Role of endogenous nitric oxide in exercise-induced airway narrowing in patients with bronchial asthma

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Background: Increased amounts of nitric oxide (NO) in expired air and induced sputum have been found in asthmatic patients, and the role of excessively produced NO in the pathogenesis of bronchial asthma is under active investigation.
Objective: This study was designed to investigate the involvement of endogenous NO in exercise-induced bronchoconstriction (EIB) in asthmatic patients by using the sputum induction method.
Methods: The concentration of NO derivatives and inflammatory indices in induced sputum were examined in 18 asthmatic subjects and 10 normal control subjects. All asthmatic subjects performed an exercise test for 6 minutes. For 8 weeks after the first exercise testing, 400 μg of beclomethasone dipropionate twice daily was administered for asthmatic subjects with EIB, and the exercise testing and sputum induction were repeated in these patients.
Results: The concentration of NO derivatives in induced sputum was significantly higher in 9 asthmatic subjects with EIB (1580 ± 280 μmol/L) than in 9 asthmatic subjects without EIB (1130 ± 210 μmol/L) and normal control subjects (510 ± 150 μmol/L). Moreover, there was a significant correlation between the concentration of NO derivatives and the percentage of maximal fall in FEV_1 ($r = 0.569$, $P = .019$). The concentration of NO derivatives was also more closely correlated with the area under the curve of the percentage fall in FEV_1 plotted against time for 30 minutes ($AUC_{0\text{-}30}$; $r = 0.812$, $P < .001$). After treatment with inhaled beclomethasone dipropionate in asthmatic subjects with EIB, there was a significant decrease in the concentration of NO derivatives in induced sputum. The change in the concentration of NO derivatives was significantly correlated with the change in the $AUC_{0\text{-}30}$ ($r = 0.896$, $P = .0114$) but not with the change in the percentage of maximal fall in FEV_1.
Conclusion: These findings suggest that excessive production of NO is associated with EIB in patients with asthma and contributes to the prolonged airway narrowing phase rather than to the maximal airway narrowing evoked by exercise. (J Allergy Clin Immunol 2000;106:1081-7.)

Key words: *Exercise-induced bronchoconstriction, nitric oxide, bronchial asthma, induced sputum*

Most patients with bronchial asthma experience exercise-induced bronchoconstriction (EIB) when they perform exercise of sufficient duration and intensity.[1] Despite the wide prevalence and clinical significance of EIB, its mechanism has not yet been elucidated. According to one hypothesis, EIB is caused by an inflammatory mediator mechanism.[2] Hyperpnea associated with exercise leads to increased airway water and heat loss in addition to hyperosmolarity of the fluid interface of the mucosal surface in airways, resulting in mast cell degranulation. However, mast cell–derived mediators, such as histamine and leukotrienes, may cause not only airway smooth muscle contraction but also airway edema. It is possible that the airway narrowing after exercise in asthmatic subjects is, at least in part, due to bronchial microvascular phenomena, such as vascular engorgement and plasma leakage, that could thicken the mucosa and thereby narrow airway diameters, which could amplify the effects of airway smooth muscle contraction.

There is increasing evidence that endogenous nitric oxide (NO) plays an important role in physiologic regulation of the airways and is implicated in the pathophysiology of airway disease.[3] The expression of constitutive and inducible NO synthase (NOS) has been demonstrated in human lung epithelial cells,[4,5] and Grasemann et al[6] recently suggested that NO that is formed by neuronal NOS is an important determinant of asthmatic airway inflammation. Indeed, increased amounts of NO in expired air in patients with asthma compared with normal subjects have been documented.[7] However, the pathophysiologic roles of excessively produced endogenous NO in asthmatic subjects have not been explored. In particular, the role of endogenous NO in EIB has been little studied, and the pathogenetic mechanisms of EIB, potentially involving mucosal, neurogenic, smooth muscle, and vascular tissues, continue to be discussed. Elucidation of induced NO as an important participant and identification of the target tissue involved would be important contributions that could lead to an increased understanding of asthma and clinical interventions for the prevention and treatment of EIB. Therefore this study was designed to determine the involvement of endogenous NO in EIB by measuring the concentration of NO in induced sputum in patients with asthma.

METHODS

Subjects

The normal control population consisted of 10 subjects (mean age, 28.5 years; mean FEV_1, 113.7%). All normal control subjects were healthy, life-long nonsmoking volunteers who had no history of

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Received for publication May 16, 2000; revised Aug 11, 2000; accepted for publication Aug 11, 2000.
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0091-6749/2000 $12.00 + 0 **1/1/110803**
doi:10.1067/mai.2000.110803

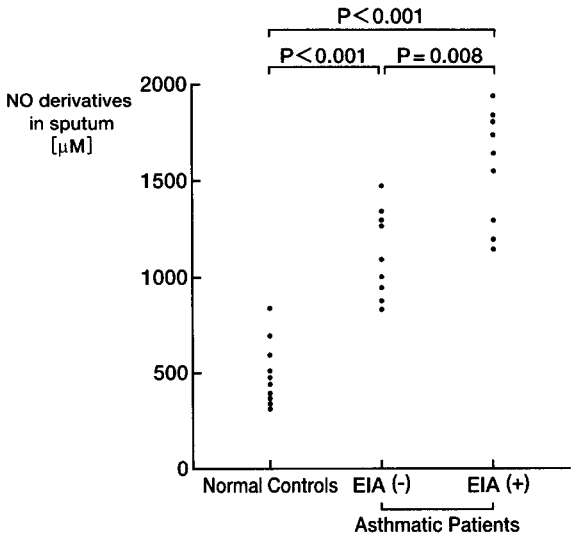

FIG 1. Comparison of the concentration of NO derivatives in induced sputum in control subjects and asthmatic patients with or without EIB.

Abbreviations used

$AUC_{0\text{-}30}$: Area under the curve of the percentage fall in FEV_1 plotted against time for 30 minutes
BDP: Beclomethasone dipropionate
ECP: Eosinophil cationic protein
EIB: Exercise-induced bronchoconstriction
NO: Nitric oxide
NOS: Nitric oxide synthase

lung disease. All asthmatic patients satisfied the American Thoracic Society criteria for asthma.[8] The clinical characteristics of these patients are shown in Table I. All asthmatic subjects were nonsmokers (mean age, 26.3 years; mean FEV_1, 93.2%). Methacholine inhalation challenge testing was performed for all asthmatic subjects. All challenge tests were performed at 1 PM to eliminate the effect of diurnal variation. After baseline spirometry and inhalation of diluent to establish the stability of FEV_1, the subjects were instructed to take slow inspirations in each set of inhalations. All asthmatic subjects in this study demonstrated bronchial hyperreactivity to methacholine. Their regular medication consisted of β_2-agonists and theophylline, and none were receiving oral or inhaled corticosteroids. Medications were not changed for a 1-month period preceding the study and were withdrawn for at least 12 hours before the methacholine challenge test and exercise test. All patients were clinically stable, and none had a history of respiratory infection for at least the 4-week period preceding the study. All subjects gave their written informed consent for participation in the study, which was approved by the Ethics Committee of Osaka City University.

Sputum induction and processing

Spirometry was performed before inhalation of 200 μg of salbutamol, which was administered through a metered-dose inhaler. All subjects were instructed to wash their mouths thoroughly with water. They then inhaled 3% saline at room temperature, which was nebulized with an ultrasonic nebulizer (NE-U12; Omron Co, Tokyo, Japan) at maximum output. They were encouraged to cough deeply after 3-minute intervals thereafter. After sputum induction, spirometry was repeated. If the FEV_1 fell, the subjects were required to wait until it returned to baseline value. The sputum samples were kept at 4°C for not more than 2 hours before further processing. A portion of the sample was diluted with PBS containing 10 mmol/L dithiothreitol (Sigma Chemical Co, St Louis, Mo) and gently vortexed at room temperature. It was then centrifuged at 400*g* for 10 minutes, and the cell pellet was resuspended. Total cell counts were performed with a hemocytometer, and slides were made by using a Cytospin (Cytospin3; Shandon, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain for differential cell counts. The supernatant was stored at –70°C for subsequent assay for NO and eosinophil cationic protein (ECP). ECP concentration was measured by using an RIA kit (Pharmacia Diagnostics, Uppsala, Sweden). All subjects produced an adequate specimen of sputum, which was defined as the patient being able to expectorate at least 2 mL of sputum. All subjects tolerated the sputum induction procedure well. There was no significant decrease in FEV_1 after sputum induction in any subject, and no subjects experienced symptoms of shortness of breath or chest tightness.

NO assay

NO derivatives (nitrate plus nitrite) in induced sputum were assayed colorimetrically after the Griess reaction, as previously described.[9] Two hundred microliters of sputum sample or standard was deproteinated by adding 20 μL of NaOH (1.0 mol/L, 4°C; Wako Chemical Co, Osaka, Japan) and 30 μL of $ZnSO_4$ (1.3 mol/L, 4°C; Wako Chemical Co). Samples were mixed and allowed to stand on ice for 15 minutes. After centrifugation (5 minutes at 4°C and 2600*g*), 100 μL of supernatant was mixed with 5×10^{-2} units of nitrate reductase (Sigma Chemical Co), 20 μL of 0.2 mol/L N-tris (hydroxymethyl) methylaminoethanesulfonic acid (pH 7.0, Sigma Chemical Co), and 20 μL of 0.5 mol/L sodium formate (Wako Chemical Co). After anaerobic incubation at room temperature for 20 minutes, 1.0 mL of water was added to the samples, and nitrite was assayed in supernatants obtained by means of centrifugation (5 minutes at 260*g*). Deproteinated samples or standards (200 μL) were mixed with 20 μL of 1% sulfanilamide (Sigma Chemical Co) in 15% phosphoric acid (Wako Chemical Co). After 10 minutes, 20 μL of 0.1% N-(1-naphtyl) ethylenediamine (Sigma Chemical Co) was added, and the absorption at 540 nm was determined.

Exercise challenge testing

Three days after sputum induction, the exercise test was performed at approximately 1 PM to eliminate the effects of diurnal variation. Exercise challenge testing was performed on an electrically driven treadmill (Q55xt, Series 90; Quinton Instrument Co, Seattle, Wash) for 6 minutes in the cardiac frequency to 90% of the maximum predicted value for the age of the patient.[10] All subjects breathed unconditioned room air (temperature, 22-25°C) and were coached to overcome hyperventilation during testing. A single-lead electrocardiogram and pulse oximetry (502-US; CSI, Tokyo, Japan) were monitored continuously. The criterion for exclusion was the presence of coronary artery disease or cardiac arrhythmias. A spirometer (Chestac –25F; Chest Co, Tokyo, Japan) was used to obtain spirometric measurements before and after exercise challenge. The higher of 2 measurements of FEV_1 obtained before exercise challenge was taken as the baseline value. Single measurements of FEV_1 were obtained 1, 3, 5, 10, 15, 20, 25, and 30 minutes after completion of the exercise challenge. The response to exercise challenge was taken to be the maximum percentage fall in FEV_1 after exercise determined as follows:

$$\%\ \text{fall in } FEV_1 = (FEV_1 \text{ baseline} - FEV_1 \text{ after})/(FEV_1 \text{ baseline}) \times 100.$$

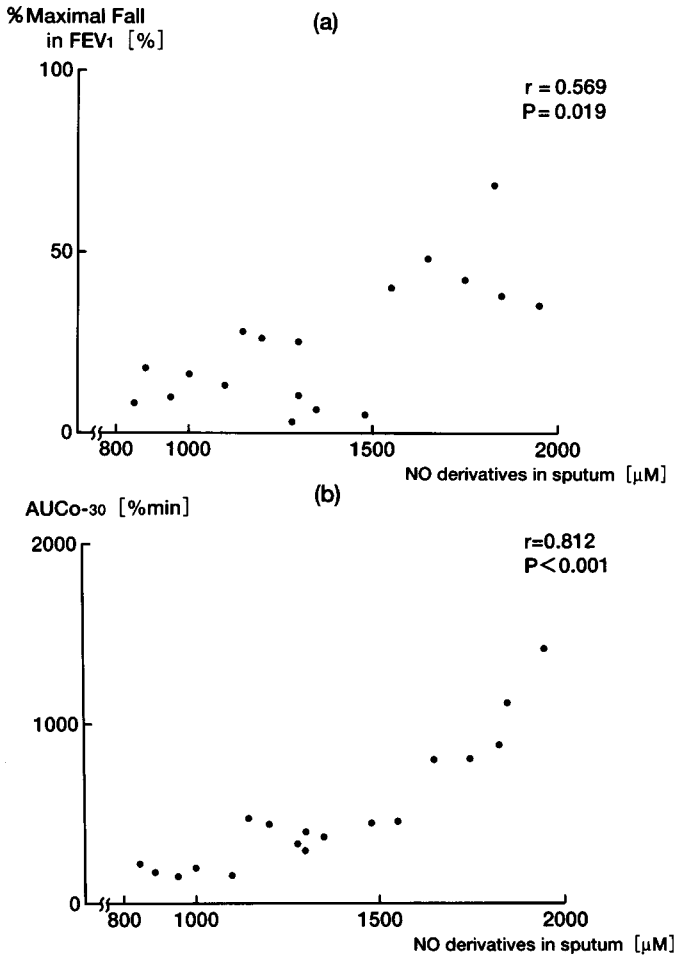

FIG 2. Correlation between the concentration of NO derivatives in induced sputum and the severity of EIB. **a,** Maximal percentage fall in FEV_1; **b,** $AUC_{0\text{-}30}$.

TABLE I. Characteristics of the study subjects

		Asthmatic patients	
	Normal control subjects	**Without EIB**	**With EIB**
Sex (M/F)	5/5	4/5	4/5
Age (y)	28.5 ± 3.5	25.0 ± 2.8	27.6 ± 2.6
IgE (IU/mL)	ND	232 ± 103	203 ± 99
FEV_1 (% predicted)	113.7 ± 5.0	93.7 ± 9.2†	92.8 ± 12.6†
PC_{20} methacholine* (μg/mL)	ND	3.19 ± 0.35	3.23 ± 0.32
Sputum			
% Eosinophils	0.5 ± 0.4	8.2 ± 5.9†	22.3 ± 8.4†‡
ECP (ng/mL)	115 ± 40	390 ± 210†	1030 ± 270†‡

All values are given as mean ± SD.
ND, Not determined.
*Geometric mean.
†$P < .01$ compared with normal control subjects.
‡$P < .01$ compared with asthmatic subjects without EIB.

Those patients whose maximum decrease in FEV_1 was greater than 20% were considered to have EIB. In addition, the bronchoconstrictor response was also assessed as the area under the curve of the percentage fall in FEV_1 plotted against time for 30 min ($AUC_{0\text{-}30}$). The $AUC_{0\text{-}30}$ was calculated by using trapezoidal integration, as described by Makker et al.[11] For 8 weeks after the first exercise testing, 400 μg of beclomethasone dipropionate (BDP) twice daily was administered for asthmatic subjects with EIB, and all of the above protocols were repeated in those BDP-treated asthmatic subjects.

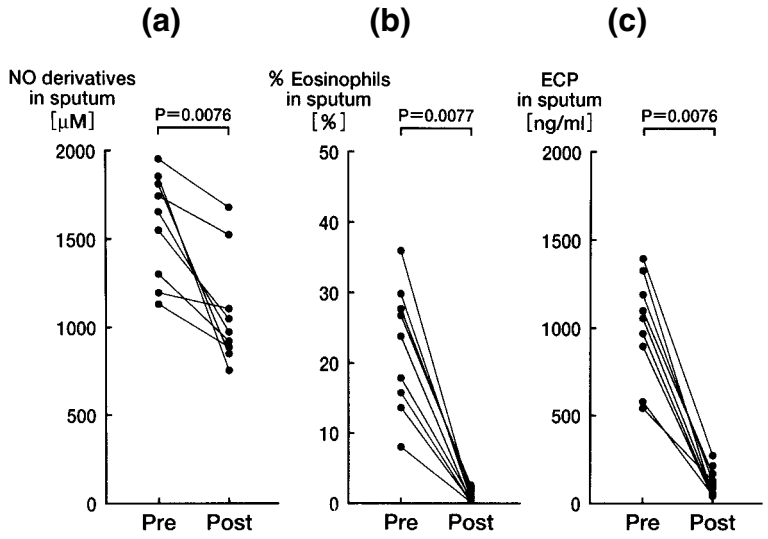

FIG 3. Change in the concentration of NO derivatives **(a)**, the percentage of eosinophils **(b)**, and the concentration of ECP **(c)** in induced sputum before and after BDP administration in asthmatic subjects with EIB.

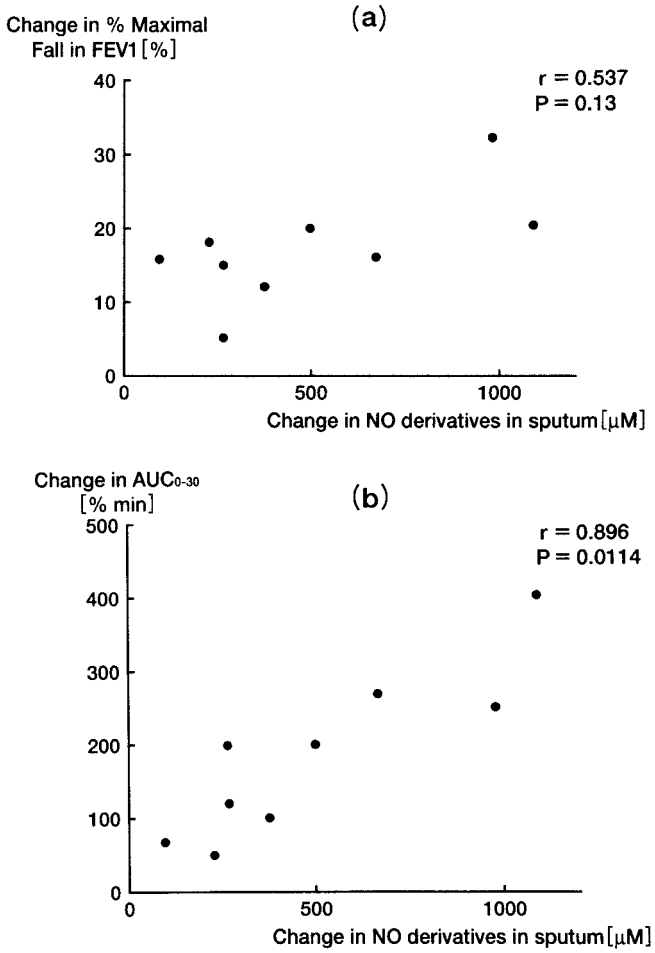

FIG 4. Correlation between the change in the concentration of NO derivatives in induced sputum and the change in the severity of EIB. **a,** Change in the percentage maximal fall in FEV_1; **b,** change in $AUC_{0\text{-}30}$. Each value was calculated as follows: value before BDP – value after BDP.

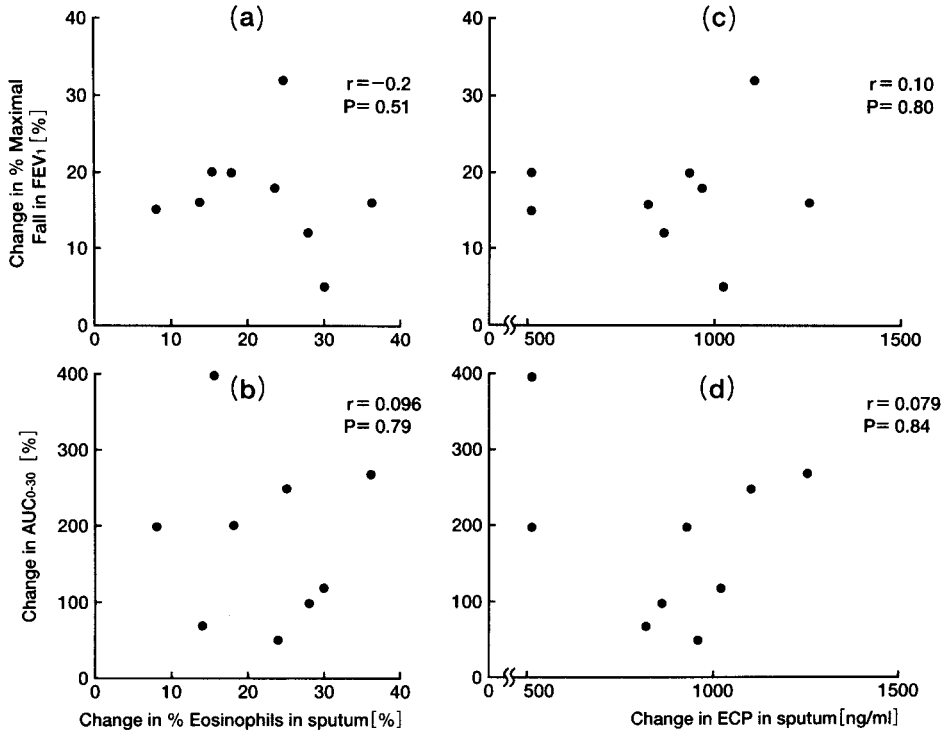

FIG 5. Correlation between the change in the percentage of eosinophils and the change in the percentage maximal fall in FEV_1 **(a)** and the change in the $AUC_{0\text{-}30}$ **(b)**. Correlation between the change in the ECP levels and the change in the maximal fall in FEV_1 **(c)** and the change in the $AUC_{0\text{-}30}$ **(d)**.

Statistical analysis

When multiple comparisons were between groups, significant intergroup variability was first established by using the Kruskal-Wallis test. The Mann-Whitney *U* test was then used for intergroup comparisons. The Wilcoxon signed-rank test was used to compare paired values. The significance of correlations was evaluated by determining Spearman rank correlation coefficients. A *P* value of less than .05 was considered significant.

RESULTS

Nine asthmatic subjects with EIB and 9 asthmatic subjects without EIB were well matched with respect to age, baseline lung function, IgE levels in serum, and hyperreactivity to methacholine (Table I). There were also no significant differences in room temperature or humidity during the exercise test between asthmatic subjects with and without EIB. However, the percentage of eosinophils and the concentration of ECP in induced sputum in asthmatic subjects with EIB (percentage eosinophils, 22.3% ± 8.4%; ECP, 1030 ± 270 ng/mL) were significantly higher than those found in asthmatic subjects without EIB (percentage eosinophils, 8.2% ± 5.9%; ECP, 390 ± 210 ng/mL) and the normal control subjects (percentage eosinophils, 0.5% ± 0.4%; ECP, 115 ± 40 ng/mL). The concentration of NO derivatives in induced sputum was also significantly higher in asthmatic subjects with EIB (1580 ± 280 μmol/L) than in asthmatic subjects without EIB (1130 ± 210 μmol/L) and normal control subjects (510 ± 150 μmol/L; Fig 1). Moreover, there was a significant correlation between the concentration of NO derivatives and the percentage maximal fall in FEV_1 (r = 0.569, P = .019; Fig 2, *A*). The concentration of NO derivatives was also more closely correlated with the bronchoconstrictor response when analyzed as the $AUC_{0\text{-}30}$ (r = 0.812, P < .001; Fig 2, *B*).

After treatment with inhaled BDP in asthmatic subjects with EIB, there was a significant decrease in the percentage of eosinophils and the concentration of ECP and NO derivatives (Fig 3). Moreover, the change in the concentration of NO derivatives was significantly correlated with the change in the $AUC_{0\text{-}30}$ (r = 0.896, P = .0114) but not with the change in the percentage of maximal fall in FEV_1 (r = 0.537, P = .13; Fig 4). In contrast, the change in the percentage of eosinophils and the concentration of ECP were not significantly correlated with the change in the percentage of maximal fall in FEV_1 and the change in the $AUC_{0\text{-}30}$ (Fig 5).

DISCUSSION

In this study higher levels of NO derivatives were found in induced sputum from asthmatic subjects with EIB than in that from asthmatic subjects without EIB and normal control subjects. Moreover, there was a significant correlation between the concentration of NO derivatives and the severity of EIB. Therefore we have taken

the next step to observe whether the blockade of NO production with inhaled BDP prevents exercise-induced airway narrowing in asthmatic subjects with EIB, as glucocorticoids prevent the induction of inducible NOS[12] and inhaled glucocorticoids decrease NO production in asthmatic airways.[13] After the treatment with inhaled BDP, there was a significant decrease in the concentration of NO derivatives, and the change in the levels of NO derivatives was correlated with the change in the severity of EIB. These findings suggest that excessive production of NO is associated with EIB in patients with asthma.

The role of excessively produced NO, if any, in the pathogenesis of bronchial asthma is under active investigation, and we know that NO is one of the most important markers in airway inflammation. We have previously found that the levels of NO derivatives in induced sputum were correlated with the magnitude of airway inflammation[14] and that the severity of EIB is related to eosinophilic airway inflammation in steroid-naive asthmatic subjects.[15] However, in this study we determined that the levels of NO derivatives were not correlated with eosinophilic airway inflammation in steroid-treated asthmatic subjects and that the change in the percentage of eosinophils and in ECP levels were not also correlated with the change in the severity of EIB after inhaled BDP therapy. Although eosinophilic inflammation was completely inhibited with inhaled BDP therapy in asthmatic subjects with EIB, the levels of NO derivatives were still higher in those asthmatic subjects than in control subjects, and those asthmatic subjects subsequently exhibited EIB. Asthmatic airway inflammation may therefore be a heterogeneous process of which sputum eosinophilia is only one part, and it may be that sputum eosinophilia and the levels of NO derivatives in sputum reflect different components of the inflammatory process. The range of levels of NO derivatives in asthmatic patients is large, with some having normal values and others having increased levels despite treatment with inhaled steroids. Little et al[16] also recently found that the range of exhaled NO levels in asthmatic patients did not return to normal in all patients, despite treatment with oral steroids. These findings require another possible explanation for the roles of endogenous NO in EIB, except as a marker of airway inflammation. Moreover, identification of the target tissue of excessively produced NO would be important for the understanding of the precise mechanism of EIB. NO is a potent vasodilator in the bronchial circulation and may mediate the hyperemia seen in asthmatic airways.[17] Endogenous NO may act to increase airway microvascular leakage, thus increasing airway edema.[18] Sensory neuropeptides also induced microvascular leakage, leading to airway wall edema and extravasation of plasma into the airway lumen.[19] Interestingly, the receptor antagonist of sensory neuropeptides improved the AUC of the percentage fall in FEV_1 and recovery time after exercise without attenuating the maximal airway narrowing evoked by exercise.[20] In this study we found that the concentration of NO derivatives is more closely related to the $AUC_{0\text{-}30}$ than the percentage of maximal fall in FEV_1. In addition, the change in the levels of NO derivatives was significantly correlated with the change in the $AUC_{0\text{-}30}$ but not with the change in the percentage of maximal fall in FEV_1. These findings indicated that NO-mediated effects contribute to the prolonged airway narrowing phase rather than to the acute onset phase, which appears to be caused by airway smooth muscle contraction. It is possible that endogenous NO is likely to play a role in modulating bronchial microcirculation, leading to the thickness of the bronchiolar wall by causing airway edema.

In this study we have not measured the concentration of exhaled NO. The measurement of exhaled NO is noninvasive and a sensitive marker for early airway inflammation[21] and can be performed repeatedly, even in patients with severe asthma.[22] Moreover, exhaled NO levels certainly reflect NOS activity. In contrast, aqueous-phase nitrite and nitrate measurements are not simply a reflection of NOS activity but also reflect intrinsic abnormalities of the airway lining fluid, including pH.[23] In this study we wished to examine inflammatory indices together with the levels of NO derivatives in induced sputum. Moreover, NO is unstable in the presence of oxygen and rapidly autooxidizes to a variety of NO derivatives. Therefore a previous report shows that one useful strategy is to use NO derivatives, including nitrite and nitrate to indicate NO formation.[24] Actually, consistent with the higher levels of exhaled NO in asthmatic subjects, nitrite and nitrate are also elevated in induced sputum and the nasal lavage fluid of patients with rhinitis.[25] In our earlier study we measured the levels of exhaled NO together with those of NO derivatives in induced sputum and found a significant correlation between the levels of exhaled NO and sputum NO derivatives in asthmatic subjects.[26]

In summary, because our results are based on treatment with inhaled BDP, further studies with specific NO synthase inhibitors, such as Nω-nitro-L-arginine methyl ester, will be required. However, our findings suggest that excessive production of NO is associated with EIB and contributes to the prolonged airway narrowing phase rather than to the maximal airway narrowing evoked by exercise.

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