentrifugation at 500 rpm for 3 min (Cytospin 3 Shandon, Southern Products Ltd., and Runcorn, England). Slides were stained with May-Grünwald Giemsa, and 500 cells were counted. Mast cells were stained with acid toluidine blue and counterstained with Mayer’s acid Haematoxyllin.

Analyses of soluble mediators were performed in cell free BW and BAL fluids. The fluids were distributed in portions and stored at -70°C until analyzed. ECP levels were measured with a fluoroimmunoassay, Pharmacia ECP Cap System FEIA, (Pharmacia & Upjohn, Uppsala, Sweden), with a detection limit <2 ng/ml.

MPO levels were measured with a competitive RIA (Pharmacia&Upjohn), which had a detection limit of <8 µg/mL. The levels of human IL-5, IL-8, eotaxin, and soluble ICAM-1 were measured by ELISA technique using Quantikine immunoassays (R&D Systems, Inc, Minneapolis, MN, USA). According to the manufacturer, the minimum detectable concentrations for substances were IL-5 3.0 pg/mL, eotaxin 5.0 pg/mL, IL-8 10 pg/mL, and soluble ICAM-1 0.35 ng/mL.

**Sputum induction**

Sputum was induced using a standardized method (97). All subjects were pre-treated with an inhaled β2-agonist (0.5 mg terbutaline). Hypertonic saline was nebulized using an ultrasonic nebulizer (De Vilbiss 2000, De Vilbiss Co, Somerset, PA, USA) with an output of 1.5 mL/min. Inhalation was performed at intervals of 7 min with increasing concentrations of saline (3%, 4% and 5%). FEV1 was monitored before commencing and after every inhalation period. All subjects received all three concentrations of saline. Following each inhalation interval, subjects were advised to rinse their mouths with water and blow their noses before trying to cough sputum into a sterile plastic container.

**Sputum processing and analyses**

Sputum was processed using a standardized method (98). The total number of cells was counted in a Bürker chamber and the viability was tested by the exclusion of trypane blue. Slides for differential counts were prepared by cytocentrifugation at 500 rpm for 3 min (Cytospin 3 Shandon, Southern Products Ltd. and Runcorn, England). Slides were stained with May-Grünwald Giemsa and 500 cells were counted. Mast cells were stained with acid toluidine blue
and counterstained with Mayer’s acid Haematoxylin. Adequate sputum specimens for cell analysis at all 6 time points were obtained from 10 subjects.

Analysis of soluble mediators was performed in cell-free supernatants from sputum. The fluids were aliquoted and stored at -70°C until analyzed. The other steps of the analysis were made as in study I and II.

**Blood samples, differential cell counts and inflammatory markers**

Differential cells were counted by routine procedure on a Coulter STKS (Coulter Electronics Ltd. Luton, Beds., England) at the Department of Clinical Chemistry (Study II, III and IV).

Serum eosinophil cationic protein (ECP) was measured by radioimmunosorbent assay using the ECP RIA kit (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), detection limit >16 µg/L. Myeloperoxidase (MPO) was measured with a competitive RIA (Pharmacia & Upjohn), detection limit of >8 µg/mL.

**Nasal lavage**

Each nasal cavity was lavaged with 10 mL warm (37°C) sterile isotonic saline with a syringe to which a tight-fitting nasal olive was adapted. The subjects sat with their head bent forward at approximately 60°, and breathed through their mouth. This position prevented the fluid from reaching the throat. The lavage fluid was passed slowly into the nasal cavity, kept there for 10 s, and withdrawn into the syringe 5 times. The lavage fluid from both nostrils was pooled into sterile plastic containers and put on ice for further analysis. The recovery from each 5 nasal lavages in both nostrils was 16±0.96 mL (mean±SD) during air exposure and 16±0.74 mL during NO₂ exposure. The nasal lavage analyses were made in the same way as the analyses of the BAL fluid.

**Symptoms**

At all chamber exposures (study I, II and III) the subjects were asked 16 questions concerning respiratory symptoms and perceptions of discomfort (i.e. tight chest, cough, headache, odor), reporting from none to maximal symptoms on a scale of 0 to 7. The questions were raised after 3 and 26 min in study I and II and after 4 and 14 min during the 2 exposures in study III. The questionnaire has been used in previous chamber exposure studies (8, 99).

In study I and IV, throughout the day, and in the following morning, the subjects kept a self-administered daily record of bronchodilator medication and symptoms from bronchi, nose, and eyes. Medication was recorded as the number of inhalations during night and day respectively. This recording of symptoms was also made in study III, but during 2 days and in the following morning. Nightly asthmatic bronchial symptoms were registered on a 5-category scale as no symptoms, woken by symptoms once or earlier than usual, awoken by symptoms more than
once, awake because of symptoms for the greater part of the night, and symptoms so severe that no sleep at all had been possible. Daytime symptoms were expressed on a 6-category scale as no symptoms, symptoms for a while, symptoms more than once, symptoms during all the day but not affecting normal activities, symptoms during the whole day affecting normal activities, and symptoms so severe that normal activities were impossible. Irritability symptoms from bronchi, nose, and eyes were noted for the last 24 hours on a 4-category scale expressed as no symptoms, tolerable symptoms, symptoms affecting normal activity, and normal activity impossible. In study II nasal symptoms as described in allergen challenge were recorded 5 times during the study days. Before exposure to NO₂/air, before allergen challenge, and 1, 3 and 18 hours after allergen.

### Table 3. Summary of methods used in Study I-IV

<table>
<thead>
<tr>
<th>Study Exposure to NO₂</th>
<th>I (500 µg/m³ 30 min)</th>
<th>II (500 µg/m³ 30 min)</th>
<th>III (500 µg/m³ 3 x 15 min)</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung function test</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Histamine challenge</td>
<td>x</td>
<td>x</td>
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<td>x</td>
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<tr>
<td>Bronchial allergen challenge</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Nasal allergen challenge</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bronchoscopy</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Induced sputum</td>
<td>-</td>
<td>-</td>
<td>x</td>
<td>x</td>
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<tr>
<td>Nasal lavage</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Symptom recording</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

x performed; - not performed

### Statistics

**Study I and III**

The Wilcoxon non-parametric signed-rank test for paired observations was used to compare data for NO₂ and filtered air before and after allergen. Probability values below 5% were considered significant. Software for Power Macintosh (version 11.0 from SPSS Inc., Chicago) was used. Pair-wise correlations of delta values were calculated for r values (Pearson’s correlation coefficient) with JMP® version 3.2.2 statistical software from SAS Institute Inc.

**Study II**

The Wilcoxon non-parametric signed-rank test for paired observations was used to compare the effects of exposure to NO₂ and filtered air, respectively, before allergen challenge. Analysis of
variance was used to compare the effects of nasal allergen challenge after NO₂ and filtered air, respectively. For analysis of variance SAS the GENMOD Procedure was used. For symptoms the Friedman’s ANOVA was used for testing changes over time followed by the Wilcoxon matched pairs test if necessary.

Study IV

Wilcoxon nonparametric signed-rank test for paired observations was used to compare data on cells and inflammatory markers in sputum and blood before and after allergen. Correlations were performed with the Pearson or Spearman test. The analyses were performed using statistical software SPSS version 11 and STATISTICA (data analysis software system), version 7 StatSoft, Inc. (2004).
RESULTS
The main results were that ECP in BW, sputum, and blood was higher after NO₂+allergen compared to air+allergen both after single 30 min and repeated 15 min exposure. Also the percentage and the absolute numbers of neutrophils were increased after NO₂+allergen in both BW and BAL after a single 30 min exposure.

Ambient level of NO₂ augments the inflammatory response to inhaled allergen in asthmatics. (Study I)
The percentage of neutrophils in both BW and BAL were higher after NO₂+allergen compared to air+allergen in both BW and BAL (BW 19 vs. 11%, P=0.05; BAL 3 vs. 1%, P=0.02, median values). The absolute numbers of neutrophils were also higher after NO₂+allergen compared to air+allergen in both BW and BAL (BW 24.0 vs. 7.0 till x10⁶/L and BAL 31.0 vs. 14.2x10⁶/L, P=0.02).

![Figure 4. Neutrophil percentage in lavage fluids after exposure to air+allergen compared to NO₂+allergen. The thick horizontal lines represent median values, boxes represent 25th-75th percentile range.](image)

Eosinophil counts in BW and BAL did not differ between the two exposures. ECP in BW was higher after NO₂+allergen compared to air+allergen (9.0 vs. 3.6 µg/m³; P=0.02, median values). ECP in BAL was higher after NO₂+allergen compared to air+allergen (2 µg/L vs. 0.0 µg/L, P=0.09) although not statistically significant.
MPO levels in the bronchial and bronchoalveolar portion were not detectable. The neutrophil counts were correlated with IL-8 levels (r=0.59, P=0.04) in BAL, but not in BW, and with ECP levels in both BAL and BW (r=0.77, P=0.01 and r=0.60, P=0.03, respectively).

The eosinophil counts in BW were associated with IL-8 levels (r=0.68, P=0.01). The eosinophil counts in BAL were correlated to the ECP levels (r=0.57, P=0.04) and ECP levels correlated with IL-5 levels (r=0.79, P=0.001) in BAL. There was no significant difference in SRaw or TGV between air and NO\textsubscript{2} exposure in the chamber or immediately after allergen challenge. There was no NO\textsubscript{2} associated effect on symptoms or pulmonary function.

**Brief exposures to NO\textsubscript{2} augment the allergic inflammation in asthmatics. (Study III)**

ECP in both sputum (28 vs. 173 µg/L, P=0.001) and blood (5.3 vs. 10.8 µg/L, P=0.004) increased more from Day 1 to Day 3 after NO\textsubscript{2}+allergen than after air+allergen, whereas eosinophil counts did not differ. The change in MPO was significantly greater after NO\textsubscript{2}+allergen than after air+allergen in blood (P=0.003) but not in sputum. These findings were not accompanied by raised levels of neutrophils in sputum and blood. Symptoms and pulmonary function were equally affected by NO\textsubscript{2}+allergen and air+allergen.
Does nitrogen dioxide affect inflammatory markers after nasal allergen challenge? (Study II)
The allergen challenge after both air and NO$_2$ exposure gave an immediate increase in the number of eosinophils, but the increase did not differ during the two exposures. Exposure to NO$_2$ followed by allergen decreased ECP in NAL fluid one hour after allergen (P=0.006) but did not affect ECP-levels at other time-points. Analysis of variance, of the whole data set from pre-allergen to 18 hours after allergen for eosinophil and neutrophil numbers, ECP and MPO...
values in NAL fluid and symptoms showed no statistically significant difference between NO2+allergen and air+allergen.

**Total eosinophil cationic protein levels in induced sputum as a marker of changes in eosinophilic inflammation in patient with allergic asthma. (Study IV)**

The increase of eosinophil counts in sputum from before allergen to after was 0.1% (0-2.5) to 7% (3-17), median and IQ range, (P=0.002). *Total ECP* increased from before allergen to after from 55.7 to 798 µg/L, (P=0.002) and *released ECP* from 21.8 to 288 µg/L, (P=0.002). Eosinophil counts in blood increased from 0.16 to 0.25x10^9/L (P=0.002).

After challenge with birch or timothy allergen all subjects developed an immediate increase in specific airway resistance. The median allergen dose PD_{SRaw,100%} was 175 SQ units. The mean decrease in FEV1 during the early phase was 13% and the mean maximal fall during the late phase was 16%. Seven of the thirteen subjects had a late asthmatic reaction (FEV1 fall ≥15%).

We found a good correlation between *total ECP* in the entire sputum sample and *released ECP* in the supernatant both before (r=0.97, P<0.01) and 24 hours after an allergen challenge (r=0.99, P<0.01). We also found a good correlation (r=0.99, P<0.01) between the changes in total and released ECP levels. There was significant but moderate correlation between *total ECP* and *released ECP* on the one hand and the change in the percentage of eosinophils on the other hand (r=0.66; P=0.02; and r=0.59; P=0.04, respectively).
DISCUSSION

The main finding of the present thesis is that exposure to an ambient level of NO₂ increases the inflammatory response to allergen in asthmatics.

People with mild asthma were chosen as they constitute a greater part of the asthma population. The subjects had stable baseline values without any anti-inflammatory medication and the risk of interfering exacerbations were minimal as the studies were performed outside the pollen season. Our results were based on a small, but well-defined group of subjects, and they are probably representative for people with mild allergic asthma. A reason for not choosing subjects with more severe asthma is their unstable baseline values, due to varying disease activity, which would make it more difficult to interpret the results. The subjects with rhinitis in study II also had asthma. Most of the subjects with asthma in studies I, III and IV also suffered from rhinitis.

The concentration of 500 µg/m³ NO₂ was chosen to study the effects of brief peak exposures which are encountered in real life situations when living in urban areas. In cities the exposures to peak levels of NO₂ usually occur several times a day. The short exposures start in the morning, when people are walking, driving or going by bus to work, and continue at midday when people are going outdoors and in the afternoon when they return home. Peak levels of about 500 µg/m³ NO₂ have been measured in Stockholm in road tunnels during rush hours (100) and in heavily polluted cities in other countries (101). Indoors even higher concentrations can occur when using unvented gas stoves and gas appliances (14). In Sweden the annual average NO₂ values are between 6 and 45 µg/m³ (mean 18,5 µg/m³) (102), but sometimes even higher levels occur.

In the current studies the exposure concentration of NO₂ was 500 µg/m³. This concentration is based on measurements in the Stockholm area (100) and is similar to previous human exposure studies on subjects with mild asthma performed in our laboratory. In 1988, Bylin noted that a 30 min exposure to NO₂ about 500 µg/m³ but not at lower or higher concentrations (260 and 1000 µg/m³ were tested) affected bronchial responsiveness, in subjects with mild asthma (99). Later the experiments were continued by measuring non-specific bronchial responsiveness at the same concentration and exposure time (500 µg/m³ NO₂ for 30 min) at different times after exposure in asthmatics. A delayed effect on the bronchial responsiveness around 5 hours after exposure was noticed (8). In study I and II we used the same concentration and time for the single exposures (500 µg/m³ NO₂ for 30 min). In study III we made a different study design where instead of a single exposure three brief repeated exposures were made. The exposures to 500 µg/m³ NO₂ were only 15 min. Our question was if one very brief exposure or three repeated brief exposures could have any enhancing effect on the inflammatory reaction. Exposure to
these very brief peak levels of NO₂ can probably occur outdoors during rush-hour in cities while travelling to and from work.

As the exposures to ambient NO₂ levels outside the laboratory could influence the results in the studies, the subject’s individual exposure to NO₂ was measured by a personal sampler during the study periods (89). Average exposure to NO₂ in ambient air was low both at the exposures to NO₂ and at the control exposure in ambient air, 18-25 µg/m³, measured with a personal sampler for 24 or 48 hours.

All the experiments in study I-III were made at rest. In many other studies exercise is used during exposure in order to increase the tidal ventilation and thereby the dose. A meta-analysis by Folinsbee shows that the effects during exercise during exposure to NO₂ levels below 1000 µg/m³ will not necessarily be greater than those at rest (41). Physical exercise is also known to cause airway obstruction in subjects with asthma. The results in the present studies are mainly applicable to exposure of people with a low exercise level, e.g. walking to a bus, sitting in a car or cooking.

We used an interval of 4 hours between exposure to NO₂ and allergen challenge. This time interval was based on the experience that there is a delayed effect of the non-specific bronchial responsiveness after NO₂ exposure (8). The time interval of 4 hours from exposure to NO₂ to allergen challenge was also taken into consideration as it enhances the allergen-induced late asthmatic reaction (88). These effects have also been confirmed when using the same model with 4 hours interval between exposure and allergen challenge in a study with repeated exposures to NO₂ (86) and in exposure to air pollutants in a road tunnel at rush-hour (100).

On some days in May you can find as many as several thousands birch pollen grains/m³ and other days the levels are far below 100 pollen grains/m³. The amount of pollen in the outdoor air can be measured by counting pollen grains per cubic meter (n/m³) in a Burkard trap normally situated at roof level. Recently, an immunochromatographic method for qualitative and quantitative determination of aero-allergens direct on sampling (ADOS) filters has been developed (103). This method makes it possible to measure the allergen concentration (SQ units/m³) and connect it to the amount of pollen grains. As an example, the pollen counts during a week in the middle of May 1997 varied between 6 and 484/m³ and the allergen concentration varied between 35 and 740 SQ units/m³ (104).

When designing the three exposure studies the purpose was to achieve situations which could occur in the outdoor surroundings in an urban area during the pollen season. We tried to estimate the amount of allergen that may be inhaled during a day in the outdoor air during the birch or grass pollen season. This is in contrast to most other allergen challenge studies, where
the given allergen doses are considerably higher than average natural exposure outdoors during the pollen season (105).

In our studies we used the same allergen extracts for allergen responsiveness as is used in clinical testing and immunotherapy. It is difficult to calculate a dose that exactly corresponds to the levels outdoors. The median bronchial allergen dose given in study I was 110 SQ, which corresponds with the lower outdoor May measurement which had a concentration of 35 SQ/m³ to an exposure time outdoors about 7.5 h. With the higher outdoor level of 740 SQ units/m³, it corresponds to about 0.5 hours exposure. The corresponding times for the exposure dose in study III were at a day with the lower level of allergens about 20 min exposure and at the higher level about 6 hours exposure. In study II the allergen dose given into the nose was about ten times higher so the corresponding times for the low a low level allergen was much longer about 2.5 days and a high level day about 3 hours.

Figure 8. Concentrations of outdoor air birch pollen obtained with the Burkard trap, reported as average values from 24-h sampling periods (dashed line) and of outdoor air birch pollen allergens determined by the DOSAFE technique (solid line) in May 1997. The figure is published with the kind permission of the publisher, Munksgaard.

Study I and III

In study I a single 30 min exposure to an ambient level of NO₂ enhanced the subsequent allergen-induced inflammatory reaction in the bronchi, as demonstrated by enhanced number of
recruited neutrophils both in the bronchial and the alveolar portion. There are no published data on the cell response in BAL after exposure to NO₂ in asthmatic subjects at a comparable time after exposure, as used in the present study. However, NO₂ at a comparable concentration does not increase neutrophils in BAL in asthmatics immediately after exposure (55). Neither does NO₂ give any change in neutrophil number in BW and BAL in normal subjects exposed to NO₂ at a comparable time (24 hours after exposure), even though the concentrations are six to fifteen-fold, higher than those used in the current study (106, 107).

Allergen bronchial challenge per se causes no increase in neutrophils in asthmatics 24 hours after challenge, although a transient increase of neutrophils in BAL fluid can be observed 2-4 hours after allergen inhalation (105). This indicates that neither exposure to low levels of NO₂ alone nor allergen inhalation alone induces a late airway neutrophilia.

We found that a combined exposure to NO₂ and a low dose of allergen mounted an additive effect of NO₂ on the neutrophilic response. However, it is plausible to assume that this potentiating effect of NO₂ could be even more pronounced with higher allergen doses.

In study I we found an increase in neutrophils in both BW and BAL after NO₂ and allergen. This suggests that at least a 30 min but not a briefer exposure is needed to recruit the neutrophils into the airways. This differs from the findings in study III, where no increase in neutrophils was found in induced sputum after a single 15 or three repeated 15 min exposures to an ambient level of NO₂ and allergen. MPO increased in blood after NO₂ exposure in study III which might indicate a priming effect of NO₂ per se on the circulating neutrophils, as discussed by others (44). We did not find any detectable levels of MPO in BW and BAL by NO₂, suggesting that the neutrophils were not significantly activated, in terms of degranulation. In some NO₂ studies an increase in MPO is associated with an increase of neutrophil counts (44), but not in others (108). We also found a significant over-all correlation between the number of neutrophils and IL-8 in BAL, although the mean levels of IL-8 were not affected. This finding suggests that chemotactic signalling for further recruitment of neutrophils was still active 19 hours after allergen challenge in this NO₂-allergen exposure model.

An essential finding in both study I and III was that NO₂ enhanced the allergen induced ECP levels. ECP levels were significantly increased in BW, and a tendency to a similar effect was seen also in BAL, although no increase of the number of eosinophils was found either in BW or in BAL. ECP levels were also raised in sputum and blood after three brief exposures but not after a single exposure to NO₂.

It is noteworthy that in the current study the levels of ECP increased although the numbers of eosinophils were unchanged. One possible explanation for this discrepancy might be a selective
effect on degranulation, but not on cell recruitment, after NO₂ exposure. Another contributing explanation is that ECP origins from both bronchial and submucosal eosinophils, though their relative contribution remains unknown. Another less likely explanation is that ECP is produced by other cells, since non-eosinophilic ECP contribution to the over-all ECP levels is not known (109).

The interpretation of the relationship between eosinophil count and ECP in sputum must be done with caution due to the fact that adequate cell samples are not produced from all subjects, a limitation with the sputum method (110). Several studies indicate that the cellular composition in sputum does not completely correlate with bronchial biopsies, which indicates that differences exist between the mucosal and luminal phase of inflammation (111). Nevertheless, whether the increase in both BW and sputum ECP levels is a consequence of increased eosinophil degranulation and/or increased recruitment of mucosal eosinophils, cannot be fully established. Mucosal biopsies are crucial to gain insight into this question.

In addition to the finding in sputum, we found a NO₂-dependent increase in blood ECP after allergen challenge. Blood ECP reflects an enhanced eosinophil secretory activity, and the propensity of eosinophils to adhere and transmigrate (75). Pre-exposure to NO₂ might increase the responsiveness of circulating eosinophils. Our data suggest that NO₂ exposure augments the allergen-induced eosinophilic inflammatory reaction, by priming circulating eosinophilic cells, thereby promoting the ECP release at the local inflammatory site.

NO₂ did not affect the allergen-induced bronchoconstriction, measured as changes in FEV₁ in the current studies, in contrast to reports in other previous studies (7, 86, 88). The NO₂ exposure dose has been the same in two of these studies. However, two factors differ, the individual exposure to NO₂ outside the chamber and the dose of allergen. In study I the individual exposure to NO₂ in ambient air (outside the chamber) during the study day happened to be higher after air than after NO₂. This reduced the difference in NO₂ exposure between the NO₂ and the control experiment settings. It might thereby have reduced the probability to detect the effect on lung function related to NO₂ exposure in the chamber. In study III, on the other hand, the exposure situations outside the chamber were similar during air and NO₂ exposure days.

The allergen doses in other studies where NO₂ affects lung function after allergen provocation are 10 and 100%, respectively, of PD₅₀ Raw100% (86, 88). In the current studies the dose of allergen was intermediate, 40% of PD₅₀ Raw100%. If this difference in allergen doses explained the different response in pulmonary function, it implied that the NO₂-induced enhancement of the asthmatic reaction to inhaled allergen was non-linear relative the allergen dose.
Study II

In study II a single exposure to an ambient level of NO$_2$ did not enhance the allergic inflammatory reaction in the nose. We found no difference in the eosinophil and neutrophil counts or the inflammatory mediators ECP and MPO after NO$_2$+allergen exposure compared to air+allergen exposure.

NO$_2$ alone did not alter ECP or eosinophil counts in the present study. This is in line with other nasal lavage studies on subjects with allergic rhinitis, where NO$_2$ does not alter the levels of ECP (49, 54). These studies and the present study suggest that short-term exposure to NO$_2$ alone at a high ambient concentration does not induce an eosinophilic inflammatory reaction in the nasal mucosa. The combined exposure to NO$_2$ and allergen had no effect on ECP-levels in NAL-fluid. This is opposed to that exposure to NO$_2$ increases the ECP levels in NAL-fluid 0.5 hours after nasal allergen challenge (54). A possible explanation of these incongruent results is differences in study design. In the study by Wang, the NO$_2$ concentration was almost 2-fold higher and the exposure time was twelve times longer than in our study. This suggests that the priming effect of NO$_2$ at ambient levels on the eosinophils in the nasal mucosa for subsequent activation by allergen is dose dependent. Pre-exposure to NO$_2$ did neither alter the neutrophil cell count nor MPO at this ambient level.

We used an established exposure model with exposure to NO$_2$ followed by an allergen challenge three to 4 hours later. The level and duration of NO$_2$-exposure and interval between exposure and allergen challenge were similar to those that induced neutrophil influx and raised levels of ECP in BW (study I). The difference between the two studies was that the allergen dose was higher (almost 10-fold) and given in the nose instead of the bronchi. Moreover, NO$_2$ 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 NO$_2$ might also explain that more is deposited in the lower airways where effects also are more visible. It should be kept in mind that the nose is a smaller target organ than the lungs, and systemic effects, such as changes in mediators in blood, are therefore not as easy to detect after nasal compared to pulmonary provocation.

Study IV

Study IV demonstrated that total ECP processed from the entire expectorate, consisting of sputum plus saliva, was a suitable marker to survey the intra-individual changes in the eosinophilic inflammation in subjects with allergic asthma. We found a significant correlation between total ECP and released ECP both before allergen and 24 hours after an allergen
challenge. There was also a high correlation between the allergen induced changes in total ECP and released ECP. These ECP changes were moderately correlated to the changes in eosinophil counts.

In the conventional sputum processing method total cell counts and differentials are determined after homogenization and centrifugation of selected sputum plugs, and various biochemical markers in the supernatant, such as released ECP, are analyzed (97, 110). An alternative method was introduced when using the entire sputum sample (98, 112). To further facilitate the application of the sputum method we treated the entire sputum sample with a mucolytic agent followed by a lysing reagent in order to release both intracellular and extracellular ECP into solution (113).

The currently described total ECP method includes fewer centrifugation steps than the conventional sputum process. Since the method assesses both the released and the cellular stored pool of ECP, the determination is presumably less sensitive for ex vivo modulation. This may affect the ratio between released ECP and intracellular stored ECP. It is well established that cell handling procedures can induce cell modulation and subsequent release of intracellular stored antigens.

An essential aim of the present study was to evaluate whether our method detected an increased eosinophilic activity in asthmatics after exposure to allergen. Indeed, induced sputum collected 24 hours after the allergen challenge had significantly higher both total and released ECP and percentage of eosinophils than induced sputum collected at baseline. The magnitude of the changes in released ECP and eosinophil counts are similar to those previously reported (114, 115). We also found a good correlation between the allergen induced changes in total ECP and released ECP, which suggests that total ECP can replace released ECP in assessment of changes in eosinophilic activity.

Sputum eosinophil counts form a direct marker of airway inflammation, but the assessments are time–consuming. It has been shown that a treatment strategy directed at normalization of the induced sputum eosinophil counts reduces asthma exacerbations (116). Our data showed that changes in total ECP correlated with changes in eosinophil counts. This indicated that our simplified method to measure ECP in the entire sputum sample may be a useful tool for monitoring the severity of asthma over time. It could be used in out-clinical settings for outdoor exposure studies. Prospective clinical studies of patients with allergic and non-allergic asthma are necessary to support this assumption.

The present studies showed that exposure to an ambient level of NO₂ increased the inflammatory response to allergen in asthmatics. These are the first studies that show that there
is an interactive effect between an ambient level of NO$_2$ and allergen on the inflammatory reactions in the lower airways. The effects were noticeable after a single exposure for 30 min but not after a single exposure for 15 min. But if the very brief exposure was repeated on the other hand, the inflammatory reaction after allergen was enhanced. The increased inflammatory reaction after NO$_2$ and allergen was noticed in the lower airways and not in the upper. The nose seemed not to be as sensitive as the bronchi when studying effects of short-term exposure to ambient levels of NO$_2$ on the allergic inflammatory reaction in the respiratory tract. As the effects on the airway inflammation were most visible in the bronchial and terminal bronchial area, both induced sputum and bronchoscopy with bronchial wash are applicable methods to study these effects. The effects of NO$_2$ in the present studies were observed on inflammatory cells and markers and were not associated with any symptoms or change in pulmonary function. Thus, we propose that short NO$_2$ exposures enhance the allergic inflammation at a sub-clinical level. The NO$_2$ dose used in the current studies and the doses of pollen allergen used are realistic for days with high pollen counts. It is therefore reasonable to assume that the exposure doses of NO$_2$, as well as those of allergen, may be commonly encountered in many urban environments.
CONCLUSION

There was an interaction between the ambient level of the air pollutant nitrogen dioxide and pollen allergen, giving rise to an enhanced allergic inflammation in the lower airways.

- A single exposure to an ambient level of NO₂ prior to a bronchial allergen challenge increased the inflammatory response by a late phase increase in the number of neutrophils and ECP levels in the lower airways.

- A single exposure to an ambient level of NO₂ prior to a nasal allergen did not enhance the inflammatory response in the nose. NO₂ and allergen did not alter the nasal lavage cells or mediators compared to air and allergen.

- Repeated brief exposures to an ambient level of NO₂ prior to a bronchial allergen challenge enhanced the inflammatory response expressed as increased levels of ECP in both induced sputum and blood.

- Total ECP seems to reflect the eosinophilic inflammatory changes in asthma, and might be a useful tool in monitoring asthma in clinical practise and in exposure studies.

This data suggests that ambient NO₂ can enhance allergic inflammatory reaction in the airways without causing symptoms or pulmonary dysfunction. The next challenge will be to perform follow-up, prospective studies in the outdoor milieu to validate this data on NO₂ mediated effect on the subclinical inflammation.
Personer med hösnuva och astma och pollenallergi, som bor i tättbebyggda områden, utsätts under pollensäsongen för både luftföroreningar och allergen. Tidigare studier har visat att pollen och luftföroreningar kan ha en samverkande effekt, som förstärker det allergiska svaret på inandat allergen. Lungfunktionen försämrar och känsligheten i luftvägarna ökar om man andas in både de luftföroreningar som finns i omgivningen och pollen. Mekanismerna bakom denna förändring av lungfunktionen är ofullständigt kända. Det här arbetet går ut på att försöka studera dessa mekanismer.

Personer med astma astma och allergi mot björk eller gräspollen exponerades i en laboratoriemiljö för omgivningshalter av en luftförorening, kvävedioxid (NO₂) och låga halter av pollen. Tanken var att i ett laboratorium åstadkomma en verklighetstrogen utomhusmiljö. Eventuellt påverkan på den allergiska inflammationen i olika delar av luftvägarna studerades med olika metoder. De nedre luftvägarna undersöktes med bronchoalveolärt lavage (lungsköljning) och inducerat sputum (upphostande av sekret) och de övre med nasal lavage (nässköljning). Lungfunktion och symptom registrerades under försöken.

Vi fann en ökning av en inflammationsmarkör (ECP), som är ett tecken på aktivering av de eosinofila granulocyterna (inflammatoriska celler inblandade i allergier) i de nedre luftvägarna. Det fann vi i både bronchalveolärt lavage och i inducerat sputum. ECP-ökning hittades också i blodet. Denna aktivering av de eosinofila granulocyterna fann vi således efter en 30 minuters exponering och efter upprepade ännu kortare exponeringar med 3x15 minuter i kombination med allergen. Efter en 30 minuters exponering fann vi också en ökning av de neutrofila granulocyterna i de nedre luftvägarna. Neutrofilökningen var inte kombinerad med en ökning av neutrofilmarkörer. Dock sågs en korrelation till IL-8, vilket kan tyda på att en neutrofilackumulering har skett. Trots eosinofilaktiveringen och ökningen av antalet neutrofiler var lungfunktion och symptom inte påverkade.

Vi fann ingen påverkan av NO₂ på inflammatoriska celler och mediatorer vid sköljningarna i näsan, varken under den tidiga eller senallergiska reaktionen. Orsaken till detta kan vara att NO₂ som har låg löslighet i vatten deponeras mer perifert i luftvägarna. Näsan är dessutom ett ytmässigt mindre organ än lungorna.

Våra studier visar att NO₂ först och främst förstärker det eosinofila svaret genom en ökning av frisättandet av ECP efter en lågdos allergen. Vi fann också en ökning av neutrofilantalet i luftvägarna. Den påverkan av NO₂ vi fann tycks vara belägen i de nedre luftvägarna och ej vara mätbar i de övre luftvägarna. En intressant fråga är vilken betydelse denna påverkan på...
inflammatoriska celler och mediatorer efter NO₂ exponeringen har, framför allt eftersom ingen påverkan noterades på lungfunktionen.

Det här är den första gången som det har påvisats en samverkanseffekt av en luftförorening (NO₂) och allergen på den inflammatoriska reaktionen i luftvägarna hos personer med astma.

En förenklad metod att följa den allergiska inflammationen i luftvägarna genom mätning av ECP i inducerat sputum har också utvärderats. Denna undersökning gjordes i samband med en allergenprovokation och tanken är att metoden ska kunna användas även vid exponeringsstudier. Den nya metoden med mätning av ECP i ofraktionerat sputum är en enklare metod än mätning av frisatt ECP. Den mäter ECP både inuti och utanför cellen.
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