Reduction of variability of exhaled nitric oxide in healthy volunteers

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Abstract Exhaled nitric oxide (eNO) is elevated in patients with asthma in contrast to healthy subjects, although the variability is high. In this study, we tried to reduce the variability of eNO in healthy subjects. We measured eNO using ERS guidelines with a fixed exhalation flow of 250 ml/s in 117 (72 women, 45 men) non-smoking healthy subjects and correlated this to anthropometric data and standard lung function measurements. Using a model previously defined by Hyde et al., we selected parameters that were likely to have a high correlation with eNO. eNO was log-normally distributed. The normal values for eNO are significantly (P < 0.001) different for men and women: in women mean ln eNO levels (SD) were 1.49 (0.34), in men 1.74 (0.41) (back-transformed value 4.43 resp. 5.73 ppb). Using multiple regression analysis, only ln DM, CO, ln TLC and ln sGaw showed a significant positive correlation with ln eNO in men, although only 20% of the variability of eNO could be explained. In women no correlation was observed and only 5% of the variability was explained. The high variability of eNO could only partly be explained in men, which makes the use of reference equations not very helpful.

Keywords exhaled nitric oxide; healthy subjects; variability; reference equation.

INTRODUCTION

In the past years many studies were conducted to unravel the role and origin of nitric oxide (NO) in pulmonary diseases (1). NO is produced by NO-synthase (NOS), of which three isoforms has been identified (2). Two of the isoforms are located in the bronchial epithelium, namely neuronal NOS (nNOS) and inducible NOS (iNOS). In the pulmonary vascular system endothelial NOS (eNOS) is expressed in the endothelium, where it produces NO, which acts as a vasodilator. As an increase in exhaled NO (eNO) is seen in asthmatics subjects (3), the research has been focussed on the airway epithelium. In transbronchial biopsies, a great increase in iNOS-expression has been seen in asthmatic subjects, induced by proinflammatory cytokines (2). Furthermore, the expression of iNOS correlates with the activity of the disease (4–6), leading to the hope that eNO could be a new measurement tool for disease activity in asthmatic patients. In all studies concerning eNO in healthy and asthmatics subjects, a substantial overlap between the two groups was seen, leading to a reduced discriminative power of the eNO measurement. Therefore, it is important to try to explain the variability of eNO in detail, in order to increase the discriminative power of the measurement. Hyde et al. (7) proposed a model which incorporates the production of NO by the lungs (VNO), the diffusing capacity for NO (DNO) that carries NO into the pulmonary capillary blood and the removal by exhalation (VA). Hyde derived relevant equations and was able to predict steady-state NO levels in subjects.

\[ P_L = \frac{V_{NO}}{D_{NO} + V_A/(P_B - P_{H_2O})} \]

The above equation shows the relationship between exhaled NO levels (PL), NO diffusion (DNO), production (VNO) and removal by exhalation (VA). P B depicts atmospheric pressure, while PH2O is the saturated water vapor pressure (47 mmHg or 6.3 kPa). (Derived from Hyde et al. (7)).

In this study, we measured exhaled NO levels in healthy subjects to determine the 'normal' levels and attempt to reduce the variability by correcting for covariates, which were derived from the 'Hyde-model'.

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METHODS

Model development

Based on the model from Hyde we determined parameters that influence eNO levels. The first parameter is the NO diffusion capacity ($D_{NO}$). Since there is no measuring method available for this parameter, we used the membrane conductance for carbon monoxide ($D_{m,CO}$) (8) because it is closely related to $D_{NO}$. Borland (9) showed that $D_{CO}$ and $D_{NO}$ are related to each other by a factor 4.3.

The production of NO depends on the NOS activity levels, which cannot be measured non-invasively. However, NOS catalyses the conversion of L-arginine into L-citrulline thereby producing NO. As the levels of eNO are influenced by the intrapulmonary (10) or intravenously (11) administered L-arginine, serum L-arginine levels could affect eNO.

The ERS criteria (12) for NO measurements request the use of a fixed exhalation flow. This results in a dependency of eNO on TLC: in larger lungs the balance between adding and removing NO shifts. Based on this approach we incorporated total lung capacity in our model. A similar line of reasoning leads to the measurement of the physiological dead space volume ($V_{D}$), which can act as a storage barrel for NO. Next to these measurements, standard lung function parameters were measured: peakflow, MEF_{75/50/25}, (forced) vital capacity, FEV_{1}, and sG_{aw}.

Subjects

Included were healthy volunteers, not on medication, non- or ex-smokers (quitted more than six months). The exclusion criteria consisted of respiratory tract infection (including rhinitis) in the past 6 weeks, atopic disorders, e.g. seasonal rhinitis, hay fever, eczema and other allergies.

Lung function

Whole body plethysmography was performed on 6200 Autobox DL (SensorMedics Cooperation, Yorba Linda, CA, U.S.A.) with the determination of static and dynamic lung volumes and airway resistance ($R_{aw}$).

Carbon monoxide diffusion capacity, including its subdivisions $D_{m,CO}$ and $Q_{CO}$, was measured on a MasterLab Pro (Erich Jaeger GmbH, Wurzburg, Germany). $D_{m,CO}$ and $Q_{CO}$ were determined with the single-breath maneuver technique according to Cotes (13). Physiological dead space ($V_{D}$) measurement was done on an Oxycon Alpha (Erich Jaeger GmbH, Wurzburg, Germany), volume and CO$_2$ calibrations were performed daily. During tidal breathing CO$_2$ was continuously measured via a side arm. $V_{D}$ was calculated using the Bohr equation. In healthy subjects the difference between $P_{a}CO_{2}$ and $P_{et}CO_{2}$ is negligible, so we used $P_{et}CO_{2}$.

Nitric oxide measurements

NO measurements were performed on a chemoluminescence analyzer (type CLD 77 AM, Eco Physics, Zurich, Switzerland). System specifications are detection limit 0.02–0.05 ppb, reaction time 0.1 s, and continuous on-line measurement of NO. Sampling flow was kept constant at 325 ml/min. Expiratory flow was measured by means of molecular mass gas analysis with ultrasonic transducers, CO$_2$ was measured via a side arm by infrared sensor (Spiroson Scientific unit, Isler Bioengineering, Dürnten, Switzerland).

eNO measurements were performed in all subjects in the morning after an overnight fast, before other tests. After the inhalation of NO-free air to TLC, the subjects inhaled against a positive pressure of 5 cm H$_2$O, thus preventing nasal air leakage. Expiratory flow was kept constant according to ERS recommendations (12) by means of a feedback system with a visual scale representing the on-line mouth pressure, leading to a constant expiratory flow of 250 ml/s. Each subject repeated the slow exhalation for at least 5 times, with reproducible curves. In all subjects end-tidal NO plateau phase was taken as the eNO value. Before each measurement a zero-point calibration was performed. Twice weekly the NO analyzer was calibrated using zero-NO air, made by room air which was led through an NO capturing filter, and NO in N$_2$ 50 ppm (Linde Ag, Unterschleißheim, Germany).

Blood samples were taken from all subjects in the morning after an overnight fast. Quantitative analysis of arginine was performed on a Biochrom 20 amino acid analyzer with an ion-exchange column (Amersham Pharmacia Biotech, Cambridge, U.K.).

Statistics

Exhaled NO levels are lognormal distributed as described earlier (14–16): a Kolmogorov–Smirnov test showed a significant departure from the normal distribution ($P < 0.001$). In consequence, all calculations were carried out with the ln-transformed values. The necessity to ln-transform was also present with the other measured (lung function) parameters. Jilma et al. (17) reported that eNO levels are higher in men than in women, therefore we compared levels in men and women by an unpaired $t$-test.

The parameters listed above and the eNO levels were incorporated into a multiple regression model. Incorporation of a multitude of (irrelevant) parameters can weaken the predictive power of any multiple regression model: we defined useful parameters as those who show
high correlation with eNO levels, but with low intercorrelations \((18,19)\). To define the multiple regression model we used forward selection of parameters. Analyses were carried out using SPSS 10 (SPSS Inc., Chicago, U.S.A.). The anthropometric data are depicted as mean and standard deviations (SD), \(z\)-levels were 0.05. The lung function was expressed as the mean (SD) of the number of standard deviations of the predicted value.

RESULTS

Of 123 subjects who volunteered in this study 6 subjects were excluded as they did not perform all pulmonary function tests, leaving 117 eligible persons, 72 women and 45 men. The women’s age was \(38.3 \pm 11.5\) (range 17–61), male age was \(40.1 \pm 10.8\) (range 25–64). The weight (in the same order) 66.0 \(\pm 8.8\) and 82.6 \(\pm 11.8\) kg, body mass index, respectively, 23.1 \(\pm 3.1\) and 24.7 \(\pm 2.9\) kg/m\(^2\), height 1.69 \(\pm 0.06\) m, respectively, 1.83 \(\pm 0.07\) m. The lung function of all subjects was shown to be within normal limits \((20)\). The FEV\(_1\) in women and men was, respectively, 0.61 \(\pm 1.1\) and 0.59 \(\pm 0.59\) standard deviations off predicted, the TLC 0.93 \(\pm 1.1\) and 0.88 \(\pm 1.15\), the residual volume 0.38 \(\pm 1.08\) and 0.35 \(\pm 1.13\). The CO-transfer values again expressed as standard deviations off predicted for women showed a small but significant departure from zero: \(-0.8\) SDS (95% CI: \(-1.1\) to \(-0.5\)). This indicates a somewhat lower CO-transfer value than expected. For men no significant departure was found: \(9.75 \pm 1.0\) SDS.

The mean eNO-levels, the 95% CI of the mean and the \(\pm 2\) SD range are depicted in Table 1. The unpaired \(t\)-test showed highly significant \((P < 0.001)\) differences in eNO levels between men and women. Mean In-transformed eNO levels were 1.49 (0.34) in women and 1.74 (0.41) ln ppb in men, which corresponds to geometric means of, respectively, 4.43 and 5.73 ppb (Table 1): further calculations were done in men and women separately. The correlation matrix between the eNO levels and the other parameters revealed that TLC and weight were significantly correlated with eNO (Table 2). The significant correlation between eNO and weight is however an artificial one because in a correlation matrix for women and men separately, the weight–eNO correlation disappeared: in women the correlation coefficient dropped to 0.049 \((P=0.685)\) and in men to 0.279 \((P=0.064)\).

Within the male and female groups no correlation was present between eNO levels and age \((P > 0.05)\). When the parameters from Table 2 were used in a multiple regression analysis none of the parameters could act as powerful predictor of eNO levels in women (Table 3). Only 5.2% of the variance present in women was explained by the regression analysis. The residual standard deviation in this case was 0.352. In men, 20% of the variance was explained, while the regression analysis showed significance \(\left(P^2=0.034\right)\). The residual standard deviation was 0.366.

Considering the large number of irrelevant parameters, but also knowing the significant correlation between TLC and eNO levels, we simplified the model to one containing \(D_{inCO}\), \(sG_{aw}\) and TLC. The outcome shows that the regression becomes stronger in male \((P=0.008)\)

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Descriptive summary of eNO levels in healthy men and women</th>
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<tbody>
<tr>
<td><strong>In-transformed NO values (ln ppb)</strong></td>
<td><strong>Back-transformed NO values (ppb)</strong></td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td><strong>Lower–upper 95% Cl</strong></td>
<td><strong>1.41–1.57</strong></td>
</tr>
<tr>
<td><strong>± 2 SD range</strong></td>
<td><strong>0.69–2.17</strong></td>
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<tr>
<td><strong>Men</strong></td>
<td><strong>Mean</strong></td>
</tr>
<tr>
<td><strong>Lower–upper 95% Cl</strong></td>
<td><strong>1.62–1.87</strong></td>
</tr>
<tr>
<td><strong>± 2 SD range</strong></td>
<td><strong>0.93–2.56</strong></td>
</tr>
</tbody>
</table>

The 95% confidence intervals depict the interval of the mean for the ln-transformed eNO values.

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>Correlations between the In-transformed values of eNO levels and explored parameters in the entire group volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Correlation coefficient</strong></td>
<td><strong>P-value</strong></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>0.113</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Significant correlations are printed in bold.
The residual standard deviation now is 0.367. In females still no significant regression was found.

Regression coefficients for $D_{m,CO}$, CO, TLC and $sG_{aw}$ in women and men showed were strongly different in either magnitude or sign. Subsequently, we tried to explain sex differences in eNO levels by incorporating these parameters in a co-variance analysis, which included the parameters mentioned and the interaction between sex and these parameters. The results indicate that the sex difference in eNO levels became non-significant ($P=0.384$). The strongest factors were $D_{m,CO}$ ($P=0.016$), $sG_{aw}$ ($P=0.005$) and the interaction between TLC and sex ($P=0.01$). The other factors or interactions were barely not or not significant. The significant interaction between sex and TLC is visualized in Fig. 1: it shows a positive correlation between TLC and eNO levels in men and a negative one in women.

**DISCUSSION**

The reduction of variability of eNO levels by using co-varyes derived from the Hyde-model (7) appeared to be small. A key factor in this model is the cellular NO production, which is not present in our approach because it can only be measured by means of invasive procedures. When a reliable (and easy) method for the measurement of cellular NO production becomes available we probably can explain more of the eNO level variability. Therefore, we cannot reject or accept the validity of the Hyde-model in explaining eNO levels. In our study serum L-arginine levels were not correlated to eNO levels and will not be a measure for cellular NO production.

Recently, another model for exhaled NO levels was published by Silkoff et al. (21). This model is based on the assumption that the alveoli produce NO, leading to a concentration $C_{alv}$. On expiration the conducting airways add NO to the alveolar amount. The amount added depends on the NO-concentration in the airway wall ($C_{aw}$), the transfer of NO from the mucosa to the air ($D_{NO}$) and the expiratory airflow. When the flow is high, not much NO-molecules can be added to a volume of air due to the short contact time. At infinite flows the eNO concentration therefore equals the alveolar concentration. The Silkoff model offers no new ways to reduce variability because the parameters in that model ($C_{aw}$, $C_{alv}$ and $D_{NO}$) are derived by non-linear regression from eNO-levels. Using these parameters to explain eNO variability thus becomes impossible.
In our study flows of approximately 250 ml/s were used, what according to Silko et al. would not reflect bronchial NO-production, but foremost alveolar NO. In this sense, we like to point at many studies which detected high eNO-levels in asthma (being a disease of the bronchi) using 250 ml/s expiratory flow (22,23).

Reference equations derived from our measurements will have a low predictive power. This implies that large differences are needed to discriminate between normal and abnormal values. Only if the changes in eNO induced by disease are substantial, the wide range of 'normal' values will not overlap with the values in diseased subjects. Simpson and co-workers (24) measured in steroid naive asthmatics mean eNO levels (exhalation flow rate 100 ml/s) of 9.09 or 17.69 ppb depending whether or not the patients recently had been exposed to indoor allergens, which probably means a considerable overlap between healthy volunteers and asthmatics. It can be calculated that, assuming an overlap of ≤5% between diseased and healthy eNO-values and based on our measurements, eNO levels in female asthmatics should show a mean (SD) of 2.84 (0.34) and in male asthmatics of 3.34 (0.41) in ppb to discriminate clearly between healthy and asthmatics (back-transformed value 170 ppb resp., 28.3 ppb).

Increasing the sample size (assuming that sampling was done in an unbiased way) would not lead to a stronger reduction of eNO levels variability because the standard deviation of a sample is not dependent on its size. The only possible conclusion is that the population standard deviation of eNO levels is relatively large. Compared to other studies in children (15) the range from the +2 SD to the −2 SD value is similar or even smaller.

Baraldi (15) reports such a 4 SD range of 14.5 ppb, while in this study we report 6.65 ppb for women and 10.3 ppb for men. In young adults, this range is reported to be 19.6 ppb (14). Kharitonov (25), pooling men and women, reported a 4 SD range of approx. 8.8 ppb, which is very similar to this study. The mean (back-transformed) eNO levels in this study are lower than that of Kharitonov, perhaps due to the fact that apparently Kharitonov did not take account of the lognormal distribution. When one calculates the straightforward mean of such a distribution, the mean is overestimated.

Jilma (17) reported higher eNO levels in men than in women (we confirm this observation) and concluded that part of the differences was attributed to body weight. We disagree with this opinion because when weight is a significant factor, this must be so in males and females, which is not the case. Other factors are responsible for the sex difference, namely TLC, $D_{m\text{CO}}$, $sG_{aw}$ and the interaction between sex and TLC. The fact that only these factors are to a great extent responsible for the sex difference rules out major sex differences in the NO synthase levels and/or activity. If a sex difference in the latter would exist, we would not be able to explain the differences using the factors described above. For arguments sake we have to recognize the possibility that NOS activity in men is lower than in women, this would lead to a decrease in sex differences in eNO levels, explained by a reduction of the differences induced by TLC, $D_{m\text{CO}}$, $sG_{aw}$. This seems unlikely to us because when we remove the influence of TLC, $D_{m\text{CO}}$ and $sG_{aw}$ a difference would remain. The remaining difference would be opposite in sign compared to that reported here.

**FIG. 1.** Scatterplot of eNO levels and TLC (both ln-transformed) showing that in men a larger TLC means higher eNO levels and the opposite phenomenon in women. The lines shown depict the regression in both groups.
The effect of $D_{m,CO}$ on eNO levels is similar in both sexes: the higher the $D_{m,CO}$ is, the lower the eNO levels are. The explanation for this could be quite straightforward: when more NO diffuses into the bloodstream, less is available for exhalation. The interaction between sex and $D_{m,CO}$ was non-significant, so we have no arguments that the effect of $D_{m,CO}$ is different in men and women. A puzzling effect can be noted for the TLC: in men a larger TLC means higher NO levels, but in women lower TLCs are equivalent to higher levels. This phenomenon explains the significant sex by TLC interaction. In men one could argue that in subjects with a large TLC the conducting airways are relatively larger, leading to a longer contact time between expired air and bronchial epithelium (with the same flow rate measured at the mouth). The opposite character in women rules out such an explanation. It is difficult to find an explanation for these phenomena, but we can rule out several factors. Men and women of all build, height, etc., entered this study in a complete random fashion, so systematic differences due to instrument bias is very unlikely. The correlation we found could be false, which is possible when a few outliers heavily influence the regression line or equation. We think that this is not the case: first of all, the variance in male and female values did not differ significantly as judged by the Levene’s test for equality of variances. Secondly, we did check for the presence of strong influencing points in the regression analysis and this check revealed that no datapoints were present which could ‘tilt’ the regression line in such a way that the TLC-effect in males is falsely generated.

A correlation between eNO and $V_d$ could not be established. The fact that in these healthy subject NO-production in the conducting airways will be low will explain this finding. Moreover, the spread in the $V_d$-values was small, thus offering few possibilities to explain eNO-variability.

In summary, we determined exhaled NO levels in a group of healthy subjects and only found a weak reduction of the variability of these levels, using parameters derived from the model of Hyde or Silkoff. Due to the low variability reduction, the use of reference equations is not very helpful. Men show higher eNO levels than women, which could be explained by the differences in TLC, $D_{m,CO}$ and $sG_{aw}$, and not by weight.

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