Exhaled nitric oxide and its relationship to airway responsiveness and atopy in asthma

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Exhaled nitric oxide (NO) has attracted increasing interest as a non-invasive marker of airway inflammation. The purpose of this study was to determine whether exhaled nitric oxide in subjects with asthma varied according to their atopic status and to examine its correlation with airway hyperresponsiveness and lung function measurements.

Forty patients with asthma and 13 controls participated in the study. Nitric oxide was measured on three occasions with intervals of at least 3 days, using a chemiluminescence method. Airway responsiveness was assessed with methacholine challenge and lung function measurements were made. All subjects recorded peak expiratory flow and kept a symptom diary during a 17-day period. There was no significant difference in lung function measurements, peak expiratory flow or symptom score between the two asthma groups. Atopic patients with asthma had a significantly higher mean amount of exhaled NO than non-atopic subjects with asthma (162 ± 68 VS. 113 ± 55 nI min⁻¹; P = 0.03) and the control group (88 ± 52 nI min⁻¹; P = 0.004). No significant difference was found in the amount of exhaled NO between non-atopic patients with asthma and the controls. In atopic subjects with asthma the mean exhaled NO was significantly correlated to the dose-response slope for methacholine (r = -0.52; P = 0.02), while no such correlation was found in the non-atopic group.

In conclusion, in this study, atopic subjects with asthma had higher levels of exhaled NO than non-atopic subjects. Atopic status should be taken into account when measuring levels of exhaled NO in subjects with asthma.
Patients and methods

SUBJECTS

Fifty-three subjects aged 17–64 years, participated in the study. The clinical characteristics of the subjects are shown in Table 1. Patients with asthma were recruited from the Department of Respiratory Medicine and Allergology at our hospital. Subjects were defined as having asthma if they had asthma diagnosed by a physician (9). All of them had had respiratory symptoms during the past 12 months, a forced expiratory volume in 1 sec (FEV₁) above 70% of the predicted value and had previously demonstrated an increased responsiveness to inhaled methacholine (10). Patients with asthma who had at least one positive skin-prick test were defined as having atopy. Skin-prick tests were carried out using Solu Prick (ALK, Denmark) standardized allergen extract against the following allergens: birch, timothy grass, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Cladosporium* and *Alternaria*. A positive test was a reaction with a mean size of ≥3 mm and no dermatographism.

Fourteen (70%) of the atopic patients with asthma and 15 (75%) of the non-atopic patients with asthma were receiving regular treatment with inhaled glucocorticoids [budesonide (n = 25) or beclomethasone (n = 4)]. There was no statistical difference in steroid treatment between the two groups. The mean daily dose of inhaled steroids was 447 pg in the atopic asthma group (n = 13) and 667 pg in the non-atopic asthma group (n = 13). Data on doses of inhaled steroids were missing in one atopic patient with asthma and in two non-atopic patients with asthma. Inhaled corticosteroid therapy was continued with no changes. All the patients but one with asthma were taking inhaled β₂-agonists as required. None of the patients was being treated with theophylline compounds. One atopic subject with asthma and eight non-atopic patients with asthma were ex-smokers (stopped smoking 2–20 years previously).

The control group comprised 13 subjects who responded to a request for volunteers or were recruited from the staff at our hospital; none had asthmatic symptoms or was on anti-asthmatic treatment and all were non-atopic (Table 1). All the investigated subjects were non-smokers and had been free from respiratory infections for at least 6 weeks prior to the study and none had a history of cardiovascular disease. All the participants received a questionnaire on the occurrence of airway symptoms in the past 12 months. The questionnaire was based on the European Community Respiratory Health Survey questionnaire (11). The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee at the Medical Faculty at Uppsala University. Verbal informed consent was obtained prior to the study.

STUDY DESIGN

Peak flow and symptom diaries were kept during a 17-day period, starting on the day of inclusion in the study. Nitric oxide was measured on three occasions at least 3 days apart and within 3 weeks in each individual. At these visits, bronchial challenges and lung function measurements were made. Bronchial challenges were performed after the NO measurements.

NITRIC OXIDE MEASUREMENTS

Nitric oxide in mixed expired gas was measured over a 5-min period using a chemiluminescence NO analyser (model 42, Thermo Environmental Instruments Inc., Franklin, MA, USA). The system was calibrated with a mixture of NO in N₂ (AGA Gas AB, Lidingö, Sweden) with a concentration of 1 ppm. A four-point calibration was thus performed (0, 10, 100, 1000 ppb). The calibration of the system was tested every morning and zero was set before each patient. The patient, with a nose clip in place, was connected to a three-way valve (Hans Rudolph, Inc., Kansas City, MO, USA) by a mouthpiece and inhaled synthetic NO-free air (AGA Gas AB) from a reservoir and exhaled into a mixing box. The volume of the mixing box was 1.5 l and the box was made of stainless steel. This material is known not to influence NO measurements. NO samples were continuously drawn into the NO analyser from the mixing box. The exhaled volume was measured by

| Table 1. Characteristics of subjects with asthma and healthy controls as mean (range) |
|--------------------------------|-------------------------------|-------------------------------|-------------------------------|
| **Atopic subjects with asthma** (n = 20) | **Non-atopic subjects with asthma** (n = 20) | **Controls** (n = 13) |
| **Age** (years) | 34 (18–63) | 40 (17–64) | 30 (22–52) |
| **Gender (M/F)** | 9/11 | 1/19 | 4/9 |
| **Disease duration (years)** | 13 (0–3–79) | 8 (0–5–42) | |
| **Inhaled steroids** | 14 | 15 | |
| **FVC (% pred)** | 97 (74–138) | 90 (71–110) | 95 (77–106) |
| **FEV₁ (% pred)** | 93 (71–124) | 88 (73–115) | 95 (70–115) |
| **SGAW (% pred)** | 68 (21–122) | 78 (38–160) | 102 (59–223) |
| **Symptom score** | 2–4 (0–4) | 1–9 (0–4) | 0 |
| **PEF variability (%)** | 7 (3–16) | 8 (3–14) | 5 (31–9) |
a Wright spirometer. The amount of NO was calculated by multiplying the exhaled minute volume by the concentration of NO in the exhaled gas and then expressing it in nl min⁻¹. NO values from the first minute were discharged and a mean value for each of the following minutes was obtained.

SPIROMETRY

Functional residual capacity (FRC), vital capacity (VC), airway resistance (RAW), specific airway conductance (SGAW), forced vital capacity (FVC) and forced expiratory volume in 1 sec (FEV₁) were measured after each challenge with a Masterlab Trans spirometer and a Masterlab body plethysmograph (Eric Jaeger AG, Würzburg, Germany). FVC, FEV₁ and SGAW were used for the study. Lung function values are presented as a percentage of reference values (12,13).

METHACHOLINE CHALLENGES

We used an automatic, inhalation-synchronized dosimeter jet nebuliser, Spira Elektro 2 (Respiratory Care Centre, Hameenlinna, Finland) (14). The bronchial challenges were performed from 0830 to 1100 hours. The subjects were instructed not to take any bronchodilatory drug 8 h prior to a test. Increasing doses of methacholine chloride (National Corporation of Swedish Pharmacies) in a 0.9% saline were given until a decrease in FEV₁ of 20% or the highest concentration was reached. Methacholine was inhaled during five breaths and lung function measurements followed in the next 3 min. Baseline values were obtained after an inhalation of saline. The methacholine was given in 10 successively increasing doses ranging from 0.0625 mg ml⁻¹ to 32 mg ml⁻¹. Patients with a provocative concentration (PC₂₀) of methacholine of ≤ 32 mg ml⁻¹ and a decrease in FEV₁ of 20% or more were diagnosed as having a positive test.

DOSE-RESPONSE SLOPE

A least-squares slope for methacholine was calculated from the regression equation for the percentage decline in FEV₁ on a cumulative dose of methacholine using all the measured points (15).

PEAK FLOW AND SYMPTOM DIARIES

Peak flow and symptom diaries were kept during a 17-day period, starting on the day of inclusion in the study. The peak expiratory flow rate (best of three measurements) was recorded four times daily with a Mini-Wright peak flow meter (Clement Clarke, London, U.K.). Daily peak flow variability was calculated using the formula: highest PEF — lowest PEF × 100/mean PEF value of the day. On awakening, the subjects filled in the symptom diary and stated whether or not they had had: breathing difficulties the previous night, wheezing in the chest, attacks of breathlessness or attacks of coughing during the previous 24 h. A symptom score (0–4) was calculated for each individual using the number of respiratory symptoms during the 17-day period.

Statistics

Comparisons between two groups were performed using the Mann–Whitney U-test. In correlations within a group, the Spearman Rank correlation test was used. ANOVA for repeated measurements was used to analyse whether there were any significant individual differences in the amount of exhaled NO between the three measurements. All the results are given as the mean ± sd. A P-value of < 0.05 was regarded as statistically significant.

Results

LUNG FUNCTION, PEAK EXPIRATORY FLOW AND SYMPTOM SCORE

There was no significant difference in lung function measurements, peak expiratory flow or symptom score between atopic and non-atopic subjects with asthma (Table 1).

No correlation was found between the amount of exhaled NO and lung function values, peak expiratory flow or symptom score in the two asthma groups or the controls.

EXHALED NO IN PATIENTS WITH ASTHMA AND CONTROLS

Atopic patients with asthma had a significantly higher mean amount of exhaled NO than non-atopic subjects with asthma (162 ± 68 vs. 113 ± 55 nl min⁻¹; P = 0.03) and the control group (88 ± 52 nl min⁻¹; P = 0.004) (Fig. 1). No
significant difference was found in the amount of exhaled NO between non-atopic patients with asthma and the control group. There was no significant difference within the subjects between the three different NO measurements. The mean range of the NO measurements within subjects was $43 \pm 31$ nl min$^{-1}$.

No significant correlation was found between the daily dose of inhaled steroids and levels of exhaled NO in either group of subjects with asthma.

EXHALED NO AND AIRWAY HYPERRESPONSIVENESS

Fifteen (75%) of the 20 atopic subjects with asthma, 12 (60%) of the non-atopic subjects with asthma and two (15%) of the controls had a positive methacholine challenge (a fall in FEV$_1$ by 20% of the predicted value or more). There was no significant difference in dose response slope (DRS) for methacholine between atopic and non-atopic subjects with asthma.

In atopic subjects with asthma the mean exhaled NO was significantly correlated to the dose-response slope for methacholine ($r = -0.52; P=0.02$), while no such correlation was found in the non-atopic group.

Discussion

We found that atopic subjects with asthma had higher exhaled NO than non-atopic subjects with asthma, while no difference was found between the two groups in terms of lung function, peak expiratory flow and symptom score. Non-atopic subjects with asthma in this study had exhaled NO levels which were not significantly different from those of the controls. Furthermore, in atopic subjects with asthma, exhaled NO was significantly correlated to the dose-response slope for methacholine, while no such correlation was found in the other two groups.

To our knowledge, this is the first study to demonstrate differences in the amount of exhaled NO in an atopic and non-atopic adult population with asthma.

Our results correspond to those recently reported by Frank et al., showing higher exhaled NO levels in atopic asthmatic children than non-atopic asthmatic children, regardless of whether or not they were receiving inhaled corticosteroids (16). Atopy therefore appears to be an important determinant of the amount of exhaled NO in asthma. In a study of healthy children, exhaled nitric oxide increased in children with a positive skin-prick test and increased as the number of positive skin-prick tests increased (17). In infants, the levels of exhaled nitric oxide are higher in those with a family history of asthma (18). Even atopic, non-asthmatic adults with seasonal rhinitis have displayed increased levels of both nasally and orally measured nitric oxide (19), indicating that atopic status independent of the asthma diagnosis may be an important determinant of increased nitric oxide production in the airways.

How can atopic status determine nitric oxide production in patients with asthma? Exhaled NO increases during the late response to allergen (4), suggesting that allergen induces an increase in iNOS expression in the airways. The mechanism for the late response is not completely understood, but it involves the release of several inflammatory mediators and cytokines. IL-1$\beta$, TNF-$\alpha$ and IFN-$\gamma$ can all increase iNOS expression and NO formation in airway epithelial cells (7, 8). Human mast cells are among the most active cells when it comes to generating pleiotropic cytokines including TNF-$\alpha$ (20), which might explain the increase in NO in atopic subjects with asthma. Furthermore, the mast cells of atopic subjects are in an activated state and may therefore be capable of releasing mediators more easily than the mast cells of non-atopic subjects.

However, it should be recognized that the majority of both atopic and non-atopic subjects with asthma were receiving treatment with inhaled corticosteroids, which can affect exhaled nitric oxide levels (21) and further studies of asthmatics who have not yet started inhaled steroid therapy are needed. This study was designed and performed before the guidelines for NO were published (22). A disadvantage with our method is the risk of mixing of NO from the nose. To limit this problem our measurements were made during exhalation with a nose clip. In this way, nasal NO will give little contribution to the exhaled gas concentration. This is supported by the fact that the NO values in our control group were of the same level as the NO values of the healthy subjects in the study reported by Högmå et al. (23).

Our data appear to be consistent with the findings of Jatakanon et al., who found a correlation between exhaled NO and airway hyperresponsiveness to methacholine (24). The correlation between exhaled NO and the dose-response slope for methacholine in atopic subjects with asthma in this study suggests that elevated NO levels or the mechanism leading to its increase may contribute to airway hyperresponsiveness.

In conclusion, in this study, atopic subjects with asthma had higher levels of exhaled NO than non-atopic subjects. Atopic status should be taken into account when measuring levels of exhaled NO in subjects with asthma.

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