

Increased S-Nitrosothiol Levels in Nonasthmatic Eosinophilic Bronchitis Compared with Cough Variant Asthma

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Key Words

S-nitrosothiol • Nonasthmatic eosinophilic bronchitis • Cough variant asthma • Airway hyperresponsiveness

Abstract

Background: Nonasthmatic eosinophilic bronchitis (NAEB) and cough variant asthma (CVA) are common causes of chronic cough. Both are characterized by eosinophilic inflammation in the airways. However, airway hyperresponsiveness, which is a characteristic feature of CVA, is not observed in NAEB. We hypothesized that endogenous bronchodilator S-nitrosothiol (SNO) levels are different between patients with NAEB and CVA. **Methods:** SNO concentrations in sputum supernatant were measured using a commercially available kit in 20 NAEB and 21 CVA patients. **Results:** The mean sputum eosinophil counts and exhaled nitric oxide values were similar in patients with NAEB ($12.4 \pm 2.3\%$, 80.6 ± 8.1 ppb) and CVA ($15.3 \pm 3.7\%$, 97.7 ± 9.2 ppb). By contrast, SNO levels in the airway lining fluid of NAEB patients were substantially higher than those of CVA patients (87.1 ± 9.8 vs. 46.8 ± 4.8 μM ; $p < 0.05$). **Conclusions:** SNOs may be an important factor in determining the development of airway hyperresponsiveness in the presence of eosinophilic inflammation.

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Introduction

Nonasthmatic eosinophilic bronchitis (NAEB) is a common cause of chronic cough [1, 2]. NAEB is characterized by eosinophilic airway inflammation [3, 4], as is cough variant asthma (CVA) [5]. However, NAEB is clinically distinct from CVA in that there is no exaggerated response to methacholine. The reason remains still unclear. Previous studies have shown that the different airway functions of NAEB and asthma are not due to differences in the localization or nature of the Th2 type inflammation [6, 7].

S-nitrosothiols (SNOs), also known as thionitrites, are biological metabolites of nitric oxide (NO). SNOs include compounds such as S-nitrosoglutathione (GSNO), S-nitrosocysteine, S-nitroso-N-cysteine and S-nitroso-BSA [8]. SNOs, predominantly GSNO, are present in the human airway lining fluid in micromolar concentrations [9]. SNOs have received much attention because they may play a central role in the development of airway hyperresponsiveness (AHR) in asthma [10]. Recently, low concentrations of airway SNOs have been reported in asthmatic patients [11–13]. These findings suggest that SNOs may regulate airway tone and are closely associated with AHR.

We tested the hypothesis that the different airway functions in patients with NAEB and CVA are due to differences in SNO levels in the airways.

Subjects and Methods

The study subjects consisted of 20 patients with NAEB and 21 patients with CVA. The subjects were recruited randomly among patients attending the outpatient allergy clinic at Samsung Medical Center from January 2007 to December 2008.

We defined NAEB as an isolated chronic cough (lasting more than 8 weeks) without any associations with symptoms suggesting variable airflow obstruction, a negative methacholine inhalation test [provocative concentration causing a 20% fall in forced expiratory volume in 1 s (FEV₁) (PC₂₀) >16 mg/ml] and increased sputum eosinophils (≥3%). We enrolled CVA patients whose main symptom was a chronic cough rather than a shortness of breath, a positive methacholine inhalation test (PC₂₀ ≤16 mg/ml) and increased sputum eosinophils. Wheezes or rhonchi were not audible on chest auscultation, and their basal lung functions were near normal (FEV₁ ≥70% of predicted value).

Exclusion criteria were the following: (1) a current smoker or an ex-smoker who had quit within the past year, (2) abnormal findings on plain chest radiographs, and (3) the use of anti-asthma medications including oral or inhaled corticosteroids, short- or long-acting β₂-agonists, theophylline and leukotriene receptor antagonists within the past 2 months. The study was approved by the local ethics committee, and all the subjects gave written informed consent.

Allergen Skin Test

Skin prick tests were done with 55 common aeroallergens including house dust mites, pollens, and fungi. Negative controls with saline and positive controls with histamine were performed concurrently. The means of the longest diameter and orthogonal diameter were measured in millimeters at 15 min. The skin prick test was considered positive if the wheal diameter of an allergen was larger than that of the positive control. Atopy was defined as one or more positive skin prick responses.

Methacholine Bronchial Provocation Test

Methacholine bronchial provocation test was performed according to the American Thoracic Society guidelines as described previously [14]. A provocative concentration of methacholine causing a 20% fall in FEV₁ (PC₂₀) was adopted as the marker for AHR, which was defined as a PC₂₀ ≤16 mg/ml.

Induced Sputum Examination

An induced sputum examination was performed as described previously [14]. In brief, sputum was induced by inhalation of 4.5% saline solution for 5 min via an ultrasonic nebulizer. Subjects were encouraged to cough up sputum into a sterile container after rinsing their mouth and throat, and this process was repeated 3 times. Sputum free of salivary contamination was treated with an equal volume of 0.1% dithiothreitol for 30 min. The mixture was further diluted with an equal volume of phosphate-buffered saline (PBS). The suspension was filtered through a nylon gauge and centrifuged at 1,000 rpm for 10 min. The cell-free supernatant of the sputum

was removed and stored at -70°C in a 2-ml sterile plastic tube until analysis. The cell pellet was resuspended in PBS, and a total cell count was taken using a hemocytometer. The cell suspension was readjusted to 1 × 10⁶ cells/ml with PBS. Slides were prepared by using a cytopsin instrument (Shandon Scientific Ltd., Shandon, UK) and were stained (Diff-Quick; American Scientific Products, Chicago, Ill., USA) for differential cell counts. We regarded the prepared sample as being adequate if squamous cells accounted for <20% of the total cells seen on microscopy. At least 300 nonsquamous cells were counted on each slide, and eosinophilic inflammation was defined as an eosinophil percentage of ≥3%.

Measurement of Exhaled NO

Exhaled NO was quantified according to the American Thoracic Society guidelines at a constant flow rate of 50 ml/s using a chemiluminescence NO analyzer (NOA 280; Sievers Instruments, Boulder, Colo., USA) as previously described [15]. Briefly, the patient inhaled NO-free air (containing <5 ppb) via a mouthpiece to total lung capacity over 2–3 s and then exhaled immediately. Each exhalation was maintained over 6 s to obtain a plateau in the NO-versus-time profile of at least 3 s. Reproducible exhalations were repeatedly performed, resulting in 3 NO plateau values. Exhaled NO was then calculated as the mean of these 3 values.

Measurement of SNOs

Concentrations of SNOs in the sputum supernatant were measured using a commercially available assay kit (Oxonon, Emeryville, Calif., USA) as previously described [16]. The kit is based on Gress and Saville reactions. First, a cleavage reaction was performed using vial 1 which breaks the S-N bond of SNOs. This resulted in the production of nitrites, which were detected spectrophotometrically (VERSAmix tunable microplate reader; Molecular Devices, USA) at 540 nm. Then, the same procedure was repeated using vial 2, which contained the cleavage reagent, and therefore, only detected free nitrites. The SNO concentration was calculated via subtraction of the vial 2 value from the vial 1 value. The sensitivity of this test was approximately 500 nM.

Statistical Analysis

Subject characteristics are described using the mean or geometric mean and the standard error of the mean. The Kruskal-Wallis test was applied to compare the data. A multiple comparison analysis with least significant difference using ranks was performed when the Kruskal-Wallis tests were significant. Correlation was determined by using Spearman's rank correlation. A difference was considered to be significant when $p < 0.05$. Statistical analyses were performed using SPSS version 17.0 for Windows (SPSS, Chicago, Ill., USA).

Results

Subject Characteristics

The clinical features of the 20 NAEB and 21 CVA patients are shown in table 1. Age, serum total IgE levels, blood eosinophil counts and basal lung functions were similar in the 2 groups. The male/female ratio and atopy prevalence were higher in NAEB patients compared with

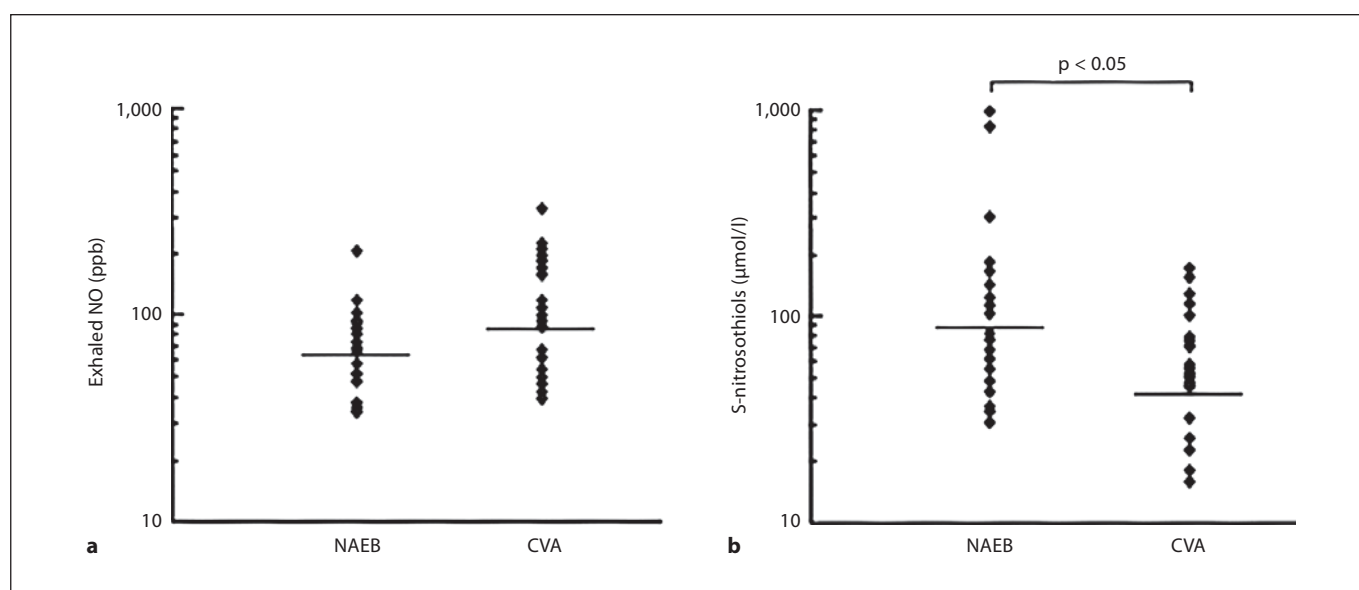


Fig. 1. A comparison of exhaled NO values (**a**) and SNO concentrations in induced sputum supernatant (**b**) from 20 NAEB and 21 CVA patients. The mean exhaled NO values were similar, but SNO levels of NAEB patients were substantially higher than those of CVA patients.

CVA patients. The percentile of eosinophils in induced sputum was similar in the NAEB ($12.4 \pm 2.3\%$) and CVA patients ($15.3 \pm 3.7\%$).

Exhaled NO Values and SNO Concentrations in Sputum Supernatant

The geographic means of exhaled NO values were similar in NAEB (80.6 ± 8.1 ppb) and CVA (97.7 ± 9.2 ppb) (fig. 1a). By contrast, SNO levels in sputum supernatant were significantly higher in the NAEB patients (87.1 ± 9.8 μM) compared with the CVA patients (46.8 ± 4.8 μM ; $p < 0.05$) (fig. 1b).

Discussion

This is the first study to investigate the difference of SNO levels in patients with NAEB and CVA. Our study shows that SNO concentrations in the airway lining fluid in patients with NAEB are significantly higher than those in patients with CVA. This suggests that SNOs, which have been proven to be a major source of bronchodilatory NO bioactivity in the airways [17], may be an important factor in determining the development of AHR in the presence of eosinophilic airway inflammation. It has been reported that SNOs relaxed human bronchial

Table 1. Characteristics of the study subjects

	NAEB (n = 20)	CVA (n = 21)
Age, years	42.4 (24–65)	36.5 (19–59)
Males	14 [70.0]*	10 [47.6]
Atopy	7 [35.0]*	14 [66.6]
Blood eosinophils/ μl	409 (134–940)	537 (144–842)
IgE, IU/ml	177.8 ± 1.2	169.8 ± 1.3
FEV ₁ , % predicted	99.8 (71.1–121.2)	98.5 (74.3–99.8)
PC ₂₀ , mg/ml	>16	3.09 ± 1.27
Sputum cell differentials, %		
Eosinophils	12.4 (5.7–19.0)	15.3 (8.0–36.9)
Neutrophils	50.5 (18.9–67.0)	39.4 (12.2–47.0)
Macrophages	28.4 (7.5–68.5)	35.7 (10.0–37.2)
Lymphocytes	6.2 (0.3–9.0)	6.5 (1.2–9.0)

Data are expressed as the mean (with ranges in parentheses) except IgE and PC₂₀ which are expressed as geographic mean values. Figures in brackets are percentages. * $p < 0.05$, compared with CVA group.

smooth muscle precontracted with methacholine [8], and SNO depletion was related to bronchial constriction in asthma [12].

NAEB is a common cause of chronic cough [1, 2], as is CVA [5]. Both conditions are associated with increased

eosinophils in the airways. However, AHR, which is a characteristic feature of CVA, is not observed in NAEB. The difference in airway function between the 2 diseases has not been fully explained. The pathophysiology of NAEB and asthma are almost identical [7], although a difference in smooth muscle mast cell infiltration has been suggested as a possible cause [18].

Increased levels of exhaled NO have been well documented not only in asthma [19] but also in NAEB [7, 15]. The role of NO in asthma and NAEB is not certain, but the smooth muscle relaxant effects of NO suggest that it may have a role as a bronchodilator. Especially in NAEB, the increase in exhaled NO may lead to the formation of sufficient SNOs that can prevent airway narrowing to external stimuli despite eosinophilic inflammation, which is well supported by our study.

SNO levels have been shown to increase with the localized production of inducible NO synthase [20, 21]. It is reasonable to assume that SNO production is also increased in asthmatic patients because elevated exhaled NO in asthma is a well-known result of inducible NO synthase upregulation in the airways [22]. Therefore, the relatively low SNO content in asthma compared with NAEB can be explained only by the accelerated catabolism of SNOs. This leads to the conclusion that the activity of GSNO reductase (GSNOR), an enzyme that catalyzes the glutathione-dependent GSNO metabolism and controls the SNO level indirectly [23], may be higher in patients with CVA compared with NAEB. Que and colleagues [17] clearly demonstrated that mice genetically lacking GSNOR do not have AHR, despite allergen-induced airway inflammation. A human study has shown that genotypic variation in GSNOR was associated with a decreased response to asthma treatment [24]. In addition, GSNOR activity measured from bronchoalveolar lavage samples was significantly increased in asthmatics compared with controls, which correlated inversely with methacholine PC₂₀ [13].

The results of the present study have potential implications for the understanding of the mechanism of AHR in asthma. AHR is defined by an exaggerated obstructive response of the airways to a variety of stimuli such as methacholine. AHR is associated with the severity of asthmatic symptoms [25] and a decline in lung function [26]. There are many factors contributing to the development of AHR, such as smooth muscle dysfunction, airway remodeling and airway inflammation [27]. However, none of these can fully explain the mechanism of AHR. The relationship between AHR and eosinophilic airway inflammation is relatively well established, but there are

still many evidences that AHR may be dissociated from airway inflammation. For example, interleukin-5 blocking monoclonal antibody causes a dramatic decrease in sputum eosinophils but does not suppress AHR at all [28]. The dosage of steroids needed to affect AHR was much higher compared with that required to affect airway inflammation [29]. In brief, the link between eosinophils and the development of AHR is still uncertain, and NAEB is a typical example of such dissociation.

There are some methodological limitations to this study. First, we did not measure the activity of GSNOR directly in sputum cells or the supernatant. Further studies are necessary to confirm the exact role of GSNOR in NAEB patients, although several studies have revealed that GSNOR is the most important factor regulating the SNO levels in the airways [17, 23]. Second, some demographic features of the NAEB patients were not similar to those of the CVA patients, such as sex or atopy prevalence, although we confirmed that SNO concentrations are not affected by sex or atopic status in this study (data not shown).

In conclusion, we have documented that SNO levels are significantly higher in NAEB patients than in CVA patients. These suggest that SNOs regulate airway tone and are closely associated with AHR in the presence of eosinophilic inflammation. Further studies exploring the factors which regulate the production or catabolism of SNOs will provide useful information for the mechanism of bronchoconstriction in asthma.

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