



## Techniques and Procedures

## Determining the Alveolar Component of Nitric Oxide in Exhaled Air: Procedures and Reference Values for Healthy Persons

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## ABSTRACT

Nitric oxide (NO) production has been described using a 2-compartment model for the synthesis and movement of NO in both the alveoli and the airways. The alveolar concentration of NO ( $C_{A_{NO}}$ ), an indirect marker of the inflammatory state of the distal portions of the lung, can be deduced through exhalation at multiple flow rates. Our objective was to determine reference values for  $C_{A_{NO}}$ . The fraction of exhaled NO ( $F_{E_{NO}}$ ) was measured in 33 healthy individuals at a rate of 50 mL/s; the subjects then exhaled at 10, 30, 100, and 200 mL/s to calculate  $C_{A_{NO}}$ . A chemiluminescence analyzer (NIOX Aerocrine) was used to perform the measurements. The mean (SD)  $F_{E_{NO}}$  was 15 (6) ppb. The mean  $C_{A_{NO}}$  was 3.04 (1.30) ppb. These values of  $C_{A_{NO}}$  measured in healthy individuals will allow us to analyze alveolar inflammatory behavior in respiratory and systemic processes.

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**Determinación de la concentración de óxido nítrico alveolar en aire espirado: procedimiento y valores de referencia en personas sanas**

## RESUMEN

La producción de óxido nítrico (NO) se describe mediante un modelo bicompartimental que relaciona la producción y la movilidad de NO desde los alvéolos hacia las vías aéreas. La espiración a múltiples flujos permite deducir la concentración alveolar de NO ( $C_{A_{NO}}$ ), marcador indirecto del estado inflamatorio de las zonas distales del pulmón. El objetivo fue determinar los valores de referencia de  $C_{A_{NO}}$ . En 33 individuos sanos se determinaron la concentración espirada de NO ( $F_{E_{NO}}$ ) a 50 ml/s y la  $C_{A_{NO}}$  a 10, 30, 100 y 200 ml/s mediante un sensor de quimioluminiscencia (NIOX Aerocrine). El valor medio ( $\pm$  desviación estándar) de  $F_{E_{NO}}$  fue de  $15 \pm 6$  ppb y de  $C_{A_{NO}}$  fue de  $3,04 \pm 1,30$  ppb. Los valores de  $C_{A_{NO}}$  obtenidos en individuos sanos permitirán analizar el comportamiento inflamatorio alveolar en procesos respiratorios y sistémicos.

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## Palabras clave:

Óxido nítrico en aire espirado

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## Introduction

Nitric oxide (NO), a lipophilic gas with a short biological half-life, is synthesized in the airway epithelium mainly by the type 2 isoform of NO synthase, also known as inducible NO synthase (iNOS), a constitutive enzyme that activates inflammatory cytokines, macrophages, and certain adhesion molecules. NO intervenes in the inflammatory process implicated in various respiratory diseases and also acts an immunomodulator, platelet activation inhibitor, and potent vasodilator. iNOS enzyme overactivity has been observed in certain processes and is reflected in increased NO production.<sup>1</sup>

The development of a simple, noninvasive way to measure the fraction of NO in expired air ( $FE_{NO}$ ) in recent years has provided an indirect, reliable way to quickly assess the degree of inflammation in certain respiratory diseases such as asthma.<sup>2</sup> Elevated  $FE_{NO}$  levels in patients with asthma have been observed to decrease with corticosteroid therapy.<sup>3,4</sup> The simplicity and reliability of this technique has made it useful in the diagnosis of asthma and for monitoring symptoms and adherence to therapy, serving to prevent exacerbations, as increased inflammation can be detected and treated quickly.<sup>3,4</sup>

However, exhaled NO has many physiologic sources in the lung and, unlike other endogenous gases such as nitrogen, its measurement depends to a large extent on expiratory flow. A model of the lung based on 2 well-differentiated compartments—the alveoli and the bronchi—has been proposed to improve our understanding of NO exchange dynamics and assessment.<sup>5-7</sup> Thus, in addition to the routine measurement of  $FE_{NO}$ , which is performed at a fixed rate of flow and which provides a marker of bronchial inflammatory activity, estimation of the alveolar concentration of NO ( $CA_{NO}$ ) has been proposed as an indicator of inflammation in the most distal portions of the respiratory system, at the alveolar-capillary membrane, as well as a reflection of endothelial events. With  $CA_{NO}$  assessment still in its preclinical stages, we sought to contribute to further development of the 2-compartment model for NO distribution as reflected in values obtained from multiple-flow measurements, to describe how the technique is carried out, and to establish reference values for  $CA_{NO}$  in healthy subjects.

## Description of the Procedure

### Determination of $FE_{NO}$ at a Constant Flow

$FE_{NO}$  is measured by a chemiluminescence analyzer in air exhaled at a constant flow of 50 mL/s according to international guidelines.<sup>8</sup> We used the NIOX analyzer (Aerocrine AB, Stockholm, Sweden), through which the patient inhales to reach total lung capacity and then exhales at a steady rate of 50 mL/s through a mouthpiece with a resistance of 20 cm H<sub>2</sub>O to ensure velum closure, thus preventing contamination by nasal NO. The analyzer discards the initial peak, basing a valid measurement on the 3-second plateau with a maximum variability of 10% from the horizontal line. The average of measurements taken from 3 valid procedures is recorded.<sup>8</sup>

### Determination of $CA_{NO}$ With the Multiple-Flow-Rate Technique

$CA_{NO}$  can be assessed in a similar fashion, by taking measurements at multiple expiratory flow rates with the same chemiluminescence analyzer described above. In the  $CA_{NO}$  procedure, the patient exhales steadily several times from total lung capacity at 3 or 4 flow rates

between 10 and 500 mL/s. The NO concentration ( $V_{NO}$ ) measured in picoliters per second is plotted for each expiratory flow rate.<sup>9</sup> The subjects in our study exhaled at 4 rates of 10, 30, 100, and 200 mL/s. The values for 100 and 200 mL/s were inserted into the formulas of Tsoukias and George<sup>6</sup> and Silkoff et al<sup>7</sup> to calculate the following flow-independent variables:  $CA_{NO}$ , maximum total airway flow ( $J'_{AW}$ ) of NO, and airway diffusing capacity ( $D_{AW}$ ) of NO.<sup>9</sup>

### Subjects

Thirty-six healthy nonsmokers (16 men, 20 women) were recruited. None had a history of atopy or had suffered illnesses in the 6 months before testing; nor had they been taking any medications. The spirometry results of all the volunteers were within reference limits.

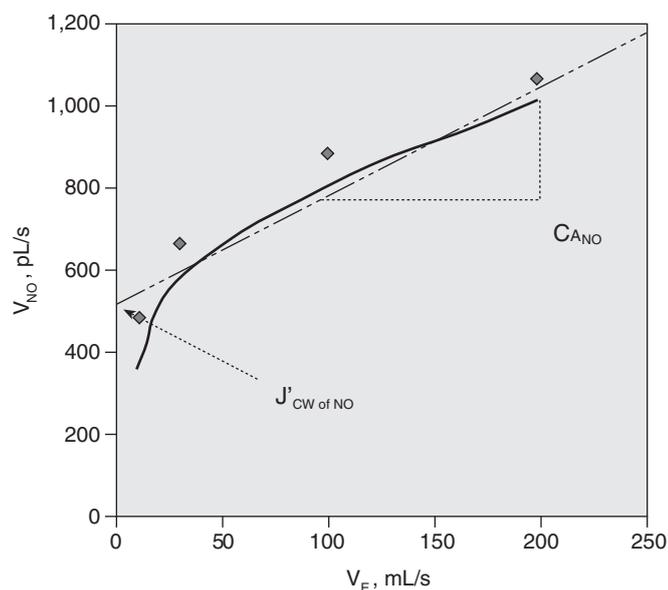
$FE_{NO}$  was measured at a fixed flow of 50 mL/s with the chemiluminescence analyzer.  $CA_{NO}$  was calculated based on readings at multiple expiratory flow rates of 10, 30, 100, and 200 mL/s. Measurements were always made at the same time of day, approximately 2 hours after a meal.

### Statistical Analysis

The mean (SD) was used to describe values of  $FE_{NO}$ ,  $CA_{NO}$ , and  $J'_{AW}$  and  $D_{AW}$  of NO. Pearson linear correlation analysis was used to compare the values. The analysis was 2-sided in all comparisons and the usual level of significance of 5% ( $\alpha = 0.05$ ) was chosen. The SPSS package, version 11.5 (SPSS Inc, Chicago, Illinois, USA) was used for all analyses.

## Results

Three of the 36 volunteers initially recruited were excluded because they did not perform the exhalation maneuver correctly. The remaining 33 subjects (17 women, 16 men), all nonsmokers, had



**Figure 1.** Derivation of the concentration of alveolar nitric oxide ( $CA_{NO}$ ).  $J'_{AW}$  indicates the maximum flow of NO in the airway compartment;  $V_E$ , expiratory flow expressed in mL/s;  $V_{NO}$ , maximum flow of NO during an exhalation maneuver at a specific flow rate expressed in picoliters per second.

a mean age of 36 (11) years and body mass index (BMI) of 23.8 (2.8) kg/m<sup>2</sup>. Mean values for lung function parameters were within the predicted ranges: forced expiratory volume in 1 second, 106% (11%); forced vital capacity, 102% (10%); and the ratio between those 2 parameters expressed as a percentage, 81% (6%).

The mean  $F_{E_{NO}}$  was 15 (6) ppb (range, 5.5-27 ppb);  $C_{A_{NO}}$ , 3.04 (1.30) ppb, (range, 1.45-6.31 ppb);  $J'_{AW \text{ of } NO}$ , 573 (145) pL/s (range, 113-1755 pL/s) (Figure 1); and  $D_{AW \text{ of } NO}$ , 4.49 (3) ppb/s. The correlations between  $F_{E_{NO}}$  and  $C_{A_{NO}}$  values on the one hand and age, sex, BMI, and spirometric values on the other were not statistically significant ( $P > .05$ ).

Nor were  $F_{E_{NO}}$  and  $C_{A_{NO}}$  levels significantly correlated in this group of individuals ( $r = 0.1$ ;  $P = .4$ ).

## Discussion

The 2-compartment model used to provide a better explanation of NO exchange dynamics assumes the existence of 2 zones or compartments that are theoretically well separated,<sup>5-7</sup> and that define the source of NO in expired air: the airways and the alveoli. The behavior of these compartments is described by 3 flow-independent parameters. The airway compartment is defined by the NO  $J'_{AW}$  and  $D_{AW}$  values. According to Fick's law, the production of NO in the airway is proportional to the difference between the concentration in the lumen and the concentration in the bronchial wall, which in turn is proportional to the  $D_{AW}$  of NO.<sup>10</sup> The concentration in the alveolar compartment, on the other hand, is defined by the third flow-independent parameter,  $C_{A_{NO}}$ , which is dynamic, changes during the breathing cycle, and reflects the balance between NO produced locally and NO that diffuses into the airway (Figure 2).

Alveolar NO is carried through the airways during expiration, such that the final exhaled concentration will be the sum of the gas transported from the alveolar space along the entire length of the airway.<sup>9</sup> With this model and these parameters, the concentration of

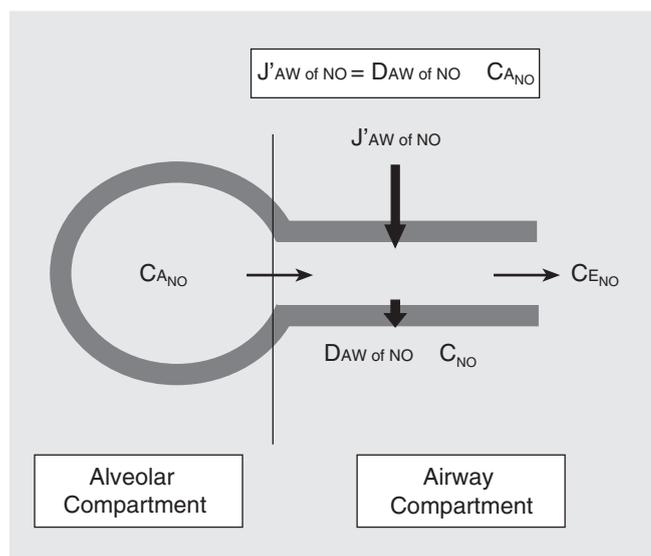
NO at any flow rate can be predicted if the paranasal sinus gas is excluded (Figure 2).

The procedure for determining the 3 flow-independent parameters involves exhaling at different constant flow rates according to a standardized method which is not used in routine clinical practice.<sup>5-7</sup> The procedure requires a chemiluminescence analyzer: the device normally used to measure  $F_{E_{NO}}$  is appropriate if it allows exhalation at different flow rates.

In the 1980s, a mathematical model applied to the measurements recorded by the analyzer was developed to reliably and reproducibly estimate the flow-independent parameters needed to apply the 2-compartment model.<sup>9</sup> Tsoukias and George<sup>6</sup> used a procedure based on measuring 2 flows (at flow rates between 100 and 500 mL/s). If the  $V_{NO}$  for each expiratory flow is plotted against the total expiratory flow for each maneuver ( $V_E$ ) expressed in milliliters per second, 2 of the parameters may be derived from the line joining the 2 points:  $C_{A_{NO}}$  will be represented by the slope of the line and the  $J'_{AW}$  of NO by the intercept (Figure 1). The equation of Silkoff et al,<sup>7</sup> meanwhile, can be used to determine the  $D_{AW}$  of NO, by multiplying the  $J'_{AW}$  by  $C_{A_{NO}}$ . The table 1 shows how other authors have performed more complex mathematical operations to calculate the same parameters.

The mean value of 3.04 (1.30) ppb we obtained for  $C_{A_{NO}}$  falls within the range of 1.0 to 5.6 ppb described in the literature for healthy persons<sup>11,12</sup> and is consistent with distal airway NO measurements ( $C_{A_{NO}}$ ) obtained by means of bronchoalveolar lavage during fiberoptic bronchoscopy in healthy individuals.<sup>12</sup> The  $C_{A_{NO}}$  values we recorded are also comparable to those obtained in healthy individuals in case-control studies on inflammatory respiratory diseases such as asthma or chronic obstructive pulmonary disease (COPD).<sup>10</sup> The values of 573 pL/s obtained for the  $J'_{AW}$  of NO and 4.49 ppb/s for the  $D_{AW}$  also fall within normal ranges in the literature (420-1280 pL/s for  $J'_{AW}$  and 3.1-9.2 ppb/s for  $D_{AW}$ ).<sup>5-11</sup>

The procedure for analyzing multiple flows by chemiluminescence is a simple, reliable, and accurate way to obtain  $C_{A_{NO}}$  values and establish reference ranges for healthy individuals. As  $C_{A_{NO}}$  reflects peripheral, distal inflammation (unlike  $F_{E_{NO}}$ , which marks bronchial inflammation), many authors have attempted to determine  $C_{A_{NO}}$  levels in relation to respiratory diseases in which such inflammation is implicated. Abnormal  $C_{A_{NO}}$  levels have been reported for patients with asthma, COPD, and interstitial lung disease associated or not with scleroderma.<sup>13-16</sup> Lehtimäki et al<sup>11</sup> conducted a case-control study in patients with asthma, patients with alveolitis, and healthy controls. The levels of NO  $J'_{AW}$  in asthma patients were higher than those in healthy individuals or patients with alveolitis (2.5 ppb for asthma patients vs 0.1 and 0.7 ppb for alveolitis patients and controls, respectively).<sup>11</sup> In contrast,  $C_{A_{NO}}$  was higher (4 ppb) in patients with alveolitis, whereas asthmatics and healthy controls had similar lower levels of around 1 ppb. The inclusion of asthmatic patients with only slight lung function impairment (mild asthma), those with untreated or recently diagnosed asthma, and those with had bronchial inflammation but little peripheral inflammation might explain the differences in  $C_{A_{NO}}$  values and the elevated  $J'_{AW}$  values in asthmatics. Consistent with that study, Brindicci et al<sup>17</sup> found that patients with severe asthma and others with exacerbated asthma had higher  $C_{A_{NO}}$  levels than healthy individuals or patients with mild asthma. The bronchial NO concentration was higher in patients with mild asthma, however. Similar results have been reported by others.<sup>14-18</sup> A negative correlation between the severity of asthma symptoms and bronchial NO, and a positive correlation between symptoms and alveolar NO has been demonstrated.<sup>18</sup> These results demonstrate that the clinical



**Figure 2.** Two-compartment model of nitric oxide (NO). The concentration of NO in expired air ( $C_{E_{NO}} = F_{E_{NO}}$ ) is the sum of NO concentrations in the alveolar and airway compartments, determined on the basis of 3 flow-independent parameters: the maximum flow ( $J'_{AW}$ ) of NO in the airway compartment, the airway compartment's NO diffusion capacity ( $D_{AW}$ ), and the concentration of NO in the alveolar compartment ( $C_{A_{NO}}$ ).

**Table**Multiple-Flow-Rate Exhalation Techniques, With Mathematical Models<sup>a</sup>

	Flow-Independent Parameters for NO			Exhalation Technique at Multiple Flow Rates
	$J'_{aw}$	$D_{aw}$	$C_{A_{NO}}$	
Tsoukias and George <sup>6</sup>	Yes		Yes	2 exhalations, at flows between 100 and 500 mL/s
Pietropaoli et al <sup>5</sup>	Yes	Yes		2 exhalations, at flows between 100 and 500 mL/s
Silkoff et al, <sup>7</sup> 2 flow rates	Yes	Yes		1 exhalation, at a flow between 15 and 50 mL/s
Silkoff et al, <sup>7</sup> 9 flow rates	Yes	Yes	Yes	9 exhalations, at flows between 4.2 and 1550 mL/s
George et al <sup>9</sup>	Yes	Yes	Yes	3 exhalations, at flows between 5 and 500 mL/s

<sup>a</sup>Based on George et al<sup>9</sup>. Equations:  $C_{E_{NO}} = (J'_{awNO} \times 1/V_E) + C_{A_{NO}}$  (from Pietropaoli et al<sup>5</sup>);  $V_{NO} = (C_{A_{NO}} \times V_E) + J'_{awNO}$  (from Tsoukias y George<sup>6</sup>);  $D_{awNO} = V_E \times (C_{E_{NO}} - C_{A_{NO}})/(C_{awNO} - C_{A_{NO}})$  (from Hogman, as cited in George<sup>6</sup>); and  $J'_{awNO} = D_{awNO} \times C_{AW_{NO}}$  (from Silkoff et al<sup>7</sup>). Two flow rates were used to determine  $C_{A_{NO}}$ . The slope of the line for  $V_E$  and  $V_{NO}$  between 2 flows (100 and 500 mL/s) indicates the value of  $C_{A_{NO}}$ .<sup>9</sup>  $C_{E_{NO}} = FE_{NO}$  indicate fractional concentration of expiratory NO at a fixed flow. Abbreviations:  $C_{A_{NO}}$ , mean alveolar nitric oxide (NO) concentration expressed in ppb;  $C_{aw}$ , mean bronchial wall NO concentration;  $D_{aw}$ , NO diffusion capacity of the airway compartment;  $J'_{aw}$ , maximum flow of NO in the airway compartment;  $V_E$ , expiratory flow recorded for each flow rate (10, 30, 100 L/s, etc)<sup>8</sup> expressed in mL/s;  $V_{NO}$ , maximum flow of NO achieved during an exhalation maneuver at a specific flow rate expressed in pL/s.

status of patients with severe or therapy-resistant asthma is more dependent on the degree of alveolar inflammation than bronchial inflammation. It could therefore be very useful to measure  $C_{A_{NO}}$  as a more precise reflection of the effectiveness of treatment than  $FE_{NO}$  in these patients.  $C_{A_{NO}}$  is a useful marker of alveolar inflammation in diseases involving the distal portions of the lung (severe asthma, interstitial pneumonia, COPD), as it quantifies alveolar damage and monitors the course of disease.

A limitation of the technique is that airway concentrations of NO are affected by smoking, with highly variable and sometimes contradictory results reported for  $FE_{NO}$ . In some studies, smokers have lower  $FE_{NO}$  values than nonsmokers; the levels rise when they quit, although normal reference values are never reached.<sup>19,20</sup> Smokers also have lower  $C_{A_{NO}}$  levels than ex-smokers (0.93 vs 1.41 ppb) and nonsmokers (1.32 ppb), explained by the association between endothelial dysfunction and the toxic effect of tobacco.<sup>19,20</sup> Cigarettes contain low concentrations of a large number of free radicals and pro-oxidant substances that diminish the activity of NO and favor a state of oxidative stress.<sup>19</sup> Smokers also have a deficit of tetrahydrobiopterin, a cofactor needed for the synthesis of NO by endothelial NOS, as a result of the absorption of aromatic amines that inhibit its synthesis<sup>21</sup>; this further contributes to the production of superoxide molecules and oxidative stress.<sup>19</sup> Tobacco also contains the amino acid N-nitrosamine, which can inhibit NO production by alveolar macrophages.<sup>20</sup> Together these mechanisms explain the low  $FE_{NO}$  and  $C_{A_{NO}}$  levels in COPD patients who are smokers or lapsing ex-smokers.<sup>20</sup> Because of these effects, these measurements will be of limited value in smokers, in whom they cannot be taken into consideration when diagnostic or therapeutic decisions are taken. Other limitations of the technique are related to the need for patient cooperation and the difficulty some have in carrying out the exhalation maneuver. Contraindications have not been described.

In summary the 2-compartment model for determining flow-independent parameters distinguishes the sources of NO and accounts for dynamic exchange in the airway and alveolar compartments, which are each anatomically distinct from one another as well as responsible for different respiratory diseases. This model also provides a better description of metabolic and structural changes that occur in diseases involving one of the compartments. The most important flow-independent parameter for describing the state of inflammation in the distal portion of the respiratory system is  $C_{A_{NO}}$ . The method of exhalation at multiple flows is an easy,

noninvasive way to measure  $C_{A_{NO}}$  quickly and reliably, allowing the 2 compartments to be modelled. Once normal reference values have been established for this parameter, the levels that can be expected in patients with distal respiratory inflammation should be determined.  $C_{A_{NO}}$  could then be used as an inflammatory marker that would be useful for monitoring symptoms and response to therapy, and even for indicating prognosis in severe asthma, therapy-resistant asthma, or interstitial lung disease.

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