Association of Alveolar Nitric Oxide Levels with Pulmonary Function and Its Reversibility in Stable Asthma

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Key Words

Airway reversibility · Alveolar nitric oxide · Asthma · Impulse oscillometry system · Peripheral airway dysfunction · Trumpet model with axial diffusion

Abstract

Background: Inflammation of peripheral airways is implicated in the pathophysiology of severe asthma. However, contributions of peripheral airway inflammation to airway caliber/function in patients with stable asthma, including those with mild to moderate disease, remain to be confirmed. **Objectives:** To determine whether peripheral airway inflammation affects airway function in patients with asthma. **Methods:** In 70 patients with mild to severe asthma, alveolar nitric oxide [CANO(TMAD)] levels were examined as a noninvasive biomarker of peripheral airway/alveolar inflammation. CANO(TMAD) and maximal nitric oxide (NO) flux in the airway compartment, J_{aw}NO, were estimated with a model that incorporated trumpet-shaped airways and axial diffusion using exhaled NO output at different flow rates. Measures of pulmonary function were then assessed by spirometry and an impulse oscillometry system, and their bronchodilator reversibility was examined. **Results:** CANO(TMAD) levels were not correlated with pre- or postbronchodilator spirometric values, but were significantly associated with prebronchodilator reactance at low frequency (X_{rs5}) ($\rho = -0.31$, $p = 0.011$), integrated area of low-frequency X_{rs} (AX) ($\rho = 0.35$, $p = 0.003$) and negative frequency dependence of resistance (R_{rs5}–R_{rs20}) ($\rho = 0.35$, $p = 0.004$). Furthermore, CANO(TMAD) levels were associated with bronchodilator reversibility of FEV₁, FEF_{25–75%}, X_{rs5} and AX ($\rho = 0.35, 0.31, -0.24$ and -0.31 , respectively; $p \leq 0.05$ for all). No variables were related to J_{aw}NO. **Conclusions:** Elevated CANO(TMAD), but not J_{aw}NO, partly reflects reversible airway obstruction originating in the peripheral airway. These findings indicate the involvement of peripheral airway inflammation in physiological abnormalities in asthma.

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Introduction

Asthma is a chronic inflammatory disorder involving proximal airways as well as small peripheral airways [1–4]. Nitric oxide (NO) is a well-established noninvasive biomarker of inflammation in asthmatic airways [5]. Recently, the alveolar fraction of exhaled NO (CANO) has been introduced to assess inflammation of peripheral airway/alveolar regions [6]. This variable has received renewed interest as a noninvasive tool for both adults [7] and children [8]. Although CANO is mathematically derived from NO output measured at multiple flow rates, a study by Berry et al. [9] has shown a strong correlation between CANO levels and eosinophil counts in bronchoalveolar lavage fluid, suggesting that CANO may reflect peripheral airway/alveolar inflammation. Moreover, two trumpet models with axial diffusion have recently been developed to correct the effect of axial contamination by small-airway NO diffusion on CANO [10, 11]. This new parameter, CANO(TMAD), precludes overestimating actual NO production in the alveolar region.

Peripheral airway dysfunction or the degree of air trapping is associated with recurrent exacerbations of severe asthma [12] or with disease severity [13]. Effective prevention of peripheral airway dysfunction requires an understanding of the contribution of peripheral airway inflammation to airway dysfunction. However, such a contribution has been confirmed only for severe refractory asthma [14, 15]. Because peripheral airway inflammation is not limited to patients with severe disease [1], we hypothesized that sustained inflammation in the peripheral airway or alveolar region may be linked to airway dysfunction in the general population of patients with asthma. In this study, we measured CANO(TMAD), pulmonary function and pulmonary function reversibility in response to treatment with a bronchodilator (BD) for patients with asthma ranging from mild intermittent to severe persistent. Using an impulse oscillometry system (IOS) [16, 17], we examined negative frequency dependence and low frequency reactance that could be proxies for peripheral airway function.

Methods

Subjects

This cross-sectional study was conducted at the outpatient asthma and cough clinic of Kyoto University Hospital from January through December 2007. We examined NO levels at different flow rates (50, 100 and 200 ml/s) in 75 adults with stable asthma.

The inclusion criteria were as follows: (1) a diagnosis of asthma as defined by the American Thoracic Society [18] on the basis of a history of recurrent episodes of wheezing and chest tightness with or without cough; (2) confirmation of airway hyperresponsiveness on past evaluations; (3) regular attendance at our clinic for >3 months; (4) no exacerbation of asthma during the last 4 weeks; (5) no changes in treatment during the last 4 weeks; (6) nonsmokers or ex-smokers who had smoked <10 pack-years but had not smoked for >1 year; (7) no evidence of other respiratory diseases on chest X-ray findings. Severity was defined according to the step classification of the 2004 version of the Global Initiative for Asthma guidelines [19]. Three patients with step 2 asthma were treated with leukotriene receptor antagonists because they refused to use inhaled corticosteroids (ICS). To examine patients with persistent airway inflammation, we measured NO levels at higher flows in patients with fraction of exhaled NO (FeNO) at 50 ml/sec (FeNO₅₀) values ≥ 25 ppb [5]. The ethics committee of our institution approved the study protocol and written informed consent was obtained from each participant.

Measurement of NO and Calculation of CANO(TMAD) and Bronchial NO Maximal Flux

NO levels were measured with a chemiluminescence analyzer (NOA 280; Sievers, Boulder, Colo., USA) according to current guidelines, apart from the expiratory flow rate [20]. The analyzer was calibrated daily with gas without NO generated by exposing ambient air to NO scavengers and a standard concentration of 640 ppb NO. The lower detection limit for NO was 2 ppb. The signal output from the NO analyzer was fed to a computer data acquisition program, and concentrations were measured using a data analysis program (NOA Analysis™ Software; Sievers). Seated subjects inserted a mouthpiece, inhaled orally to total lung capacity, exhaled immediately against a resistance and maintained mouth pressure at 20 cm H₂O, displayed on a pressure gauge. The steady-state NO plateau was taken as the FeNO value. By varying expiratory resistances, we measured FeNO levels at three expiratory flows of 50, 100 and 200 ml/s in that order. If an adequate NO plateau was not reached at 200 ml/s, patients were then instructed to exhale at a flow of 150 ml/s instead ($n = 4$).

CANO(TMAD) was estimated using a model recently developed by Condorelli et al. [10] that incorporated trumpet-shaped airways and axial diffusion rather than simply assuming that the lung was comprised of two separate regions with a rigid airway compartment and a well-mixed expandable compartment [6]. The equation used was the following: $\text{CANO(TMAD)} = \text{slope} - \text{intercept}/740$. The slope and intercept of this equation were determined from a linear regression line after plotting NO output (that is, exhaled NO level times expiratory flow) as a function of expiratory flow. When CANO(TMAD) values were below zero, they were assigned a value of zero. Bronchial NO maximal flux (J_{aw}NO), a marker of inflammation in the airway compartment, was also determined by multiplying the intercept by 1.7. Because this trumpet model with axial diffusion was developed based on the assumption that NO flux from the airway tree was a constant and this simplified equation was valid at an expiratory flow rate between 100 ml/s and 250 ml/s, we applied FeNO levels at 100 and 200 ml/s to obtain a linear regression line between NO output and exhalation flow. Five patients whose results had negative slopes were excluded from further analysis [10].

Table 1. Patients' characteristics (n = 70)

Gender, M/F	33/37
Age, years	57.4 ± 15.6
Disease duration, years	12.2 ± 10.4
Asthma severity ^a , 1/2/3/4	2/35/17/16
Atopy, positive ^b , %	66
IgE, IU	190 (5, 6,800)
Ex-smoker, %	33
Sinusitis/rhinitis, %	66
Daily dose of ICS ^c , µg	400 (0, 1,600)
%FEV ₁ , %	89.4 ± 16.5
%FEF _{25–75%} , %	57.4 ± 26.8
Rrs20, kPa·s·l ⁻¹	0.32 ± 0.11
Rrs5–Rrs20, kPa·s·l ⁻¹	0.06 (–0.03, 0.43)
Xrs5, kPa·s·l ⁻¹	–0.13 (–0.56, 0.17)
AX, kPa·l ⁻¹	0.44 (0.01, 5.51)
FeNO50, ppb	44.9 (25.0, 224.4)
J'awNO, pl·s ⁻¹	3,638 (0, 15,300)
CANO(TMAD), ppb	3.9 (0, 34.9)

Values are means ± SD or medians (with ranges in parentheses).

^a According to the Global Initiative for Asthma guidelines [19].

^b Considered atopic when one or more specific IgE antibodies against HDM, cat dander, dog dander, molds, weed, cedar pollen or grass pollen were positive.

^c Equivalent dose of fluticasone.

Pulmonary Function Tests

After NO measurements, subjects underwent pre- and post-BD (that is, inhalation of 200 µg salbutamol) pulmonary function testing. Respiratory impedance was measured by IOS followed by spirometric testing. Reversibility of the impedance and spirometric values were calculated as follows: (post-BD value – pre-BD value)/pre-BD value.

Spirometry was performed according to the standards of the American Thoracic Society [21] using a ChestGraph HI-701 spirometer (Chest, Tokyo, Japan). FEV₁ and mid forced expiratory flow (FEF_{25–75%}) were determined.

Respiratory impedance was measured using a Jaeger MasterScreen, IOS™ (Erich Jaeger, Hoechberg Germany) that met standard recommendations [22]. The IOS is different from the classical forced oscillation technique by the use of an impulse rather than a pseudorandom noise signal and in data processing. In brief, rectangular mechanical impulses containing a continuous power spectrum ranging from 0 to 100 Hz, generated by a loudspeaker at intervals of 0.2 s, were applied to the respiratory system through a mouthpiece during tidal breathing. The resulting pressure and flow signals were measured next to the mouthpiece, and were analyzed for amplitude and phase differences using a fast Fourier transform to determine resistance (Rrs) and reactance (Xrs) of the total respiratory system. To reduce loss of energy in the upper airways, the chin and cheeks were supported by the subjects' hands. As proxies for peripheral airway function [16, 17, 23, 24], we used the negative frequency dependence of Rrs be-

tween 5 and 20 Hz (Rrs5–Rrs20), Xrs at 5 Hz (Xrs5) and reactance area (AX) that is the integral of Xrs from 5 Hz to the resonant frequency at which Xrs crosses zero. Rrs20 was used as a measure of proximal airway resistance, as oscillatory pressure components at frequencies ≥ 20 Hz are thought to be damped out before reaching the peripheral airways [16].

Statistical Analysis

Data were analyzed using GraphPad Prism 4.00 (GraphPad Software Inc., La Jolla, Calif., USA) and StatView software 5.0 (SAS Institute Inc., Cary, N.C., USA). When data were normally distributed, linear regression analysis was used. Otherwise, Spearman's rank-correlation test was used. The correlation coefficient from linear regression analysis is denoted as R, whereas Spearman's correlation coefficient is given as rho. Results are expressed as means ± SD or medians (with ranges in parentheses). We considered p values of ≤ 0.05 to indicate statistical significance.

Results

Patients' Characteristics

The characteristics of the 70 subjects are shown in table 1. Measures of pulmonary function and FeNO50 of the 70 subjects did not differ significantly from those of the 5 patients whose slopes were negative (data not shown).

Correlations between Patients' Characteristics and CANO(TMAD) or Bronchial Flux

Patients with more severe asthma showed a trend for higher J'awNO levels than those with milder disease (rho = 0.23, p = 0.056), but not for CANO(TMAD) levels (rho = 0.17, p = 0.17). The presence of atopy, rhinitis and smoking history were not associated with CANO(TMAD) or J'awNO (data not shown).

Correlations of CANO(TMAD) or Bronchial Flux with Pre- and Post-BD Pulmonary Function Measures

The associations of CANO(TMAD) and J'awNO with pulmonary function measures are summarized in table 2. Correlations of pulmonary function measures with FeNO50 that were strongly correlated with J'awNO levels (rho = 0.83, p < 0.0001) are also shown in this table. There was no association of CANO(TMAD) with pre-BD %FEV₁, %FEF_{25–75%} or Rrs20. CANO(TMAD) was significantly associated with pre-BD Rrs5–Rrs20, Xrs5 and AX (fig. 1). The association between Xrs5 and CANO(TMAD) remained significant even when two obvious outliers (one with the highest Xrs5 and another with the lowest Xrs5) were excluded (rho = –0.36, p = 0.003, n = 68). There was no association between

Fig. 1. Relationships between CANO(TMAD) levels and pre-BD Xrs5 (left) and Rrs5-Rrs20 (right).

CANO(TMAD) and any measure of post-BD pulmonary function. J'awNO was not related to any indices of pulmonary function.

Measures of pre-BD Rrs5-Rrs20, Xrs5 and AX were moderately to strongly correlated with each other ($|\rho|$ ranged between 0.75 and 0.93, $p < 0.0001$). These measures of pre-BD IOS were correlated with pre-BD %FEV₁ ($|\rho|$ ranged between 0.33 and 0.40, $p < 0.01$).

Correlations between CANO(TMAD) or Bronchial Flux and Reversibility of Pulmonary Function Measures

Reversibilities of FEV₁ and FEF_{25-75%}, as well as of Xrs5 and AX, were significantly associated with CANO(TMAD) (table 2). J'awNO was not related to any indices of reversibility.

Reversibilities of FEV₁, FEF_{25-75%}, Xrs5 and AX were associated with pre-BD levels; reversibility of FEV₁ was correlated with the pre-BD FEV₁ level ($R = -0.56$, $p < 0.0001$); for %FEF_{25-75%}, $\rho = -0.25$, $p = 0.042$; for Xrs5, $\rho = 0.48$, $p < 0.0001$; for AX, $\rho = -0.40$, $p = 0.001$.

Discussion

To the best of our knowledge, this is the first study to demonstrate that in adult patients with stable asthma, CANO(TMAD), but not J'awNO, is significantly associated with Rrs5-Rrs20, Xrs5, and AX levels, which are thought to reflect peripheral airway function. Our novel findings are that the increase in CANO(TMAD) associates with greater reversibility of FEV₁, FEF_{25-75%}, Xrs5 and AX in response to treatment with a BD. These findings suggest that peripheral airway/alveolar inflamma-

Table 2. Correlation coefficients of NO levels with pulmonary function values

	CANO(TMAD)	J'awNO	FeNO50
Pre-bronchodilator			
%FEV ₁	-0.18	-0.03	-0.12
%FEF _{25-75%}	-0.09	-0.05	-0.10
Rrs20	0.22	-0.17	-0.05
Rrs5-Rrs20	0.35**	-0.09	0.11
Xrs5	-0.31*	-0.05	-0.19
AX	0.35**	-0.03	0.14
Reversibility			
Δ FEV ₁	0.35**	-0.007	0.09
Δ FEF _{25-75%}	0.31*	0.09	0.21
Δ Rrs20	-0.23	-0.007	-0.01
Δ (Rrs5-Rrs20)	-0.22	0.05	0.01
Δ Xrs5	-0.24*	-0.16	-0.25*
Δ AX	-0.31*	-0.16	-0.10

$\Delta = (\text{post-BD value} - \text{pre-BD value})/\text{pre-BD value}$; * $p \leq 0.05$; ** $p < 0.01$. Correlation coefficients of Spearman's correlation coefficients are shown.

tion may contribute to reversible airway obstruction originating in the peripheral airway.

Before the introduction of CANO(TMAD), CANO had been used as a reasonably well-validated marker of inflammation of the peripheral airway/alveolar region [9]. Histological studies have suggested that peripheral airway inflammation has a similar or greater impact on disease severity than proximal airway inflammation [1, 23, 24]. Studies examining CANO have drawn similar conclusions; CANO in refractory asthma is significantly greater than that in mild to moderate asthma [9], and pa-

tients with oral steroid-dependent asthma have higher CANO levels than patients with mild-to-moderate or severe asthma [14, 25]. In this study, J'awNO showed a trend toward increased values in patients with more severe asthma than those with milder disease. However, when CANO(TMAD) was determined using a trumpet model with axial diffusion that estimated NO concentrations exiting the respiratory bronchioles and alveolar region, CANO(TMAD) was not correlated with disease severity, which is consistent with a recent finding by Mahut et al. [26]. Given that CANO is not adjusted for NO axial diffusion from conducting airways and could be more contaminated by small-airway NO diffusion than CANO(TMAD), this discrepancy between previous findings with CANO and those with CANO(TMAD) in this study may indicate that small-airway inflammation influences asthma severity to a greater extent than the actual alveolar inflammation. Indeed, a histological study of severe asthmatics showed that small airways with perimeters less than 6 mm were the most significantly infiltrated by inflammatory cells across the entire airway tree, from the large airways to the alveolar tissue [23].

van Veen et al. [14] found significant correlations of CANO with peripheral airway obstruction, as assessed by residual volume, functional residual capacity and closing capacity, in patients with severe asthma. However, when patients with mild-to-moderate asthma were included, these correlations disappeared, and only a significant association between CANO and the slope of the single breath nitrogen washout curve remained. Although CANO was correlated with $FEF_{25-75\%}$ in children with refractory asthma [15], CANO was not correlated with $FEF_{50\%}$, $FEF_{25\%}$ or residual volume in adults with asthma [25]. These studies provide little or no evidence of a link between peripheral airway inflammation and dysfunction in the population of adults with asthma.

In the current study, however, CANO(TMAD) was modestly, but significantly associated with small-airway reactance/capacitance (Xrs5, AX) and negative frequency dependence of airway resistance (Rrs5–Rrs20). These variables obtained by IOS, a noninvasive and effort-independent tool, have been proposed as relevant and sensitive indices of peripheral airway function [16, 17, 23, 27, 28]. Previous oscillation studies have demonstrated that as peripheral resistance increases, the resistance at low frequencies increases compared to the higher frequencies, thereby resulting in greater negative frequency dependence of Rrs [16, 29]. Xrs at low frequency is also considered to reflect the capacitative properties of peripheral airways and to detect peripheral airway narrowing [30].

In our recent study of patients with asthma, Rrs5–Rrs20 and AX, but not Rrs20, are significantly associated with residual volume [23]. Another recent long-term study of controller therapy for pediatric asthma also demonstrates that AX may be sensitive to changes in peripheral airway function [27]. The nature of these indices of IOS may support our premise that Rrs5–Rrs20, Xrs and, particularly, AX may reflect peripheral airway function. However, as discussed below, the data should be interpreted with caution because IOS indices are indirect measures and no associations of IOS indices with pathological changes have been demonstrated thus far.

Airway variability is another feature of asthma, implying instability of the disease. Endobronchial and transbronchial biopsy studies of nocturnal asthma have demonstrated a significant contribution of peripheral airway inflammation to nocturnal decrement of pulmonary function as compared with proximal airway inflammation [31]. In studies of pediatric asthma, higher FeNO levels are associated with greater FEV_1 reversibility [32–34]. Tsuburai et al. [35] show associations between FeNO levels and FEV_1 reversibility in adult asthmatics with airflow limitation, but not in those without airflow limitation. Our study of adult asthmatics extended the previous findings by showing that smoldering inflammation in the peripheral airway/alveolar compartment is responsible for greater BD response in FEV_1 , $FEF_{25-75\%}$, Xrs5 and AX, and that reversibility to a BD depends on the degree of pre-BD obstruction. Given that CANO(TMAD) was correlated with pre-BD Xrs5 and AX, but not with FEV_1 or $FEF_{25-75\%}$ values, we speculate that peripheral airway/alveolar inflammation may contribute to reversible airway obstruction originating in the peripheral airway.

In sharp contrast to CANO(TMAD), bronchial NO flux (that is, J'awNO levels) was not correlated with any indices of pulmonary function and FeNO50 was only correlated with reversibility of Xrs5, which was consistent with other studies [7, 36]. This discrepancy between CANO(TMAD) and J'awNO could be attributed to differences in their responses to ICS treatment. When patients are treated with an ICS, J'awNO readily decreases, whereas CANO is resistant to them [37]. Therefore, we speculate that any correlation of J'awNO or FeNO50 with pulmonary function might have been modified by ICS treatment. Indeed, in steroid naïve patients, exhaled NO levels are correlated with airway hyperresponsiveness [38], asthma symptom scores [39] or BD response [34], whereas such correlations disappear in patients on ICS. Intriguingly, a recent study shows significant associations between exhaled NO levels and FEV_1 or BD re-

sponses in pediatric asthmatics receiving ICS treatments when they are classified according to their ICS doses [40], which may suggest that stratifying patients by ICS doses could provide deeper insights into the roles of exhaled NO in patients on ICS.

The role of CANO(TMAD) in peripheral airway obstruction should not be overestimated because the associations were modest; elevated CANO(TMAD) is not a strong indicator for low Xrs5 or increased AX. Also, IOS variables were not compared with closing volume or closing capacity, other proxy measures of peripheral airway obstruction. As mentioned above, IOS measurements have several limitations and should be interpreted cautiously [16, 17] because of issues that include artifact effects of upper airway compliance and leaks at the mouthpiece. Increases in Rrs5–Rrs20 may imply heterogeneity of peripheral airway narrowing in addition to an absolute increase in resistance [41]. Xrs5 is relatively noise sensitive, whereas AX is less so. Resistance and reactance in the peripheral airway may indicate different properties, given that pre-BD values of Rrs5–Rrs20 were not correlated with reversibility to a BD, whereas pre-BD values of AX and Xrs5 were, although the precise mechanisms underlying this discrepancy are unknown.

We may have missed some patients with normal FeNO50 and high CANO(TMAD) by excluding those with FeNO50 <25 ppb. When pediatric patients with asthma were grouped based on their J'awNO and CANO(TMAD) levels, Puckett et al. [42] found that 11% of the patients had normal J'awNO and high CANO(TMAD) levels. However, when we examined another 21 patients with stable asthma who had FeNO50 <25 ppb, their ratios of CANO(TMAD) levels to FeNO50 levels did not differ from those of the currently studied population (data not shown), suggesting that patients with normal FeNO50 and high CANO(TMAD) are not

commonly observed in our patient population. Therefore, we believe that the possibility of missing patients with normal FeNO50 and high CANO(TMAD) may not have severely affected our findings.

Five patients were excluded from the analysis because their results had negative slopes in plots of NO output versus expiratory flow between 100 and 200 ml/s. NO axial diffusion from the airway tree increases as bronchial flux increases and its relative impact over NO conductive transport exiting the alveolar region is greater at lower expiratory flows [10]. Therefore, the negative slopes for 5 patients may indicate that, in these patients, the relative impact of NO axial diffusion on NO output with exhalation flows around 100 ml/s might have remained either because of increased bronchial reflux or because of relatively small NO production in the alveolar region. Measurements with higher expiratory flows would have been desirable, but it was difficult for our patients to obtain a steady-state NO plateau with higher flows, such as at 250 or 300 ml/s. Despite these limitations, our findings support the superiority of a multiple flow technique to a conventional single flow measurement, as the former provides information on peripheral airway/alveolar inflammation.

In conclusion, inflammation in the peripheral airway/alveolar region as assessed by CANO(TMAD) partly contributes to the development of peripheral airway obstruction and to reversibility of pulmonary function in response to BD. Monitoring peripheral airway/alveolar inflammation may provide higher-quality information for patients with asthma.

Financial Disclosure and Conflicts of Interest

The authors have no conflicts of interest to disclose.

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