Nitric Oxide Airway Diffusing Capacity and Mucosal Concentration in Asthmatic Schoolchildren

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ABSTRACT

Asthmatic patients show increased concentrations of nitric oxide (NO) in exhaled air (F_{NO}). The diffusing capacity of NO in the airways (D_{awNO}), the NO concentrations in the alveoli and the airway wall, and the maximal airway NO diffusion rate have previously been estimated noninvasively by measuring F_{NO} at different exhalation flow rates in adults. We investigated these variables in 15 asthmatic schoolchildren (8–18 y) and 15 age-matched control subjects, with focus on their relation to exhaled NO at the recommended exhalation flow rate of 0.05 L/s ($F_{NO,0.05}$), age, and volume of the respiratory anatomic dead space. NO was measured on-line by chemiluminescence according to the European Respiratory Society's guidelines, and the NO plateau values at three different exhalation flow rates (11, 99, and 382 mL/s) were incorporated in a two-compartment model for NO diffusion. The NO concentration in the airway wall ($p < 0.001$), D_{awNO} ($p < 0.01$), and the maximal airway NO diffusion rate ($p < 0.001$) were all higher in the asthmatic children than in control children. In contrast, there was no difference in the NO concentration in the alveoli ($p = 0.13$) between the groups. A positive correlation was seen between the volume of the respiratory anatomic dead space and $F_{NO,0.05}$ ($r = 0.68, p < 0.01$), the maximal airway NO diffusion rate ($r = 0.71, p < 0.01$), and D_{awNO} ($r = 0.56, p < 0.01$) in control children, but not in asthmatic children. $F_{NO,0.05}$ correlated better with D_{awNO} in asthmatic children ($r = 0.65, p < 0.01$) and with the NO concentration in the airway wall in control subjects ($r < 0.77, p < 0.001$) than *vice versa*. We conclude that $F_{NO,0.05}$ increases with increasing volume of the respiratory anatomic dead space in

healthy children, suggesting that normal values for $F_{NO,0.05}$ should be related to age or body weight in this age group. Furthermore, the elevated $F_{NO,0.05}$ seen in asthmatic children is related to an increase in both D_{awNO} and NO concentration in the airway wall. Because D_{awNO} correlates with the volume of the respiratory anatomic dead space in control subjects and $F_{NO,0.05}$ correlates with D_{awNO} in asthmatic children, we suggest that D_{awNO} partly reflects the total NO-producing surface area and that a larger part of the bronchial tree produces NO in asthmatic children than in control children. (*Pediatr Res* 54: 496–501, 2003)

Abbreviations

NO, nitric oxide
 F_{NO} , fraction of NO in exhaled air, plateau value
 $F_{NO,0.01, 0.05, 0.1, \text{ and } 0.5}$, F_{NO} at the targeted expiratory flow rate of 0.01, 0.05, 0.1, and 0.5 L/s
 $\dot{V}_{NO}$, NO diffusion rate, calculated as the F_{NO} multiplied by the expiratory flow rate
 $\dot{V}_{NO,0.01, 0.1, \text{ and } 0.5}$, $\dot{V}_{NO}$ at the targeted expiratory flow rate of 0.01, 0.1, and 0.5 L/s
 $\dot{V}_{NO,max}$, maximal NO diffusion rate
 F_{ANO} , alveolar NO concentration/fraction
 F_{awNO} , airway wall NO concentration/fraction
 D_{awNO} , diffusing capacity of NO of the airway wall
 V_D , volume of respiratory anatomic dead space
 $\dot{Q}$, exhaled flow rate
iNOS, inducible nitric oxide synthase

NO can be measured in exhaled air, and high levels of NO are believed to reflect allergic inflammation in the airways of

asthmatic patients (1–3). Correlations between exhaled NO and other markers of airway inflammation such as eosinophils in sputum and blood (4–6), bronchial hyperresponsiveness (7), and bronchial eosinophilia (5, 8) have been demonstrated. In the lower airways, NO is thought to be produced by iNOS in

Received July 8, 2002; accepted December 3, 2002.

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Supported by the Swedish Heart Lung Foundation, Swedish Asthma and Allergy Association's Research Foundation, Samariten Foundation, Swedish Foundation for Health Care Science and Allergy Research, Consul ThC Bergh Foundation, and the

Swedish Research Council-Medicine (Project No. 10354).

DOI: 10.1203/01.PDR.0000081761.33822.36

the epithelial cells (9). The mechanisms that regulate iNOS induction are not fully understood, but there is evidence for both transcriptional and posttranslational regulation of NO synthesis in human epithelial cells (10). Because NO is produced in the conducting airways, the concentration of NO in exhaled gas will be flow-dependent (11). Therefore, a targeted exhalation flow rate of 50 mL/s has been suggested by the American Thoracic Society (12) to make data comparable. The NO plateau value is usually presented as the F_{ENO} in parts per billion.

However, a few investigators have put a lot of effort into measuring exhaled NO at multiple exhalation flow rates, to gain more information on NO formation in the lower respiratory tract (13–17). Some of these investigators have proposed models for NO diffusion in which alveolar and airway mucosal NO concentrations can be estimated. The general concept of these models is basically the same, and the equations do not differ extensively. The airways are approximated to a two-compartment model: the alveolar region and the conducting airways. NO is primarily produced within the airway wall, diffusing in a concentration-dependent manner into the airway lumen during exhalation. If F_{ENO} is determined at several suitable exhalation flow rates, effective F_{AWNO} , F_{DawNO} , F_{ANO} , and $\dot{V}_{\text{ENOmax}}$ can be estimated (13).

The aim of our study was to estimate these variables in a group of schoolchildren with and without asthma. We used a method of approximate linear regression analysis with a computerized iteration process recently described by Högman *et al.* (17). This method has been shown to be numerically robust and allows estimation of the above-mentioned variables with only three different exhalation flow rates. Furthermore, to learn more about NO diffusion in human airways, we also wanted to study the relation of these variables to $F_{\text{ENO}0.05}$ and subject characteristics such as age and V_D .

METHODS

F_{ENO} and exhalation flow rate data from 15 asthmatic schoolchildren (age 8–18 y; mean, 14 y) with elevated F_{ENO} levels and 15 age-matched control children were used in the model.

The children were randomly recruited from the Allergy Clinic at Astrid Lindgren Children's Hospital, Karolinska Hospital.

Thirteen of these 15 asthmatic children had atopic asthma and were sensitized to furry pets, pollen, or house dust mite. Atopy was evaluated by skin-prick testing. Five children were steroid-naïve. Median dose of inhaled steroids was 100 $\mu\text{g/d}$ in steroid-treated patients. A brief structured interview regarding symptoms was performed on the day of examination. Fifteen nonatopic, nonasthmatic children (age 8–18 y; mean, 12 y), six girls and nine boys, without history of atopy or heredity for atopic disease served as control subjects. They were selected regardless of NO levels. Subjects with either a recent history of respiratory tract infection (7 d prior to the study) or signs of respiratory tract infection on the day of examination were not accepted in the study. Pubertal girls were not examined during menstruation. All children were nonsmokers. All subjects completed the study. Medication and other subject characteristics are described in Table 1. V_D was calculated as the body weight (in kilograms) multiplied by 2 mL (18).

NO measurements. Measurements were performed in accordance with international recommendations for on-line NO measurements previously described in the European Respiratory Society Task Force Report (19). The data used in the study were, however, originally obtained to study reproducibility of F_{ENO} at different flow rates (20). Therefore, individual variations in exhalation flow rate and F_{ENO} levels were accepted and registered, as long as the flow rate was kept constant and an F_{ENO} plateau was achieved during the exhalation. We used the Aerocrine NO system (Aerocrine AB, Stockholm, Sweden), including the CLD 77 AM chemiluminescence analyzer from Eco Physics AG (Dürnten, Switzerland) for on-line NO measurements and monitoring of pressure. The sensitivity of the analyzer is 0.1 ppb, the rise time from 0 to 90% is 0.1 s, the sampling flow rate 110 mL/min, and the machine lag time from the mouthpiece 0.7 s. The children were comfortably seated, inhaled NO-free air from a reservoir, and subsequently exhaled against different linear resistors (Hans Rudolph Inc, Kansas City, MO, U.S.A.). The target oral pressure at the different flow rates was 10 cm H_2O . As support in this maneuver, the children

Table 1. Subject characteristics

Asthmatics						Controls		
Age (y)	Sex	V_D (mL)	Atopy	Medication	Inhaled steroids ($\mu\text{g/d}$)	Age	Sex	V_D (mL)
17.2	M	200	yes	No medication	0	15.9	M	132
18.3	M	118	yes	No medication	0	10.8	M	56
11.0	M	72	yes	Budesonide	100	18.7	M	128
14.1	M	78	yes	Budesonide	100	16.5	M	134
15.8	M	106	yes	Fluticasone propionate	250	10.2	M	64
13.1	M	124	yes	Budesonide	400	8.1	M	48
15.0	M	168	no	Fluticasone propionate	400	13.8	M	138
13.3	M	126	yes	Fluticasone propionate	500	8.2	F	62
13.0	M	100	yes	Fluticasone propionate (and salmeterol 100)	500	11.0	M	86
13.1	F	96	yes	Fluticasone propionate (and salmeterol 100)	100	16.1	M	126
11.9	F	90	yes	Chromoglycate	0	13.1	F	96
17.5	M	148	yes	Fluticasone propionate (and montelukast 10 mg)	500	9.1	F	58
10.1	F	112	yes	No medication	0	11.1	F	96
15.8	M	116	yes	Budesonide	200	10.7	F	76
8.1	M	64	yes	Chromoglycate	0	11.3	F	96

were given a target pressure between two indicator lines at 8 and 12 cm H₂O. Resistors were changed between exhalations. We used the following resistors: 20, 100, 200, and 1000 cm H₂O · mL · s to get target flow rates of 500, 100, 50, and 10 mL/s.

NO and flow calculations. Mean F_{ENO} was calculated during an F_{ENO} plateau of 3 s. A plateau was approved when F_{ENO} did not vary more than 10% or ± 1 ppb.

Flow was calculated as the ratio between pressure and resistance and was corrected for analyzer sample flow rate. Values from three consecutive exhalations at each targeted flow rate were registered and fitted into the model.

Extended NO analysis. The model used in this study has been described in detail elsewhere (17). Briefly, it is based on the relationship between F_{ENO} and the NO formation in the two compartments: the alveolar region and the conducting airway tube. Four variables are estimated in the model: Faw_{NO}, Fano, Daw_{NO}, and $\dot{V}_{\text{ENO}_{\text{max}}}$. The mathematical elaboration based on standard physics of diffusion gives the basic equation for F_{ENO} as a function of $\dot{Q}$: Equation, msp 8

$$F_{\text{ENO}} = F_{\text{awNO}} - (F_{\text{awNO}} - F_{\text{ano}})e^{-D_{\text{awNO}}/\dot{Q}}$$

For three unknown variables at least three data points are required. As suggested by Tsoukias and George (13), Fano and $\dot{V}_{\text{ENO}_{\text{max}}}$ are calculated from the almost linear slope between, for example, $\dot{V}_{\text{ENO}_{0.5}}$ and $\dot{V}_{\text{ENO}_{0.1}}$. Then Daw_{NO} is solved by finding the intercept of the $\dot{V}_{\text{ENO}}$ axis and the line used for defining the slope, and thereafter a computer-powered iterative algorithmic process is used to solve Daw_{NO}. In a final step the accuracy is improved further still by using a second-order iteration to correct for the minimal alinearity in the Fano slope (17). Once Fano and Daw_{NO} have been estimated, Faw_{NO} can be calculated. This approach is a robust alternative to the sometimes more problematic least-square fit method in which more data points are needed at more extreme exhalation flow rates (<10 and >400 mL/s) in the F_{ENO}-flow plot curve (16). Because the reproducibility of the F_{ENO} data are known to be decreased at more extreme flows (20), it can severely affect the curve fit quality.

Statistical procedures. We used Prism 3 software (Graph Pad Software Inc, San Diego, CA, U.S.A.) for statistical analyses. Nonparametric tests were applied as the distribution of these variables is not known and our material was too small for normal distribution analysis. Data are expressed as arithmetic mean $\pm$ SEM unless otherwise stated. To study correlations we used the Spearman rank correlation test. A *p* value of less than 0.05 was considered significant.

Ethics. The study was approved by the regional ethics committee at the Karolinska Institute, and the children and their parents gave their informed consent before inclusion.

RESULTS

General results. All subjects completed the study. The F_{ENO} values were highly flow-dependent, and the difference between groups was significant at all flow rates (*p* < 0.001, all).

Daw_{NO}, Faw_{NO}, Fano, and $\dot{V}_{\text{ENO}_{\text{max}}}$ Asthmatic children showed significantly higher levels for all these variables except

for Fano (Daw_{NO}, 26.3 ± 3.2 versus 13.2 ± 1.7 nL · s⁻¹ · ppb⁻¹ × 10⁻³; Faw_{NO}, 154.2 ± 23.3 versus 54.6 ± 8.4 ppb; Fano, 1.5 ± 0.2 versus 1.2 ± 0.1 ppb; $\dot{V}_{\text{ENO}_{\text{max}}}$, 3.5 ± 0.4 versus 0.7 ± 0.1 nL/s; asthmatic and control children, respectively). The scatter diagrams for these variables and F_{ENO_{0.05}} are shown in Figures 1 and 2. Even though there was no overlap in F_{ENO_{0.05}} between the groups, Faw_{NO} and Daw_{NO} overlapped considerably. However, the combination of both a high Faw_{NO} and a high Daw_{NO} was not seen in the control group. We could not find any difference in F_{ENO_{0.05}} values or in the estimated variables between steroid-treated and steroid-naïve asthmatic children (not shown).

Correlations. A summary of all the correlations is shown in Table 2. We found a strong correlation between F_{ENO_{0.05}} and $\dot{V}_{\text{ENO}_{\text{max}}}$ in both groups. However, when Faw_{NO} and Daw_{NO} were studied separately, F_{ENO_{0.05}} correlated better with Daw_{NO} in asthmatic children and with Faw_{NO} in control children. Fano showed no correlation at all with F_{ENO_{0.05}}, the estimated variables, or V_D and age (not shown). A positive correlation between V_D and F_{ENO_{0.05}}, $\dot{V}_{\text{ENO}_{\text{max}}}$, and Daw_{NO}, was seen in the control group but not in the asthma group. Even though V_D and

Figure 1. Levels of F_{ENO_{0.05}} (left) and the estimated level of $\dot{V}_{\text{ENO}_{\text{max}}}$ (right) in 15 asthmatic schoolchildren and 15 age-matched control subjects. ****p* < 0.001 (Mann-Whitney *U* test). The bars indicate median values.

Figure 2. Estimated levels of FawNO (left) and Fano (center) and the DawNO (right) in 15 asthmatic schoolchildren and 15 age-matched control subjects. ** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney U test); ns, not significant. The bars indicate median values.

FawNO did not seem to be related to each other in either group, a significant correlation between FawNO and age could be demonstrated in control children.

To validate the model and the mathematical solutions, we checked for a correlation between expected (see "Methods,"

Figure 3. The correlation between expected and measured $F_{ENO_{0.05}}$ ($n = 89$ exhalations in 30 asthmatic and healthy schoolchildren). The expected values were calculated by using the estimated variables and measured flow at the targeted exhalation flow rate of 50 mL/s in the equation described in "Methods" (extended NO analysis). *** $p < 0.001$ (Spearman rank).

extended NO analysis) and measured F_{ENO} at the targeted exhalation flow rate of 0.05 L/s. Even though these measured values had not been used to solve the estimated variables, the correlation was highly significant ($r = 0.99$, $p < 0.001$).

DISCUSSION

A few previous studies in adults have demonstrated that FawNO, F_{ANO} , DawNO, and $\dot{V}_{ENO_{max}}$ can be estimated noninvasively by measuring NO in exhaled air at multiple exhalation flow rates (13–15, 17, 21). The physiologic model for these calculations is basically the same even though the mathematical solutions can vary among different research groups. The aim of our study was to investigate whether such variables could be estimated in schoolchildren and how asthmatic children differ from healthy control children. We also studied the influence of age and airway size on these variables and their relation to $F_{ENO_{0.05}}$.

The asthmatic children showed higher values in all estimated variables except Fano. This is in good agreement with previous findings in adults (14, 17). An increase in FawNO in asthmatic subjects seems logical inasmuch as iNOS is induced in the bronchial epithelium in asthma (9). However, even though the groups were completely separate with respect to $F_{ENO_{0.05}}$, both FawNO and DawNO overlapped considerably. The discriminatory power of these two variables therefore seems to be lower than that of $F_{ENO_{0.05}}$. Interestingly, Lehtimäki *et al.* (22) found increased Fano in patients with allergic alveolitis, using a similar model for NO diffusion. This shows that NO measurements at multiple flow rates can be of interest in other airway diseases as well because it seems possible to distinguish between alveolar and bronchial inflammation. Their data also validate the NO diffusion model.

The increase in DawNO, seen in asthmatic children, has been more of a challenge to explain. Jörres (15) speculated about influences of NO biochemistry, the total surface area excreting NO, and the amount and quality of mucosal secretions. How-

Table 2. Correlations between variables under study

	Asthmatics			Controls		
	<i>r</i> Value	95% CI	<i>p</i> value	<i>r</i> value	95% CI	<i>p</i> value
FENO _{0.05} vs $\dot{V}_{\text{ENO}_{\text{max}}}$	0.95	0.85 to 0.98	<0.001	0.98	0.93 to 0.99	<0.001
FENO _{0.05} vs C _{aw} NO	0.47	-0.08 to 0.80	0.08	0.77	0.41 to 0.92	<0.001
FENO _{0.05} vs D _{aw} NO	0.65	0.18 to 0.87	<0.01	0.48	-0.05 to 0.80	0.07
V _D vs FENO _{0.05}	0.05	-0.48 to 0.56	0.85	0.68	0.24 to 0.89	<0.01
V _D vs $\dot{V}_{\text{ENO}_{\text{max}}}$	-0.004	-0.53 to 0.52	0.99	0.71	0.29 to 0.90	<0.01
V _D vs F _{aw} NO	0.40	-0.16 to 0.76	0.14	0.42	-0.13 to 0.78	0.12
V _D vs D _{aw} NO	-0.30	-0.71 to 0.27	0.28	0.56	0.05 to 0.84	<0.05
Age vs FENO _{0.05}	0.02	-0.51 to 0.54	0.94	0.74	0.34 to 0.91	<0.01
Age vs $\dot{V}_{\text{ENO}_{\text{max}}}$	-0.12	-0.60 to 0.44	0.70	0.76	0.40 to 0.92	<0.001
Age vs F _{aw} NO	0.28	-0.28 to 0.70	0.31	0.52	0.00 to 0.82	<0.05
Age vs D _{aw} NO	-0.34	-0.74 to 0.22	0.21	0.40	-0.15 to 0.77	0.14

CI, confidence interval.

ever, the influence of mucous secretions is probably small as NO diffuses rapidly into the gas phase (23).

Silkoff *et al.* (14) argued that an increase in NO release from inhibitory nonadrenergic noncholinergic nerve fibers could be involved. This notion was based on the observation that DawNO seemed unaffected by inhaled steroids and that DawNO, FawNO, and $\dot{V}_{\text{ENO}_{\text{max}}}$ were negatively correlated with airway hyperresponsiveness. However, no other study has demonstrated a negative correlation between exhaled NO and airway hyperresponsiveness. Instead, previous investigators have found either a positive correlation (7, 24, 25) or no correlation at all (26, 27) between exhaled NO and airway inflammation. In addition, nonadrenergic noncholinergic nerves are not found in the epithelium (28, 29), and because NO is rapidly metabolized within the tissue (30) or absorbed by Hb (31–33), it is doubtful that neuronal NO has the ability to reach the airway lumen. Instead, it is believed that iNOS, which is primarily found apically in the epithelial cells, is responsible for mucosal NO release in the airways (34).

In our study, children of various ages and with different lung volumes were investigated. A positive correlation between DawNO and V_D could be demonstrated, but only in control children. Because V_D is defined as the airway volume not involved in gas exchange, V_D relates to the conducting airway surface area. We therefore suggest that the main determinant of DawNO is the total NO-releasing surface area. Even though the main source of NO is the upper airways (2), there is evidence of NO release from the lower airways in both asthmatic and healthy humans (35, 36). In healthy subjects, it is mostly the larger bronchi that release NO (36). We believe that the proportion of the airway surface area that is involved in NO release remains fairly constant during childhood, but we suggest that if the child becomes asthmatic, the formation of NO is extended to the peripheral airways. Consequently, the relationship between DawNO and V_D disappears.

The relationship between DawNO and age, body size, or V_D has not previously been studied, but it has been shown that adult men have higher FENO levels than women (37), even after the adjustment for body size (38). This might be explained by our finding that DawNO was associated with V_D and that the V_D in relation to body weight is greater in men (39).

Our data are also supported by observations from Franklin *et al.* (40), who found a positive correlation between FENO and age

in 137 healthy children, and Kissoon *et al.* (41), who more recently showed similar results. In addition, we found that FENO_{0.05} correlated better with DawNO in asthmatic children and with FawNO in control subjects, which indicates that the expansion in total NO-releasing airway surface area overrides the association between FENO_{0.05} and FawNO in asthma. However, we believe that the FENO_{0.05} value, measured in an asthmatic or nonasthmatic individual, is determined by both FawNO and DawNO. Therefore, it is not surprising that such a strong correlation between FENO_{0.05} and $\dot{V}_{\text{ENO}_{\text{max}}}$ was found, as $\dot{V}_{\text{ENO}_{\text{max}}}$ depends on FawNO and DawNO.

It has been shown that FENO_{0.05}, FawNO, and $\dot{V}_{\text{ENO}_{\text{max}}}$ are decreased after treatment with inhaled steroids (14). However, DawNO seemed unaffected by this treatment (14). The reason for this is unclear. Future studies may reveal whether DawNO, in combination with FENO_{0.05}, would give a better measure of the degree and extension of asthmatic inflammation before and after the introduction of antiinflammatory treatment.

FENO values at three different exhalation flow rates were successfully incorporated in a two-compartment model for NO diffusion recently described by Högman *et al.* (17). The correlation between measured and expected FENO_{0.05} was very strong, indicating that the accuracy of the measurements, the physiologic model, and the mathematical solutions should be adequate. Nevertheless, our data were originally obtained to study the reproducibility of $\dot{V}_{\text{ENO}}$ at different exhalation flow rates (20). Consequently, no reproducibility criteria for $\dot{V}_{\text{ENO}}$ were followed. FENO measurement under strict flow control may further improve $\dot{V}_{\text{ENO}}$ reproducibility and, thus, the accuracy of flow-independent variables estimated using mathematical solutions involving $\dot{V}_{\text{ENO}}$. Furthermore, future studies should determine the optimal exhalation flow rates used in NO diffusion model in different age groups to improve reproducibility.

CONCLUSIONS

We conclude that a more detailed picture of NO formation in the lower airways can be achieved noninvasively in children by measuring NO at multiple exhalation flow rates. Future investigations will clarify the clinical usefulness of these variables. In our hands, DawNO seems to reflect the extension of inflammation into the peripheral airways.

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