Buffering airway acid decreases exhaled nitric oxide in asthma

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Background: The human airway is believed to be acidified in asthma. In an acidic environment nitrate is converted to nitric oxide (NO).

Objective: We hypothesized that buffering airway lining fluid acid would decrease the fraction of exhaled NO (FENO).

Methods: We treated 28 adult nonsmoking subjects (9 healthy control subjects, 11 subjects with mild intermittent asthma, and 8 subjects with persistent asthma) with 3 mL of 10 mmol/L phosphate buffered saline (PBS) through a nebulizer and then serially measured FENO levels. Six subjects also received PBS mouthwash alone.

Results: FENO levels decreased after buffer inhalation. The maximal decrease occurred between 15 and 30 minutes after treatment; FENO levels returned to pretreatment levels by 60 minutes. The decrease was greatest in subjects with persistent asthma (−7.1 ± 1.0 ppb); this was more than in those with either mild asthma (−2.9 ± 0.3 ppb) or healthy control subjects (−1.7 ± 0.3 ppb, P < .001). Levels did not decrease in subjects who used PBS mouthwash.

Conclusion: Neutralizing airway acid decreases FENO levels. The magnitude of this change is greatest in persistent asthma. These data suggest that airway pH is a determinant of FENO levels downstream from NO synthase activation.

Clinical implications: Airway biochemistry modulates FENO levels. For example, nitrite is converted to NO in the airway, particularly the inflamed airway, by means of acid-based chemistry. Thus airway pH should be considered in interpreting clinical FENO values. In fact, PBS challenge testing integrates airway pH and FENO analysis, potentially improving the utility of FENO as a noninvasive test for the type and severity of asthmatic airway inflammation. (J Allergy Clin Immunol 2006;118:817-22.)

Key words: Buffer, airway acid, nitric oxide, nitrite, asthma

Several pulmonary diseases are characterized by acidic exhaled breath condensate (EBC) collected both from patients breathing orally and from patients who are endotracheally intubated.1,4 The acidity of these specimens is believed to reflect acidity in airway lining fluid. However, direct assays of airway lining fluid pH are challenging and invasive. Therefore we have tested a novel index of airway acidification that makes use of the facts that (1) nitrite (NO2−) is normally present in airway lining fluid,5,6 (2) nitric oxide (NO) is readily measured in exhaled air,7 and (3) NO2− protonation to form nitrous acid (acid dissociation constant [pKa] of approximately 3.3) can rapidly evolve NO2,8 according to the following equations:

\[ 3H^+ + 3NO_2^- \rightarrow 3HNO_2 \]

\[ 3HNO_2 \rightarrow 2NO + NO_3^- + H^+ + H_2O. \]

Of note, there are additional reactions in the lung, including the major pathway downstream from nitrite protonation, in which reduced thiols—such as glutathione—are converted to S-nitrosothiols, which can evolve NO.1,2,5,8 We specifically hypothesized that, to the extent that airway lining fluid is acidified, buffering H+ would inhibit NO2− protonation and decrease the fraction of exhaled NO (FENO).

Introduction of acid into the airway, whether through local formation in the airway, aspiration of gastric acid, or inhalation of acidic aerosols or air pollutants, can cause the cough, bronchoconstriction, and airway inflammation characteristic of asthma.1,5 Of note, acute asthma exacerbations are associated with acid breath (acidopnea) that resolves.
with corticosteroid therapy. Indeed, Ahmed et al. have shown that inhalation of sodium bicarbonate can markedly decrease airflow obstruction in certain patients experiencing an acute asthma exacerbation. These observations suggest the possibility that acidification of the airway lining fluid could contribute to the pathophysiology of asthma and that corticosteroid therapy upregulates airway buffering mechanisms. Here we show that inhalation of PBS causes a decrease in FENO levels, that this decrease in FENO levels is greater in subjects with asthma than in control subjects, and that the magnitude of this decrease is greater in subjects with persistent asthma than in those with mild intermittent asthma.

METHODS

Subjects

Subjects aged 18 to 50 years were recruited by advertisement. Subjects were categorized by the United States National Heart, Lung, and Blood Institute guidelines as having either mild intermittent asthma or moderate or severe persistent asthma based on an established history of physician-diagnosed asthma and a history, examination, and baseline spirometry performed by one of the investigators at the time of study entry. Exclusion criteria included a smoking history, FEV1 of 40% of predicted value or less, age less than 18 years, FENO level of less than 5 ppb, or an acute asthma exacerbation requiring rescue medication more than 3 times per day, an acute increase in corticosteroid dosing, or both. We also enrolled control patients with no history of asthma or atopy, a FENO level of less than 35 ppb, and no acute or chronic medical condition. Not all patients were enrolled in the Severe Asthma Research Program: some were enrolled for this protocol only. The protocol was reviewed and approved by the University of Virginia Human Investigation Committee.

Lung function testing

Baseline spirometry (Collins/Ferraris Respiratory, Louisville, Colo) was performed according to American Thoracic Society standards, with the average of 3 sequential measurements within 10%.

FENO levels were measured at an expiratory flow rate of 50 mL/s by using NIOX (Aerocrine AB, Solna, Sweden), according to American Thoracic Society guidelines.

EBC was collected with the R-Tube (Respiratory Research, Inc, Charlottesville, Va) and deaerated, as previously described, before pH measurement.

Clinical protocol

After initial history and physical examination, subjects completed baseline FENO measurement, followed by spirometry and EBC collection. Subjects were given a 3.0-mL nebulized inhalation of 10 mmol/L neutral pH sterile PBS prepared aseptically by the investigational drug pharmacy in accordance with a United States Food and Drug Administration Investigational New Drug exemption. This was delivered through a Hudson RCI micromist nebulizer with a mouthpiece. The nebulization lasted approximately 10 minutes. In control experiments subjects performed a 2-minute mouthwash with an equal volume of PBS. At the completion of the PBS treatment, the subjects repeated EBC collection and then performed FENO measurements at 15, 30, 45, and 60 minutes.

In vitro model

A film of Krebs-Henseleit buffer (NaCl, 118 mmol/L; KCl, 5.4 mmol/L; NaH₂PO₄, 1.10 mmol/L; glucose, 11.1 mmol/L; NaHCO₃, 25.0 mmol/L; MgSO₄, 1.38 mmol/L; and CaCl₂, 2.32 mmol/L) containing 1 μmol/L albumin and 1 μmol/L NaNO₂ was produced in a rotating glass custom tonometer purged with 5% CO₂ in N₂. This system approximates the contents and the CO₂/HCO₃⁻ balance of airway lining fluid and of EBC, allowing us to titrate an acid dose-response study that could not be performed in vivo. To this tonometer, we added increasing amounts of 1N HCl and measured (1) the changes in headspace and NO signal, (2) the reversibility of this change in NO signal with an addition of 10 mmol/L PBS, and (3) the deaerated pH at which these changes occurred.

Statistical analysis

We calculated the changes of FENO (repeated) measurements at 15, 30, 45, and 60 minutes from baseline measurement (at 0 minute). A mixed model was used to compare the changes with respect to group, time, and their interaction. We also computed the maximal changes and compared them by means of 2-sample t testing among different groups, with the Bonferroni adjustment for multiple comparisons. Regression models were used to describe the effect of baseline FEV₁ on changes in FENO levels and to describe the in vitro dose-response. Statistical computations were performed with SAS 9.1 software (SAS Institute, Inc, Cary, NC).

RESULTS

Subjects

Twenty-eight subjects were given PBS through a nebulizer, including 9 healthy control subjects, 11 subjects with mild intermittent asthma, and 8 subjects with persistent asthma (Table 1). An additional 6 subjects (2 control subjects and 4 with asthma) were given PBS as a mouthwash.

Exhaled NO levels decrease after PBS inhalation

Inhaled PBS was well tolerated in all subjects: there were no adverse events. FENO levels decreased in all control and asthmatic subjects after PBS inhalation (Figs 1 and 2). The maximal change occurred between 15 and 30 minutes after PBS challenge, and levels returned to baseline by 60 minutes (Fig 1). The maximal change was greater in subjects with persistent asthma (−7.2 ± 1.0 ppb, mean ± SEM) than in those with mild asthma (−2.9 ± 0.3 ppb, P < .001) and was greater in both groups of asthmatic patients than in control subjects (−1.7 ± 0.4 ppb, P < .0001, Fig 2). Six additional subjects performed the study after only a mouthwash with PBS. The change in

Abbreviations used

EBC: Exhaled breath condensate
FENO: Fraction of exhaled nitric oxide
NO: Nitric oxide
NO₂⁻: Nitrite
NOS: Nitric oxide synthase
PBS: Phosphate buffered saline
pKa: Acid dissociation constant

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FENO levels (−0.7 ± 0.4 ppb, Fig 2) was not significant, and there was no difference between control (n = 2) and asthmatic subjects (n = 4) after mouthwash. Starting mean deaerated EBC pH was neutral (7.68 ± 0.09) and increased only modestly after PBS (to 7.76 ± 0.04); this change was not linearly related to a change in FENO level ($R^2 = 0.18$). Mean FEV1 did not change after buffer inhalation. Starting FENO levels were associated with maximum change in FENO levels ($R^2 = 0.64, P < .0001$), as was starting FEV1 ($R^2 = 0.27, P = .005$, Fig 3).

**Modeling the relationship between change in NO levels and change in deaerated buffered pH**

We modeled the airway acid-base buffer dose response in vitro. Breath condensate pH decreases to less than 6 in the context of acute asthma exacerbations,\(^1^,\(^2\) rather than in the stable patients we enrolled in our studies. In the deaerated pH range from 6.0 to 8.5 in vitro, although NO level always increased with the addition of acid, there was not a linear association between change in NO level and either change in pH ($R^2 = 0.03$) or post-HCl deaerated pH ($R^2 = 0.11$). The relationship between pH and change in NO level before and after PBS was not linear in our range of interest because of the complexities of buffer chemistry (differing pKa values of HCO$_3^-$, H$_2$CO$_3$, H$_2$PO$_4^-$, and albumin). On the other hand, at lower pH, the changes in headspace NO were related to both the post-HCl deaerated pH ($R^2 = 0.71$) and the change in deaerated pH ($R^2 = 0.87$). Each change in NO signal was attenuated after HCl injection by subsequent injection of PBS. Headspace NO levels increased with HCl, even when the postdeaerated (CO$_2$-free) pH was greater than 8, reflecting the fact that CO$_2$ is an important component of airway lining fluid acid-base balance, as it is an important component of EBC pH balance.\(^1^,\(^2\) These data suggest that the relationship between PBS-inhibitable NO$_2$ protonation and deaerated EBC pH is complex and reflects (1) the presence of multiple buffers with different pKa values; and (2) the fact that pH does not report proton concentration linearly but rather logarithmically. Therefore it is not surprising that change in deaerated EBC pH was not linearly related to change in FENO level at higher (healthier) pH values in vivo.

**DISCUSSION**

The regulation of airway epithelial pH is incompletely understood. Airway epithelial cells can respond to the introduction of exogenous acid by neutralizing surface pH through activation of enzymes similar to those in the renal tubular epithelium, including carbonic anhydrases and glutaminase.\(^1^,\(^1^,\(^1^,\(^1^,\(^6\) Recent data suggest that there might also be mechanisms that lead to endogenous airway acidification. Specifically, EBC pH is low in a variety of lower respiratory tract diseases that are not specifically associated with gastric aspiration,\(^1^,\(^4\) and direct measurement data suggest that airway pH regulation is substantially abnormal in human airways diseases, such as cystic fibrosis and bronchopulmonary dysplasia.\(^1^,\(^5^,\(^7\) Indeed, this endogenous acidification appears to be initiated by TH1 cytokines during infection\(^1\) and might serve to augment antimicrobial host defense in the lung, as it does in the stomach.\(^5\) In asthma, however, airway acidification triggered by TH1 cytokines during a viral respiratory tract infection could exacerbate cough, bronchoconstriction, and mucous plugging.\(^1^,\(^2^,\(^5^,\(^7\)
FENO level does not consistently distinguish severe asthma from less severe asthma. Indeed, there might be many determinants of the level of FENO in a given patient. Most of these effects are downstream of NO synthase (NOS) activation and/or regulated by NOS substrates, inhibitors, or products, accounting for the robust effect of NOS inhibition on FENO levels. Determinants include (1) NOS expression and activity; (2) enzymes regulating levels of NO-consuming superoxide; (3) enzymes regulating airway levels of S-nitrosothiols that are produced by NOS and can, in turn, evolve NO; and (4) prokaryotic denitrification enzymes in the context of airway infection. We now suggest that mechanisms involved in the regulation of airway pH can also affect FENO downstream from NOS activation. Indeed, the complexity of interpreting FENO levels recapitulates the complexity of asthma itself, which is not one disease but rather represents the interface between airway inflammation and a range of pulmonary biochemical abnormalities. In the past, FENO levels have been used to measure S-nitrosothiol catabolism in airways disease; here we have used it to classify the asthmatic airway response to buffer challenge. These modifications might ultimately add value to FENO testing, allowing characterization of specific asthma phenotypes to target specific treatments, such as S-nitrosothiol replacement or airway buffering, to specific patients.

Of note, determinants of airway pH, like those of FENO, might be many, and there can be explanations for our data other than simple inorganic pH buffering by phosphate. We have recently shown, for example, that phosphate-dependent glutaminase is active in human airway epithelial cells, suggesting that phosphate could increase airway buffering capacity by increasing ammonia production. Our data do not exclude this indirect buffering effect.
Repeated spirometry can also affect F_{ENO} levels. In our hands, however, repeated F_{ENO} testing after spirometry did not substantially alter F_{ENO} levels, as evidenced by the lack of change in the subjects who used mouthwash. Further, the coefficient of variation for the test is 5% or less, making repeatability issues unlikely to account for the consistent decrease we observed after PBS in patients with asthma.

F_{ENO} levels decreased somewhat in healthy subjects after buffer challenge. This suggests the possibility that at least some of the NO in the normal airway might be derived from NO_{2}⁻ protonation. The distal airway is likely to be acidic. Surfactant is secreted in lamellar bodies at a pH of approximately 3, and macrophages and high PCO₂ (approximately 40 mm Hg) might also lower the pH. Indeed, data suggesting that inhaled NO_{2}⁻ is converted to NO in vivo might reflect simple NO_{2}⁻ protonation in acidic parts of the airway.29 However, airway pH mapping studies will be required to confirm this hypothesis.

Oral irrigation with buffer had a minimal effect on F_{ENO} levels, likely because both buffer and saliva were at neutral pH. This observation does not exclude the possibility, however, that a previous study in which various mouthwashes altered F_{ENO} levels did so on the basis of oral acidification or alkalinization.30

Consistent with previous reports, we found that subjects with asthma who were not experiencing an acute exacerbation had relatively neutral EBC pH.1,2,4 Although EBC pH increased somewhat with PBS inhalation, the change in F_{ENO} levels with PBS was substantially more dramatic, pH increased somewhat with PBS inhalation, the change in acidic parts of the airway.29 However, airway pH regulation might be a determinant of F_{ENO} levels. Testing with inhaled buffer in the lung function laboratory might prove to be informative with regard to characterizing asthma phenotypes, particularly those of more severely affected patients. Furthermore, pH regulatory mechanisms might provide new therapeutic targets for patients with asthma.

REFERENCES


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