Alveolar Nitric Oxide and Effect of Deep Inspiration During Methacholine Challenge*

Christophe Delclaux, MD, PhD†; Françoise Zerah-Lancner, MD; Bruno Mahut, MD; Stephan Ribeil; Armelle Dubois; Christian Larger; and Alain Harf, MD, PhD‡

Study objectives: To assess whether the dual anatomic origin of exhaled nitric oxide (NO), namely alveolar and bronchial, could explain the link between exhaled NO and airway responsiveness, and could participate in the bronchodilatory effect of deep inspiration (DI) that may be evidenced during methacholine challenge.

Design and setting: Prospective study in a laboratory performing pulmonary function tests of an academic hospital.

Patients and interventions: Patients underwent multiple flow analysis of exhaled NO, allowing calculation of total maximum airway NO flux (J'awNO) and NO concentration of expansible compartment (CANO), and received a cumulative methacholine dose of 2,000 μg. DI effect was assessed by continuous measurement of the resistance of respiratory system using the forced oscillation technique before and after DI.

Results: In a first phase involving 23 patients, a positive correlation between log values of J'awNO and CANO was demonstrated with the degree of airway responsiveness (percentage of FEV1 decrease). In a second phase involving 38 patients, only log CANO was correlated with responsiveness, and no significant relationship was demonstrated between J'awNO or CANO and the effect of DI. Patients with smaller airways and/or distal airflow limitation exhibited a constrictive response to DI.

Conclusion: Airway responsiveness is mainly associated with an increase in distal origin of NO output, and no relationship between exhaled NO and the effect of DI was evidenced.

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Key words: concentration of NO from the expansible compartment; forced oscillation technique; total maximum flux of NO from airways

Abbreviations: AHR = airway hyperresponsiveness; CANO = concentration of expansible compartment; DI = deep inspiration; FENO = fractional exhaled concentration of NO; FENO,0.2 = fractional exhaled concentration of NO at 200 mL/s; J'awNO = total maximum airway nitric oxide flux; MEF25–75 = maximal expiratory flow between 25% and 75% of vital capacity; NO = nitric oxide; PFT = pulmonary function test; ppb = parts per billion; Rrs = resistance of the respiratory system

Airway hyperresponsiveness (AHR) is a condition in which the airway responds both too much and too easily to various stimuli, and its presence in asymptomatic subjects is a risk factor for subsequent development of asthma.1 The pathogenesis of AHR involves both airway inflammation and remodeling.2 Along this line, the degree of AHR has been shown to correlate both with an increase in airway inflammatory cells and in some altered structural components, such as deposition of subepithelial collagen or proteoglycans in the airway wall.2 Exhaled nitric oxide (NO) is a marker of airway inflammation and atopy, two conditions associated with AHR. Previous studies3–6 have evidenced a statistically significant relationship between exhaled NO and the degree of...

*From Unité INSERM U492-Université Paris XII (Drs. Delclaux and Harf), Faculté de Médecine de Créteil; and Service de Physiologie (Drs. Zerah-Lancner and Mahut, and Mr. Ribeil, Ms. Dubois, and Mr. Larger), Explorations Fonctionnelles, Hôpital Henri Mondor, AP-HP, Créteil, France.
†Currently at Service de Physiologie-Radio-Isotopes, Hôpital Européen Georges Pompidou, Paris, France.
‡Deceased at the time of article submission.

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Correspondence to: Christophe Delclaux, MD, PhD, Service de Physiologie, Explorations Fonctionnelles, Hôpital Henri Mondor, 51, avenue du Maréchal de Lattre de Tassigny, 94 010 Créteil, France; e-mail: christophe.delclaux@creteil.inserm.fr
airway responsiveness, whereas some investigators found a very weak or not significant relationship. We may hypothesize that the double origin of exhaled NO, namely bronchial and alveolar, could account for the discrepant results that have been obtained by these previous investigators. However, NO has both bronchodilatory and bronchoprotective effects that could seem contradictory with its link with AHR and inflammation. Consequently, the aim of this study was to evaluate whether the origin of NO could explain these apparent contradictions. Multiple flow measurement of exhaled NO allows differentiating NO output from expansible distal (alveoli and bronchioles) and conducting airways; this analysis is based on a two-compartment model that has been validated both in healthy and pathologic conditions. Therefore, the first aim of this study was to assess which compartment is responsible for the potential relationship between exhaled NO and airway responsiveness. The response during methacholine challenge can be measured by either a decrease in FEV₁ or an increase in airway resistance. Physician taking care of patients in the pulmonary function laboratory know that despite a correlation between both methods of assessment of airflow limitation some frank discrepancies are observed. These discrepancies can be attributed to the bronchomotor effect of deep inspiration (DI) [either dilator or constrictor] that is made during the forced expiration. The second aim of this study was to evaluate whether NO originating from either alveoli or bronchial tree could participate in the bronchodilatory effect of DI that may be evidenced during methacholine challenge.

**Materials and Methods**

**Study Design**

This study was conducted in two main phases (Fig 1). The aim of the first phase was to assess whether relationships between AHR and NO parameters obtained from multiple flow measurements (total maximum airway NO flux [JawNO], and NO concentration of the expansible compartment [CANO]) could be established. In the second phase of the study, the effect of DI during methacholine challenge was assessed.

The relationship between the degree of AHR and exhaled NO has been established using the most commonly used criterion of response during methacholine challenge testing: a 20% decrease in FEV₁. Inasmuch as the goal of the study was to assess the effect of DI in the second phase using the forced oscillation technique, our study protocol was adapted from the one described by Orehek and colleagues, who used airway resistance assessment, namely using both the resistance of the respiratory system (Rrs) and FEV₁ measurements. Since only a single FEV₁ measure was performed at the end of the bronchial challenge, the degree of responsiveness was analyzed in terms of percentage of FEV₁ decrease for the highest dose of methacholine administered, ie, 2,000 μg (the provocative dose causing a 20% fall in FEV₁ cannot be calculated). Consequently, only patients who received this highest dose were included in the statistical analysis (the number of patients who received lower cumulative doses are shown for information), which emphasizes that included patients had no AHR or mild-to-moderate AHR.

**Preliminary Experiment of Phase 1:** In order to determine which level of Rs augmentation should be reached to detect all patients with a reduction of FEV₁ > 20% (specificity approximately 100%), we compared the two measures (FEV₁ decrease and Rs increase) during methacholine challenge testing.

**Preliminary Experiment of Phase 2:** One potential confounding factor is the fact that methacholine administration uses a DI for each dose. Therefore, the duration of DI effect could be an important bias. Consequently, in a limited number of patients (the first 20 patients in whom DI modified significantly Rs), the effect of DI was continuously monitored until the Rs value returned to the baseline value (value of Rs without DI). The goal of this experiment was to determine the length of time to wait after methacholine administration before measuring resistance.

**Phases 1 and 2:** Consecutive patients, all with baseline FEV₁ > 80% of predicted value, were screened. The study design is described in Figure 1. The first phase allowed the evaluation of the relationship between the distal and proximal origin of NO and the degree of airway responsiveness. In the second phase of the study, the effect of DI was assessed during methacholine challenge and compared with the results of NO measurements. The sole modification was that after Rs measurement (without DI) after challenge with 2,000 μg methacholine, patients were asked to perform a DI, and Rs (with DI) was immediately measured.

**Subjects**

All consecutive patients referred for methacholine challenge testing were eligible for the study. Consequently, patients with miscellaneous conditions were enrolled as previously done in

![Figure 1. Study design. Arrows refer to a dimension of time; all tests were done during a single day.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/22025/ on 06/24/2017)
studies that have demonstrated a significant relationship between exhaled NO and AHR. Only patients who received a 2,000 µg methacholine dose were included (see “Study Design”). Since exhaled NO concentration is influenced by smoking status and its effect on AHR remains debated, current smokers were not included. The protocol was approved by our local ethical committee. All subjects gave their informed consent.

**Pulmonary Function Tests**

All patients were studied in the morning between 9 AM and 12 noon since a circadian dependency of DI effect has been previously shown in both asthmatic and healthy subjects.\(^1\)

**NO Measurement**

Fractional exhaled concentration of NO (FENO) measurement was performed before basal spirometry and methacholine challenge. FENO was measured using a chemiluminescent NO analyzer (EVA4000; Seres; Aix en Provence, France) as previously described.\(^10,14,15\) J’awNO and CANO were calculated after obtaining several FENO measurements at different expiratory flow rates, using the linear approach.\(^7–10\) The criteria used for each FENO measurement were those recommended in the American Thoracic Society guidelines.\(^10\) The reproducibility of a single FENO value has been shown to be good (coefficient variation < 10%). Each patient performed three to five expiratory flow rates, in a range of 50 to 250 mL/s. The plateau values of FENO obtained at the lowest expiratory flow rates were not used if a loss of linearity of the relationship between NO output and expiratory flow rate was evidenced (in our experience, the linearity of this relationship is evidenced for expiratory flow rates > 100 mL/s in patients with increased total maximum flux from airways, and > 50 mL/s in healthy subjects).\(^10\) FENO at 50 mL/s and FENO at 200 mL/s (FENO\(_{0.2}\)) were then computed as follows:

\[
\text{FENO}(V) = \text{CANO} + J’\text{awNO/}V \times 0.06
\]

for V equal to 50 mL/s (fractional exhaled concentration of NO, 50 mL/s) and 200 mL/s (FENO\(_{0.2}\)), respectively, as described elsewhere. \(V = \text{air flow}\).\(^10\)

**Functional Respiratory Tests**

Flow-volume curves were obtained using a spirometer (SensorMedics Corporation; Yorba Linda, CA). Three technically acceptable measurements were performed for each test, accordingly to international guidelines. The values were expressed as percentages of predicted values.

**Forced Oscillation Technique**

Respiratory impedance was determined using the standard forced oscillation technique (Oscilink; Datalink-MSR; Rungis, France), as described elsewhere and according to recent recommendations.\(^17\) The real component of Rrs, which is related to the resistive properties of the respiratory system, was submitted to linear regression analysis over the 4- to 16-Hz frequency range to obtain the intercept (R\(_0\), resistance extrapolated to 0 Hz). Three 17-s measurement periods were averaged to yield the final value of these parameters, allowing calculation of the mean value of Rrs during approximately the first minute after a DI. Modifications of Rrs are related to either bronchodilation or bronchoconstriction and cannot be ascribable to modifications of lung volumes. An increase in functional residual capacity due to methacholine challenge would have only minor effects on Rrs because the relationship between Rrs and lung volume is nearly linear in this range of volumes.\(^19\)

**Methacholine Challenge Testing**

Bronchoprovocation test and withdrawal of medications were in accordance with American Thoracic Society guidelines; no patient received inhaled or oral steroids at the time of study. Methacholine aerosol was delivered using a nebulization dosimeter (FDC 58; Médiprom; Paris, France). The forced oscillation technique was used to measure Rrs at baseline, after diluent, and after methacholine challenge (100 to 2,000 µg of cumulative doses of methacholine in different steps). Rrs was measured 2 min after each step (this lag time allowed to ensure that deep inhalations that were performed during methacholine challenge would have not influenced importantly the subsequent measurement of Rrs, (see “Preliminary Experiment of Phase 2”). The study was terminated when Rrs increased by 100% (see “Preliminary Experiment of Phase 1”) at any concentration or when the maximum dose of methacholine had been administered.

**Statistical Analysis**

Results are expressed as median (25th to 75th percentile). FEV\(_1\) and Rrs indexes, computed as the ratio of the difference between postbronchoconstriction and prebronchoconstriction values over the prebronchoconstrictive value, were used to express airway hyperreactivity.

A receiver operating characteristic curve was realized to determine which level of Rrs increase should be reached to detect all patients with a reduction of FEV\(_1\) ≥ 20%. It showed the true-positive rate (sensitivity) vs the false-positive rate (1 – specificity) at various levels of Rrs and FEV\(_1\) indexes, and made it possible to determine the cut-off value corresponding to the largest number of well-classified patients (see "Preliminary Experiment of Phase 1").

Inasmuch as log values of NO parameters were normally distributed as described elsewhere, \(^21\) correlations between log values of NO parameters and the degree of responsiveness were evaluated using least-square linear regression techniques. For all comparisons, \(p < 0.05\) was considered significant.

**RESULTS**

**Phase 1**

**Preliminary Experiment:** A significant relationship (\(p < 0.0001, R = 0.71\)) was found between the percentage of Rrs increase and the percentage of FEV\(_1\) decrease obtained for each single dose of methacholine done for 25 patients (94 pairs of measures), the equation for which is as follow: Rrs (percentage increase) = 1 + 2.57 × FEV\(_1\) (percentage decrease). Receiver operating characteristic curve analysis demonstrated that a 100% increase in Rrs represented the best cut-off value in term of specificity (99%). As a consequence, in subsequent experiments, methacholine challenge was continued until the Rrs increase reached a 100% augmentation above baseline value, since we wanted to detect all patients with AHR based on the “gold standard”...
method (FEV1 decrease). Furthermore, this preliminary experiment confirmed that an approximate 50% increase in Rrs was the adequate cut-off value corresponding to a 20% decrease in FEV1 in term of sensitivity (100%; specificity, 92%).

Thirty patients were then prospectively studied, of whom 23 patients who received 2,000 μg methacholine were included; their characteristics are described in Table 1. A significant relationship was evidenced between the degree of FEV1 decrease and both log values of CANO and J'aw NO (Fig 2). The percentage of FVC decrease was also correlated with log values of CANO and J'aw NO (p = 0.009, R = 0.53; and p = 0.006, R = 0.56, respectively). A highly significant relationship was logically evidenced between the degree of FEV1 decrease and the log value of FENO calculated for an expiratory flow rate of either 50 mL/s (p = 0.0003, R = 0.68) or 200 mL/s (Fig 2). The median value of J'aw NO was significantly higher in patients with AHR than in patients without AHR (174 nL/min [range, 82 to 327 nL/min] vs 35 nL/min [range, 16 to 44 nL/min] respectively, p < 0.001); conversely CANO was not significantly different whether of not patients had AHR (8.7 parts per billion [ppb]; range, 6.0 to 12.0 ppb; vs 7.9 ppb [range, 3.7 to 9.3 ppb], respectively).

**Phase 2**

Forty-four consecutive patients were prospectively screened, of whom 38 patients were included in phase 2. In the first 20 patients in whom a significant effect of DI was evidenced (modification of Rrs > 20% as compared with the value of Rrs before DI, inasmuch as the coefficient of variation of Rrs measure is 10%).18,22 the median duration of DI effect (either bronchodilatory or bronchoconstrictive) was

| Table 1—Characteristics of the Patients Enrolled in the Two Phases of the Study* |
|-----------------|-----------------|-----------------|
| Characteristics  | Phase 1 (n = 23) | Phase 2 (n = 38) |
| Age, yr         | 41 (27–53)      | 44 (32–52)      |
| Male/female     | 7/16            | 15/23           |
| Challenge test indication |
| Asthma symptoms | 13              | 12              |
| Chronic cough   | 5               | 16              |
| Nasal polyposis | 5               | 10              |
| Baseline PFT FEV1, % predicted | 97 (92–106) | 103 (91–110) |
| PFT at 2,000 μg methacholine, % | |
| FEV1, decrease | 22 (16–36)      | 19 (8–26)       |
| Rrs increase    | 52 (41–87)      | 45 (20–88)      |
| AHR among all tested† | 17/30         | 21/44          |
| AHR among included† | 12/23        | 19/38          |
| Total dose of methacholine, μg | |
| 100             | 0               | 0               |
| 400             | 2               | 2               |
| 1,000           | 5               | 4               |
| 2,000 (inclusion criterion) | 23            | 38              |

*Data are presented as median (25–75th percentile) or No.†FEV1, decrease ≥ 20% as compared to diluent effect.

**Figure 2.** Relationship between airway responsiveness and exhaled NO. Airway responsiveness was defined by the percentage of decrease in FEV1 in the 23 patients of phase 1 of the study who inhaled a total dose of 2,000 μg methacholine. Upper panel: The relationship of responsiveness with CANO (log CANO) calculated using the multiple flow analysis of exhaled NO. Middle panel: Relationship of responsiveness with J'aw NO (log J'aw NO) calculated using the multiple flow analysis of exhaled NO. There was no significant relationship between CANO and J'aw NO. Lower panel: Relationship of responsiveness with FENO0.2 (log FENO0.2).
65 s (range, 35 to 120 s) [see “Preliminary Experiment of Phase 2”]. This result allowed considering that the mean value of $R_{rs}$ that is measured over approximately 1 min (see “Materials and Methods”) is representative of the global effect of DI. When considering only the first 17-s sequence of $R_{rs}$ measurement, only two patients shifted from the group without effect of DI to the group with a bronchodilatory effect, suggesting a very transient effect in some patients.

The characteristics of the 38 consecutive patients who were prospectively enrolled in the phase 2 are shown in Table 1. There was no significant relationship between the effect of DI and the degree of AHR (percentage of $FEV_1$ decrease). The effect of DI was not correlated with either alveolar NO concentration or $J_{aw}$. The effect of DI was negatively correlated with distal airflow limitation (predicted value of maximal expiratory flow between 25% and 75% of vital capacity [$MEF_{25–75}$]; Fig 3, upper panel) and with an index of airway size ($MEF_{25–75}/FVC$; Fig 3, lower panel).

No significant difference in term of effect of DI, NO parameters ($CANO$ and $J_{aw}$), and pulmonary function test (PFT) results were evidenced considering the challenge testing indication (Kruskal-Wallis test). In this phase, a significant relationship was evidenced between the degree of $FEV_1$ decrease and the log value of $CANO$ ($p = 0.012, R = 0.42$), but no significant relationship was evidenced with the log value of $J_{aw}$ ($p = 0.18$). A significant relationship was evidenced between the degree of $FEV_1$ decrease and the log value of FENO calculated for an expiratory flow rate of 200 mL/s ($p = 0.028, R = 0.36$), but the relationship was not significant using the log value of FENO calculated for an expiratory flow rate of 50 mL/s ($p = 0.55$).

**DISCUSSION**

The main result of this study is to suggest that the degree of airway responsiveness is positively correlated with an increase in concentration of NO from the expansible compartment of the lung. This result could partly account for the discrepant results that have previously been obtained by other investigators since the contribution of NO output from distal airways among total NO output depends on the level of expiratory flow rate (for instance, 31% at 50 mL/s and 61% at 200 mL/s in healthy subjects). This hypothesis is further suggested by our results in the second phase demonstrating a significant relationship between the percentage of $FEV_1$ decrease with FENO calculated for an expiratory flow rate of 200 mL/s (the value recommended by European Respiratory Society task force) but not with FENO value calculated at 50 mL/s (the value recommended in American Thoracic Society guidelines). It has to be noted that the values of alveolar NO concentration in patients without AHR tended to be higher than our normal values (approximately 5 ppb). No definite explanation can be given, but unsuspected alveolitis may account for minimal distal inflammation in patients who were referred for asthma symptoms or chronic cough.

Franklin and coauthors demonstrated that exhaled NO was raised in children with a combination of atopy and increased airway responsiveness independent of symptoms. Raised exhaled NO seems to be associated with an underlying mechanism linking atopy and responsiveness; we may hypothesize that exhaled NO produced by distal airways could consti-
stitute this link. We recently described correlations between parameters of exhaled NO, ie, CANO and JawNO, with both inflammation and airway remodeling, both underlying AHR physiopathology, in children with refractory asthma. It remains to determine whether the increase in distal NO output constitutes a protective response or a detrimental factor due to alveolar inflammation. The capability of endogenous NO to modulate airway responsiveness is well demonstrated. All of these data are confirmed by in vitro studies in airways with intact epithelium, but not in epithelium-denuded airways, indicating a role for the epithelium as a source of bronchoprotective NO in the airways. Despite a modest bronchodilatory effect of endogenous or exogenous NO, epithelial-derived NO and resulting exhaled NO may have more subtle effects such as modifications of bronchomotor tone. Therefore, we hypothesized that different amounts of basal NO output could be associated with variable effects of DI during methacholine challenge inasmuch as DI has demonstrated both bronchodilatory and bronchoprotective effects of short duration, the latter not being assessed in this study.

The evaluation of DI effect has been made by several means, as airway resistance assessment or partial flow-volume curves. These methods are not easily performed by all patients, and could miss some DI effect of short duration (few seconds). By contrast, measurement of DI effect using Rs allows an immediate and continuous monitoring of DI effect and has been utilized by several authors even if some bias, due to modifications of chest wall resistance, have recently been discussed by Black and coauthors.

In these patients referred for methacholine challenge testing, we selected a subgroup of patients exhibiting no AHR or a mild degree of AHR who received the same cumulative dose of methacholine (2,000 μg), and we compared the degree of responsiveness assessed by both Rs increase and FEV1 decrease. The effect of DI varied from bronchoconstriction to bronchodilation in these patients with various degrees of responsiveness. The factors that determine either bronchodilatory or bronchoconstrictive effect of DI during methacholine challenge remain still debated. We were unable to demonstrate a significant relationship between basal NO output from proximal or distal airways and the effect of DI. This may be ascribable to the fact that patients with miscellaneous conditions were enrolled in our study, which constitutes the main limitation of this study.

It has been shown that a substantial number of patients may have a significant increase in resistance of airways without significant reduction in FEV1 in response to methacholine, a fact that has been attributed to either a dilatory effect of DI or larger intrinsic airway size relative to lung size. Parker and McCool demonstrated that MEF25–75/FVC ratio, which is thought to be an index of airway size relative to lung size, was negatively associated with the degree of methacholine airway responsiveness. Interestingly, in our study both the percentage of predicted value of MEF25–75 and the MEF25–75/FVC ratio were correlated with the effect of DI. This could suggest that patients exhibiting a mild degree of distal airflow limitation (decreased MEF25–75) or smaller airways (low MEF25–75/FVC ratio) are more prone to have constriction due to their DI. Along this line, Lutchen and coauthors have conjectured that inflammation and wall remodeling facilitate a dangerous degree of heterogeneous constriction inclusive of airway closures or near closures, and contribute to the prevention of a DI from having a residual bronchodilatory effect. In conclusion, airway responsiveness is mainly associated with an increase in distal origin of NO output, and no relationship between exhaled NO and the subsequent effect of DI was evidenced.

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