Analysis of Exhaled Nitric Oxide by the Helium Bolus Method*

Masaharu Shinkai, MD; Shunsuke Suzuki, MD; Akira Miyashita, MD, PhD; Hideo Kobayashi, MD, FCCP; Takao Okubo, MD, PhD, FCCP; and Yoshiaki Ishigatsubo, MD

Study objectives: The precise anatomic sites contributing to exhaled nitric oxide (eNO) are still unknown. The present study was designed to analyze profiles of eNO by referring to the He exhalation curve and examining the effects of breath-holding and expiratory flow rates on eNO.

Participants: Healthy volunteers and patients with stable asthma.

Measurement and results: We used the He bolus method of the closing volume, and simultaneously analyzed the concentrations of exhaled He and nitric oxide (NO). By referring to the He exhalation curve, the expired gas was divided into three parts: airway dead space (phase 1), a mixture of airway and alveolar gas (phase 2), and alveolar gas (phase 3 and phase 4). The eNO profiles showed a peak in phase 2 (peak eNO) and decreased gradually to a plateau in the latter half of phase 3 (plateau eNO). The levels of peak eNO were higher than those of plateau eNO in both normal subjects and asthmatic patients. Breath-holding increased levels of peak eNO 2.5-fold in both normal subjects and asthmatic patients, but it did not affect the levels of plateau eNO. The levels of peak eNO increased as the expiratory flow rate decreased, and the levels of plateau eNO showed a similar flow dependency.

Conclusion: A peak value of eNO concentration profiles may directly express the production of NO in the airway.

Key words: airway; asthma; breath-holding; exhaled nitric oxide; expiratory flow rate; helium bolus; nitric oxide; peak nitric oxide; plateau nitric oxide

Abbreviations: eNO = exhaled nitric oxide; NO = nitric oxide; ppb = parts per billion; RV = residual volume; TLC = total lung capacity; VC = vital capacity

High levels of exhaled nitric oxide (eNO) have been observed in patients with various pulmonary diseases, such as bronchial asthma, viral respiratory infections, and bronchiectasis.1–3 However, the precise anatomic sites contributing to eNO are still unknown. Theoretically, all tissues adjacent to the respiratory tract could excrete nitric oxide (NO) into the exhaled gas. Earlier studies4,5 have indicated that a large portion of eNO arises from the nose. By analysis of NO with CO₂, eNO has been reported to be produced in the airways, but not at the alveolar level.6 Some investigators7,8 have reported that eNO is mainly derived from the upper airway, while studies9,10 using bronchoscopy or intubation have suggested that the lower airway is the source of the increased eNO in patients with bronchial asthma.

A particular concern is that NO measured in exhaled air may be contaminated by NO derived from the nose.11,12 The heterogeneity of the sources of eNO complicates the interpretation of eNO profiles, and profiles of the NO exhalation curve have not been fully characterized. The profiles of eNO have been described as an initial peak (peak eNO) followed by a plateau, and the plateau level of the eNO curve (plateau eNO) has been used for analysis.11,13,14 After breath-holding, the levels of peak eNO increased to greater than plateau levels.15 However, this increase in peak eNO has been regarded to be the result of nasal contamination.4,10 The measurement of eNO has been improved by using a low flow rate and a positive airway pressure without the wearing of a nose clip.11,16 It has been also demonstrated that breath-holding does not allow any contamination of eNO by nasal NO.16,17 It is therefore possible that peak eNO represents the production of NO in the airway wall and is useful in...
evaluating airway inflammation. In the present study, to further characterize the peak eNO, we used the He bolus method of closing volume, in which the He exhalation profile divides the expired gas into a mixture of dead space and airway (phase 1), a mixture of airway and alveolar gas (phase 2), and alveolar gas (phase 3 and phase 4), and analyzed profiles of eNO by referring to the He exhalation curve. We also studied the effects of breath-holding and expiratory flow rates on eNO in normal subjects and asthmatic patients.

**Materials and Methods**

**Subjects**

Patients with bronchial asthma (age range, 22 to 75 years) were enrolled at our outpatient clinic, and healthy volunteers (age range, 23 to 46 years) were also recruited from hospital employees. Patients with asthma were all in stable condition, and its severity was mild to moderate (Table 1). All patients and subjects were nonsmokers. Written informed consent was obtained from all participants.

**Measurement of eNO**

eNO was measured according to the method of Silkoff et al. Levels of eNO were measured by a rapid-response chemiluminescence analyzer (NOA 270B; Sievers; Boulder, CO), and a calibration was performed between 520 parts per billion (ppb) and 0 ppb. The linearity of the analyzer response was verified by repeated calibrations. The apparatus of eNO measurement consists of a one-way valve (model 1400; Hans-Rudolph; Kansas City, MO), of which the inspiratory port is connected to a Douglas bag containing NO-free air, and of which the expiratory port is connected to a pneumotachograph and a spring-loaded valve (Threshold PEP; Healthscan Products; Cedar Grove, NJ) to give a constant positive pressure (14 cm H2O) in relation to the mouth. The expiratory line. eNO was continuously sampled via the side port of the mouthpiece with a mass spectrometer (WSMR-1400; Westron; Chiba, Japan). To keep the flow rate constant, the flow rate was displayed in the front of the subject.

**He Bolus Method of Closing Volume**

Fifty milliliters of He was injected to the mouthpiece at the beginning of inhalation from residual volume (RV) to total lung capacity (TLC), and subjects were then asked to exhale to RV through the mouthpiece at a constant flow rate by observing the flow rate on the oscilloscope. He concentrations were measured via a side port close to the mouthpiece with the mass spectrometer. The expired volume was obtained from the integration of the pneumotachograph signal and was displayed, along with the He concentrations, on an XY recorder.

Flow rate, mouth pressure, and concentrations of NO, CO2, and He were simultaneously recorded at a sampling rate of 100 Hz by the MacLab System (AD Instruments; Castle Hill, Australia). Expiratory flow rate studied in the present study was 60 mL/s, which was slightly higher than the recommended flow rate by the American Thoracic Society, because the present study was performed before the publication of this recommendation.

**Study Protocol**

**Test of Nasal NO Contamination:** We studied whether the nasal cavity is in communication with the airway during a slow exhalation against positive airway pressure or during breath-holding. While the nasal cavity was gently flushed with He, the subjects were asked to exhale from TLC without breath-holding at a flow rate of 60 mL/s against positive pressure. He concentrations were monitored simultaneously with both NO and CO2 throughout the exhalation. In addition, during a slow exhalation from TLC after breath-holding for 10 s, nasal contamination was examined in the same way as described above.

**Simultaneous Measurement of eNO and the He Exhalation Profile:** Before the eNO measurement, the subjects breathed NO-free air for 1 min and then performed two vital capacity (VC) maneuvers. At RV, the subjects inhaled an He bolus of 50 mL and NO-free air to TLC and then, with or without breath-holding for 10 s, exhaled at a flow rate of 60 mL/s against positive airway pressure. By referring to the He exhalation curve, we divided the eNO curve into phases 1, 2, 3, and 4 (Fig 1). The typical eNO trace showed a peak in phase 2 and then a slight decrease in phase 3, forming a plateau. We sampled the peak and plateau eNO levels, the latter of which was measured for at least 5 s during the latter part of phase 3.

**Effects of Expiratory Flow Rate on eNO:** To examine the effects of the expiratory flow rate on eNO, the subjects were asked to exhale at a flow rate of 60 mL/s, 120 mL/s, or 240 mL/s against positive airway pressure. The subjects controlled the expiratory flow rate by visual feedback. The order of flow rate was randomized. Before the NO measurement, the subjects breathed NO-free air for 1 min and then performed two VC maneuvers. Next, during exhalation from TLC to RV, eNO was measured.

**Effects of Breath-Holding on eNO:** After breathing NO-free air for 1 min and carrying out two VC maneuvers, subjects were asked to hold their breath at TLC for 10 s and then to exhale to RV at a flow rate of 60 mL/s. The order of eNO measurements with or without breath-holding was randomized.

All measurements were performed with subjects in a seated position. The subjects did not wear a nose clip. After eNO

<table>
<thead>
<tr>
<th>Groups</th>
<th>Gender, M/F</th>
<th>Age, yr</th>
<th>VC, % Predicted</th>
<th>FEV1, % Predicted</th>
<th>Atopy/Nonatopy</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>6/11</td>
<td>47 ± 18</td>
<td>95 ± 18</td>
<td>80 ± 22</td>
<td>13/4</td>
<td>13/17</td>
</tr>
<tr>
<td>Normal</td>
<td>14/3</td>
<td>34 ± 8</td>
<td>114 ± 15</td>
<td>111 ± 16</td>
<td></td>
<td>13/17</td>
</tr>
</tbody>
</table>

*Data are presented as No. or mean ± SD. F = female; M = male; ICS = inhaled corticosteroid; Theo = theophylline; Anti-LT = antileukotrienes.
measurement, spirometry was performed, and VC and FEV1 were obtained with a dry-seal spirometer (CHESTAC-25V; Chest; Tokyo, Japan).

**Statistical Analysis**

Results are expressed as the mean value ± SD. Where appropriate, data were analyzed using a two-way analysis of variance with repeated measures, followed by a post hoc comparison using the Newman-Keuls test. For the comparison between healthy subjects and asthmatic patients, a Mann-Whitney U test was used, and the paired data were examined with a paired t test. p < 0.05 was considered significant.

**RESULTS**

**Evidence of NO Contamination From Nasal NO**

When the subjects exhaled from TLC without breath-holding while applying positive airway pressure without a nose clip, He injected into the nasal cavity was not detected in the expired gas. Figure 2 shows the trace concentrations of eNO, CO2, and He as well as the flow and mouth pressures after breath-holding for 10 s, and no He was detected in the expired gas. Thus, the present method can prevent any contamination from nasal NO in the eNO measurement with or without breath-holding for 10 s.

**Effects of Sampling Site on eNO: Peak eNO vs Plateau eNO**

After washout of the airway dead space, the He concentration rose sharply in phase 2 (Fig 1); phase 2 consists of gas from both the airways and alveoli. Profiles of eNO showed a peak in phase 2 and a drop to a plateau in phase 3. The mean value of peak eNO at 60 mL/s was 20.8 ± 5.3 ppb and that of the plateau eNO was 12.9 ± 3.8 ppb in normal subjects; this difference was statistically significant (p < 0.001; Fig 3). In asthmatic patients, peak eNO was 91.1 ± 52.9 ppb and plateau eNO was 62.3 ± 41.6 ppb. This difference in eNO was also statistically significant (p < 0.001).

**Effects of Flow Rate on eNO**

In both normal subjects and asthmatic patients, as the expiratory flow rate increased, the levels of peak...
eNO decreased, with a similar decline in plateau eNO (Fig 4). Peak eNO levels at 60 mL/s and 240 mL/s in the normal subjects were 20.8 ± 5.3 ppb and 13.0 ± 3.6 ppb, respectively, and this difference was statistically significant (p < 0.001). In asthmatic patients, peak eNO was also different between 60 mL/s and 240 mL/s (113.0 ± 100.3 ppb and 56.0 ± 39.9 ppb, respectively, p < 0.001). When eNO was normalized to the value of the lowest flow rate, both peak eNO and plateau eNO at the middle and highest flow rates decreased similarly in both normal subjects and asthmatic patients. At any flow rate (60 mL/s, 120 mL/s, or 240 mL/s), peak eNO was higher than plateau eNO in both normal subjects and asthmatic patients (both p < 0.05, analysis of variance). We therefore decided to adopt the eNO values at the lowest flow rate of 60 mL/s.

**Effects of Breath-Holding on eNO**

The peak eNO concentrations after breath-holding for 10 s in normal subjects was 54.3 ± 14.6 ppb, which was higher than the value without breath-holding (20.9 ± 5.0 ppb; p < 0.001). In asthmatic patients, peak eNO after breath-holding increased significantly from 90.1 ± 52.9 to 226 ± 162 ppb (p < 0.001). The increases in peak eNO in response to breath-holding were 2.6-fold in normal subjects and 2.5-fold in asthmatic patients, and were similar between these two groups (Fig 5). Plateau eNO was not changed by breath-holding in either normal subjects or asthmatic patients.
DISCUSSION

We have demonstrated that by referring to the He exhalation curve, a peak value in the early part of the eNO curve comes from the airway. Breath-holding for 10 s causes an increase in peak eNO levels, but does not affect the plateau eNO levels. Therefore, the peak eNO levels after breath-holding are suggested to represent the actual concentrations of NO in the airway walls.

Several eNO measurement techniques have been suggested as standard methods. There are several key points in measuring eNO, including the expiratory flow rate and positive airway pressure. Silkoff et al demonstrated that eNO levels are dependent on the expiratory flow rate. However, the flow rate has varied among investigators, from 4 to 250 mL/s. Our lowest flow rate of 60 mL/s is close to the recommended flow rate (50 mL/s) from the American Thoracic Society. Positive airway pressure during expiration is critical to closing the soft palate, thus preventing nasal contamination. We confirmed that no nasal eNO contamination occurs during exhalation against a positive airway pressure of 14 cm H₂O at any flow rate. In previous studies in which nasal contamination was suspected, the subjects were wearing nose clips. Rubinstein et al demonstrated that the use of a nose clip opens the nasopharyngeal velum. It has also been recommended regarding NO measurement, that no nose clip be worn. Thus, in the measurement of eNO, a slow exhalation without the wearing of a nose clip is actually ideal.

In early studies, the peak eNO value was reported as an eNO concentration. However, the peak eNO was regarded as a result of contamination from nasal NO of high concentrations. Thereafter, the peak eNO has been substituted for a plateau eNO. However, it has been revealed that the initial peak of eNO is derived from the airway wall, not from nasal NO. Theoretically, the initial rise of eNO in the first 200 mL is considered to be from the airway compartment and is due to diffusion from the airway wall to the airstream. We found that by referring to the He exhalation curve, eNO had a peak in phase 2 and then decreased to a plateau in the phase 3 or phase 4 in the VC maneuver of NO measurement. Thus, the peak eNO levels at a low flow rate express chiefly the NO level of the airway wall.

The maneuver of breath-holding had been reported to increase the levels of eNO. In a study insufflating an indicator gas to the nose, however, breath-holding itself did not cause any gas leak from the nose to the airway. Silkoff et al also confirmed that breath-holding does not allow nasal contamination. In the present study, we confirmed that during breath-holding for 10 s, no gas leak from the nose to the airway occurred. Persson et al reported that breath-holding increases the peak eNO in a duration-dependent manner. Massaro et al found a significant increase in eNO after breath-holding in patients with endotracheal intubation. Theoretically, a diffusion of NO occurs both during breathing and breath-holding in the airway. When the expiratory flow is fast, the plateau eNO level is determined primarily by NO levels of the alveolar gas (phase 3), irrespective of breath-holding time. When the expiratory flow rate is slow, phase 3 gas passes through the airway for a longer time, resulting in a substantial amount of airway NO possibly diffusing into the exhaling air, thus increasing eNO levels. In our normal subjects, the peak eNO level after breath-holding was 54 ppb, which is very close to the equilibrium concentration (56 ppb) in trachea during breath-holding reported by DuBois et al. The magnitude of the increase in peak eNO in response to breath-holding in our asthmatic patients was comparable to that of normal subjects, although no change in the plateau eNO was observed. During breath-holding, NO in the airway wall may diffuse into the exhaling gas, but after breath-holding, alveolar gas (phase 3) may pass through the airway for a given time, suggesting that the amount of NO diffusing from the airway may depend on the flow rate alone.

In conclusion, the present study has demonstrated that peak eNO, especially after breath-holding, more directly expresses NO levels in the airway wall.

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REFERENCES