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Effect of Atmospheric Nitric Oxide (NO) on Measurements of Exhaled NO in Asthmatic Children

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Summary. The measurement of exhaled nitric oxide concentrations [NO] may provide a simple, noninvasive means for measuring airway inflammation. However, several measurement conditions may influence exhaled NO levels, and ambient NO may be one of these. We measured exhaled NO levels in 47 stable asthmatic children age 5 to 17 years and in 47 healthy children, gender and age matched. Exhaled [NO] in expired air was measured by a tidal breathing method with a chemiluminescence analyzer, sampling at the expiratory side of the mouthpiece. NO steady-state levels were recorded. In order to keep the soft palate closed and avoid nasal contamination, the breathing circuit had a restrictor providing an expiratory pressure of 3–4 cm H_2O at the mouthpiece. To evaluate the effect of [NO] in ambient air, measurements were randomly performed by breathing ambient air or NO-free air from a closed circuit.

Breathing NO-free air, exhaled [NO] in asthmatics (mean ± SEM) was 23.7 ± 1.4 ppb, significantly higher ($P < 0.001$) than in healthy controls (8.7 ± 0.4 ppb). Exhaled NO concentrations measured during ambient air breathing were higher (49 ± 4.6 ppb, $P < 0.001$) than when breathing NO-free air (23.7 ± 1.4 ppb) and were significantly correlated (r = 0.89, $P < 0.001$) with atmospheric concentrations of NO (range 3–430 ppb). These findings show that 1) exhaled [NO] values of asthmatic children are significantly higher than in healthy controls, and 2) atmospheric NO levels critically influence the measurement of exhaled [NO]. Therefore, using a tidal breathing method the inhalation of NO-free air during the test is recommended. **Pediatr Pulmonol. 1998; 26:30–34.**

Key words: nitric oxide; asthma; ambient nitric oxide; inflammatory marker; normal children.

INTRODUCTION

Nitric oxide (NO) plays a role in many aspects of normal pulmonary function, and is involved in airway inflammatory responses of several airway diseases.[1,2] The finding of increased concentrations of exhaled NO in asthmatics[3] has led to the suggestion that the measurement of exhaled NO may provide a simple noninvasive means of measuring airway inflammation and of monitoring whether treatment is effective.[2,4,5] NO has been detected in exhaled air by a number of investigators in humans but the amounts reported differ considerably.[6] Conditions of measurement critically affect the NO levels obtained,[6] and among these are fluctuations in ambient NO concentrations.[7] In urban areas, atmospheric environmental NO may rapidly change over a few hours within a day, probably in relation to traffic, and during the winter it can often exceed 100–200 ppb. The possible influence of ambient NO was considered and has been debated since the first discovery of NO in exhaled air by Gustaffson et al.[8] Other reports have excluded its influence.[4,9,10] The reasons that may explain the apparent disappearance of ambient inhaled NO in the lung are not clear. One of the major factors contributing to the partial pressure of NO in the lungs is ventilatory exchange between NO in the lower airways and NO in room air.[11] A recent paper[7] indicated that exhaled NO concentrations correlate significantly with the inhaled ambient concentrations of NO. It is, therefore, important that measurements of exhaled NO are made under standardized conditions, considering all factors that could influence this measurement.

The principal aim of this study was to define the contribution of natural background NO on exhaled NO measurements in children, using a tidal breathing technique.

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We also compared NO values of asthmatics to those of healthy controls in order to confirm previous reports[5,12,13] in which asthmatic children presented with elevated exhaled NO concentration.

MATERIALS AND METHODS

Subjects

Asthmatic Patients

Forty-seven children with stable asthma (12 females, 35 males, age 5 to 17 years, mean 10.2 ± 0.3 years) attending the Pediatric Pulmonology/Allergy Out-Patient Clinic of the Children's Hospital of Padova were included in the study. The diagnosis of asthma was based on clinical history and examination, pulmonary function parameters, and pulmonary function response to inhaled beta-adrenergic agents, according to international guidelines.[14] All children had a history of mild to moderate asthma for at least 2 years. Thirty-seven children were under continuous treatment: 29 subjects with inhaled steroids at low doses (200–400 mcg/day of beclomethasone dipropionate, or equivalent doses of fluticasone or budesonide), four with cromones, and four with oral theophylline. Ten patients were not under continuous therapy. All patients used an inhaled bronchodilator on demand. At the time of the study, the disease was well controlled in all children. The presence of allergy was demonstrated in 45 out of 47 children by positive skin-prick tests (dust allergy was present in 71%, grass pollen allergy in 54%, and alternaria allergy in 21%).

Healthy Controls

Forty-seven healthy children, age and gender matched with asthmatics (12 females, 35 males, age 6 to 15 years, mean 10 ± 0.4 years) were recruited in two public schools of Padova. Their parents were asked to complete a health questionnaire regarding respiratory or allergic diseases. Healthy controls had no history of airway disease, allergy, or cardiac disease and were not taking any medication. None of the subjects of the study (asthmatics and controls) had any history or signs of respiratory tract infection in the 3 weeks before the study.

Protocol

After a screening visit, each child performed a spirometry, and a NO measurement was taken. In order to evaluate whether ambient NO may influence values of exhaled NO, two separate measurements of exhaled NO were made in all asthmatic patients: children were randomized to start breathing either ambient air or NO-free air. Measurements were performed sequentially on each subject on the same day. Ambient NO levels were always recorded before the test. Comparisons of exhaled NO concentrations between asthmatics and controls were performed with the subjects breathing NO-free air. The protocol was approved by our Institutional Review Board, and informed consent was obtained from both the parents and the children.

Abbreviations

FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
iNOS	Inducible NO synthase
NO	Nitric oxide
ppb	Parts per billion

Determination of Exhaled NO Levels

Exhaled NO in expired air was measured by a tidal breathing method (Fig. 1) using a chemiluminescence analyzer (CLD 700 Al-Med, Ecophysics, Switzerland) and modifying the experimental set-up proposed by Alving et al.[3] according to the recommendations of the European Respiratory Society[6] for measurement in children. The analyzer is sensitive to NO concentrations between 1 and 1,000 ppb. All measurements were performed in the morning between 9 AM and 1 PM. Children were seated wearing a nose clip and were asked to breathe at tidal volume through a mouthpiece and a two-way valve to avoid rebreathing. The mouthpiece was connected by a Teflon tube (5 mm internal diameter) to the analyzer. Children were randomized to inhale ambient air or NO-free air. To exclude the effect of NO in ambient air, the children breathed NO-free air obtained by passing ambient air through a purafill converter. NO-free air was stored in a 14 L collapsible reservoir maintained inflated to approximately 70–80%. NO levels were analyzed continuously by the chemiluminescence analyzer, sampling at a constant flow of 0.7 L/min. [NO] reached a steady-state level during mouth breathing after 1–2 min without significant further fluctuations. NO levels were recorded between the third and the sixth minute of mouth breathing. The subjects were asked to breathe without speaking or swallowing. The exhaled air that was not withdrawn by sampling was scavenged through a tube with a one-way valve to prevent contamination with ambient air. In order to keep the soft palate closed,[6] the breathing circuit had an internal restrictor that created a low resistance and provided a positive pressure of 3–4 cm H_2O at the mouthpiece. Before entering the analyzer, exhaled air was passed through a water vapor trap. The accuracy of our method of measuring exhaled NO has been previously reported in 22 healthy children.[15] The chemiluminescence analyzer operates over a wide range of ambient temperatures (5–33°C) and humidity (0–95%) without an adverse effect on measurement accuracy. Before each study, the chemiluminescence analyzer was calibrated with a certified calibration mixture (300 ppb)

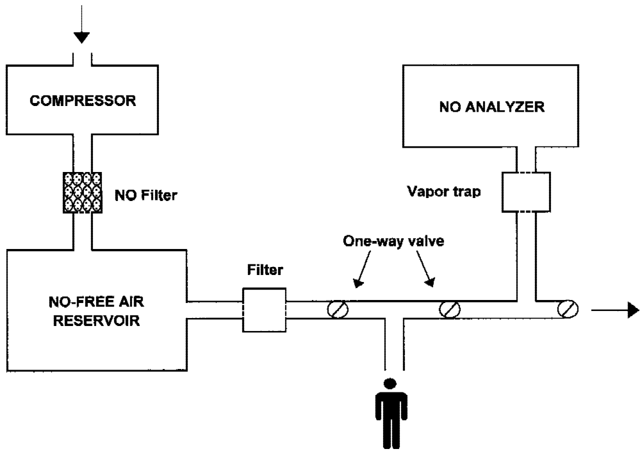

Fig. 1. Schematic illustration of the experimental set-up to measure exhaled NO.

of NO in nitrogen (SIAD, Bergamo, Italy) with guaranteed stability. The mixtures are prepared by a gravimetric method using NO which has a purity of >99.9% certified both by wet analysis and gaschromatographic analysis. The mixtures are contained in aluminum cylinders whose internal surfaces are treated to reduce porosity and improve stability. Total NOx within the cylinder did not exceed by more than 10 ppb the value of NO itself.

Spirometry

Spirometric parameters were measured with a 10 L bell spirometer (Biomedin, Padova, Italy), and the best of three maneuvers was recorded as a percentage (%) of

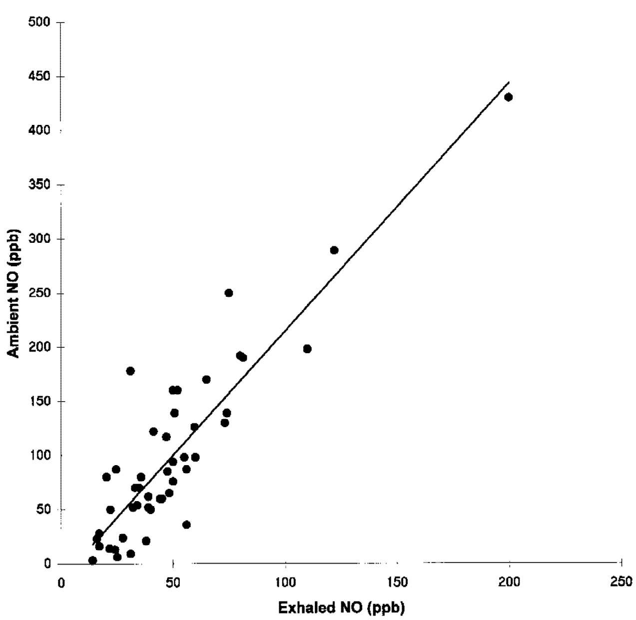

Fig. 2. Relationship between ambient NO and orally exhaled NO values in 47 asthmatic children (r = 0.89, *P* < 0.001).

predicted reference values.[16] The spirometer was regularly calibrated with a certified volume syringe.

Statistical Analysis

All exhaled NO concentrations are reported in ppb. The results are shown as mean ± SEM. For comparison of NO measurements between children with asthma and healthy children, a Mann-Whitney rank sum tests was used. The relation between exhaled NO concentrations breathing ambient air or NO-free air was evaluated by the linear correlation coefficient. A *P*-value of less than 0.05 was considered significant.

RESULTS

One hundred and forty-one measurements of exhaled NO were performed between September 1996 and April 1997. All children completed the protocol easily. Inhaling NO-free air before the measurement, the mean value of orally mixed exhaled NO in 47 asthmatic children was 23.7 ± 1.4 ppb (range 8–60 ppb); exhaled NO concentrations were significantly higher compared to healthy controls (8.7 ± 0.4 ppb, range 3.5–15 ppb) ($P < 0.001$). Breathing ambient air with different concentrations of atmospheric NO, the mean value of exhaled NO in the same asthmatic children was 49 ± 4.6 ppb (range 14–200 ppb), which was significantly higher in comparison to the value found breathing NO-free air (23.7 ± 1.4 ppb, $P < 0.001$). The ambient background level of NO measured before the tests during the study period ranged from 3–430 ppb. We observed a significant correlation (r = 0.89, $P < 0.001$) between exhaled NO values and inhaled ambient concentrations of NO. Figure 2 shows the relationship between inhaled ambient and exhaled NO values in 47 asthmatic children. This demonstrates that ambient [NO] has an important influence on the measurements of orally exhaled NO.

Spirometric measurements in asthmatic children were in the normal range (mean FEV_1 92.3 ± 1.4% pred, FVC 100 ± 1.3% pred). Control subjects also had normal lung function values (mean FEV_1 92.6 ± 2% pred, FVC 93.3 ± 1.6% pred). There was no significant correlation (*P* = ns) between exhaled NO and FEV_1 or FVC in asthmatic or control children.

DISCUSSION

The major finding in this study was that natural background NO affects the measurement of orally exhaled NO concentrations. Based on this observation we recommend that the inhalation of NO-free air is of utmost importance in standardizing measurements of exhaled NO when a tidal breathing method is used. In northeast Italy, ambient NO levels present wide fluctuations during the year, varying from 2–3 ppb during the summer to up to 300 ppb during the winter. Similar NO atmospheric values have been reported in Finland[6] and probably are present in many industrialized urban areas. In order to evaluate whether ambient NO may influence values of exhaled NO concentrations, we performed sequentially two separate measurements of exhaled NO levels, breathing either ambient air or NO-free air. Orally mixed exhaled NO concentrations were higher when breathing ambient air and significantly correlated with the inhaled ambient concentrations of NO, as shown in Figure 2. This observation proves that when breathing ambient air, measured exhaled NO levels are dependent on the atmospheric concentration of NO in inspired air. Our findings are in accordance with recent data of Dotsch et al. [7] who, in a large number of subjects, found a clear impact of ambient NO on orally exhaled NO concentrations. This stresses the impact of measurement conditions on the results and confirms the need to perform measurements in a carefully standardized manner. Hyde et al.[11] developed equations to calculate NO production by the lower airways, taking into account the transport of NO into the perfusing blood as well as into the expired air. These equations are valid when NO concentrations in room air are negligible or the subject inhales NO-free air, but they require a more complex solution when the partial pressure of NO in the inspired air (i.e., NO in ambient air) is significantly above zero. The threshold above which measurements of exhaled NO may be affected by environmental NO is not known at this time. From several studies in healthy controls it can be deduced that values of 7–8 ppb represent the normal concentrations of exhaled NO in humans.[6,10,17,18] It is, therefore, likely that ambient NO concentrations less than 8–10 ppb may not significantly influence exhaled NO, but this still needs to be demonstrated.

Previous reports have not shown a significant relationship between ambient NO levels and exhaled NO levels, nor did they consider the importance of ambient NO levels.[9,10,19] Using the method of a single slow exhalation, inhalation of a single breath of NO calibration gas (100–1,000 ppb) did not affect the exhaled NO in a small group of normal subjects.[4,9,20] Because NO is a highly reactive gas, a rapid absorption from the lung and binding of inhaled NO to hemoglobin could be one explanation of its rapid disappearance after a single high dose inhalation. However, this experimental exposure to NO does not reflect the condition in which moderately high concentrations of atmospheric NO are inhaled for several hours. In this latter condition, it is indeed possible that the capability of the lung to absorb atmospheric inhaled NO is limited. Recently, Byrnes et al.[21] using a single slow exhalation method in adults, demonstrated a clear influence of ambient background [NO] on exhaled NO concentration, and they concluded that a high inspired level of NO makes the exhaled NO concentrations difficult to interpret.

The second finding of the present study was that, consistent with previous observations,[3,5,7,12] exhaled NO levels in asthmatic children were significantly higher than those of healthy age and gender matched controls. As previously reported,[7,9,15] we found no relationship between the degree of airway obstruction, as measured by percentage of predicted FEV_1, and the levels of exhaled NO, however, the asthmatic children included in this study had normal values of FEV_1 and FVC.

In 1993, Alving et al.[3] reported increased NO levels in exhaled air of asthmatics. Current understanding suggests that an upregulated expression of iNOS in bronchial epithelium of asthmatic patients[22] is the most likely explanation of this phenomenon. Glucocorticoids are known to inhibit the expression of iNOS and this has been confirmed by studies in which both systemic[10,12,15] and inhaled steroid treatment[23] can significantly reduce exhaled NO concentrations in a few days. In asthmatic children treated with different doses of inhaled steroids a dose dependency of exhaled NO has been reported, and only those treated with the highest doses presented [NO] values similar to those of healthy controls.[5] Patients in our study were treated with low doses of inhaled steroids and this may explain why they still presented increased values of NO with respect to controls.

In conclusion, our study provides evidence that atmospheric NO may critically affect measured exhaled NO concentrations, and we suggest the inhalation of NO-free air during the tidal breathing test. In addition, we have confirmed previous observations that exhaled NO levels are significantly higher in children with asthma than in healthy controls.

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