

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 nitrite is converted to nitric oxide (NO).

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

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 F_{ENO} levels. Six subjects also received PBS mouthwash alone.

Results: F_{ENO} levels decreased after buffer inhalation. The maximal decrease occurred between 15 and 30 minutes after treatment; F_{ENO} 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 F_{ENO} levels. The magnitude of this change is greatest in persistent asthma. These data suggest that airway pH is a determinant of F_{ENO} levels downstream from NO synthase activation.

Clinical implications: Airway biochemistry modulates F_{ENO} 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 F_{ENO} values. In fact, PBS challenge testing integrates airway pH and F_{ENO} analysis, potentially improving the utility of F_{ENO} 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

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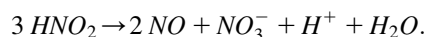
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Several pulmonary diseases are characterized by acidic exhaled breath condensate (EBC) collected both from patients breathing orally and from patients who are endotracheally intubated.¹⁻⁴ The acidity of these specimens is believed to reflect acidity 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 NO^{2,8} according to the following equations:



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 (F_{ENO}).

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.^{1,9} Of note, acute asthma exacerbations are associated with acid breath (acidopnea) that resolves

Abbreviations used

EBC:	Exhaled breath condensate
F _{ENO} :	Fraction of exhaled nitric oxide
NO:	Nitric oxide
NO ₂ ⁻ :	Nitrite
NOS:	Nitric oxide synthase
PBS:	Phosphate buffered saline
pKa:	Acid dissociation constant

with corticosteroid therapy.² Indeed, Ahmed et al¹⁰ 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.^{1,2,11} Here we show that inhalation of PBS causes a decrease in F_{ENO} levels, that this decrease in F_{ENO} 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 spirometry¹² 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, F_{ENO} 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 F_{ENO} 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,¹³ with the average of 3 sequential measurements within 10%.

F_{ENO} 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.¹⁴

EBC was collected with the R-Tube (Respiratory Research, Inc, Charlottesville, Va) and deaerated, as previously described,² before pH measurement.

Clinical protocol

After initial history and physical examination, subjects completed baseline F_{ENO} 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 F_{ENO} 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,^{1,2,11,15} 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 F_{ENO} (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 F_{ENO} levels and to describe the *in vitro* dose responses. 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 I). 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. F_{ENO} 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 \pm 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

TABLE I. Subject characteristics

	Control	Mild intermittent	Persistent	ANOVA
N (M/F)	9 (4/5)	11 (3/8)	8 (3/5)	NS
Age (y ± SEM)	38 ± 3.4	32 ± 2.7	31 ± 3.4	NS
FEV ₁ (%)	101 ± 16	94 ± 2.7	85 ± 6.9	<i>P</i> < .01
FVC (%)	106 ± 14	96 ± 8.3	94 ± 5.9	<i>P</i> < .05
FEV ₁ /FVC (± SEM)	0.82 ± .02	0.80 ± 0.026	0.72 ± 0.023	<i>P</i> < .05
Starting F _{ENO} level (ppb)	11.7 ± 1.2	22.7 ± 2.8	61.9 ± 10	<i>P</i> < .001
On chronic inhaled corticosteroids	0%	45%	88%	

M, Male; F, female; NS, not significant; FVC, forced vital capacity.

F_{ENO} 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 F_{ENO} level ($R^2 = 0.18$). Mean FEV₁ did not change after buffer inhalation. Starting F_{ENO} levels were associated with maximum change in F_{ENO} 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 HCO₃⁻/H₂CO₃, H₂PO₄, 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 postdeaeration (CO₂-free) pH was greater than 8, reflecting the fact that CO₂ 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 NO₂⁻ 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 F_{ENO} level at higher (healthier) pH values *in vivo*.

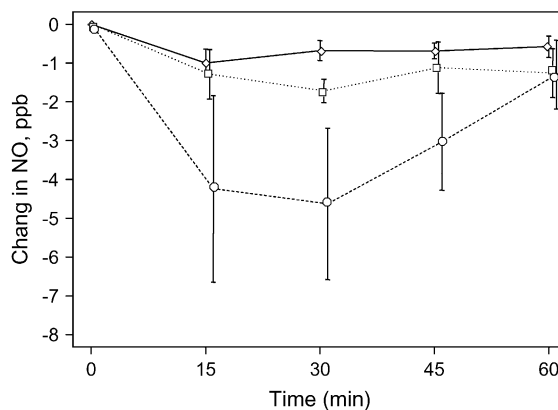


FIG 1. F_{ENO} levels decrease after buffer inhalation. F_{ENO} levels were measured serially before (time 0) and after inhalation of 10 mmol/L PBS. Subjects with persistent asthma (circles) had a greater decrease at 15, 30, and 45 minutes than did control subjects (diamonds); $P < .01$, adjusted for multiple comparisons by using the Bonferroni method. Subjects with mild asthma (squares) did not differ from control subjects.

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.^{1,11,16} 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.^{15,17} Indeed, this endogenous acidification appears to be initiated by T_H1 cytokines during infection¹¹ and might serve to augment antimicrobial host defense in the lung, as it does in the stomach.⁸ In asthma, however, airway acidification triggered by T_H1 cytokines during a viral respiratory tract infection could exacerbate cough, bronchoconstriction, and mucous plugging.^{1,2,17,18}

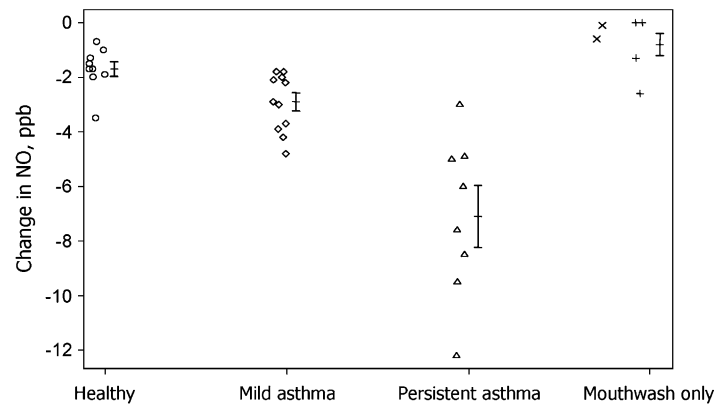


FIG 2. The maximum change in F_{ENO} levels after buffer challenge is greatest in subjects with persistent asthma. The differences between baseline F_{ENO} levels and the lowest F_{ENO} level for each individual subject were compared among 4 groups: control subjects inhaling PBS, subjects with mild intermittent asthma inhaling PBS, subjects with persistent asthma inhaling PBS, and subjects performing a mouthwash with PBS (2 control subjects and 4 subjects with persistent asthma). Change in F_{ENO} levels in control subjects was less than that in those with mild intermittent asthma ($P = .04$) or persistent asthma ($P < .0001$). Of note, the change in subjects with persistent asthma was greater than that in subjects with mild intermittent asthma ($P < .0001$).

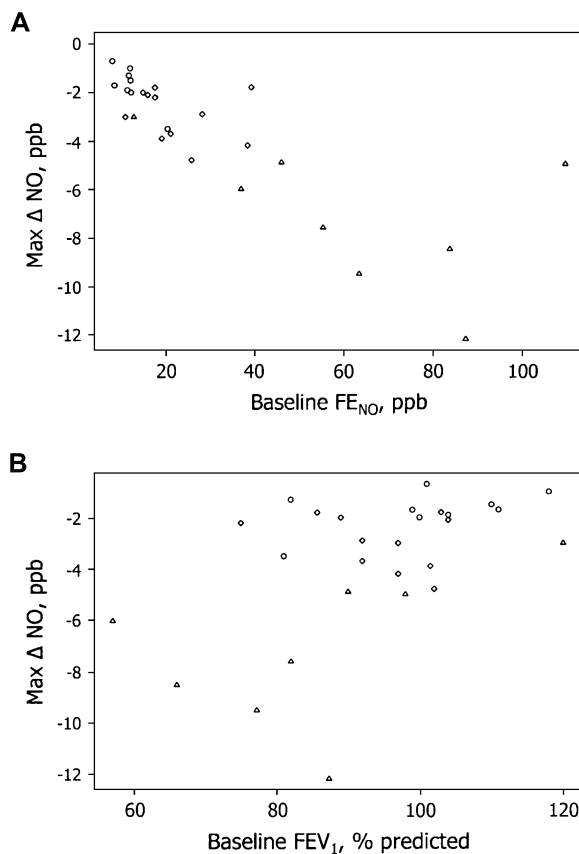


FIG 3. Relationship between maximum change in F_{ENO} level and starting F_{ENO} level and FEV_1 . Starting F_{ENO} level was associated with change in F_{ENO} level (**A**; $R^2 = 0.64$, $P < .0001$), as was starting FEV_1 (**B**; $R^2 = 0.27$, $P = .005$). Circles represent control subjects, diamonds represent subjects with mild intermittent asthma, and triangles represent subjects with persistent asthma.

F_{ENO} level does not consistently distinguish severe asthma from less severe asthma.¹⁹ Indeed, there might be many determinants of the level of F_{ENO} 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 F_{ENO} levels.²⁰ Determinants include (1) NOS expression and activity^{7,20}; (2) enzymes regulating levels of NO-consuming superoxide^{19,21}; (3) enzymes regulating airway levels of S-nitrosothiols²²⁻²⁴ that are produced by NOS and can, in turn, evolve NO²⁴; and (4) prokaryotic denitrification enzymes in the context of airway infection.^{15,25} We now suggest that mechanisms involved in the regulation of airway pH can also affect F_{ENO} downstream from NOS activation. Indeed, the complexity of interpreting F_{ENO} 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, F_{ENO} levels have been used to measure S-nitrosoglutathione catabolism in airways disease²⁶; here we have used it to classify the asthmatic airway response to buffer challenge. These modifications might ultimately add value to F_{ENO} 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 F_{ENO} , might be many, and there can be explanations for our data other than simple inorganic pH buffering by phosphate.¹ 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.¹⁸ 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,^{18,27} 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 3,²⁸ and macrophages and high PCO_2 (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 studies, 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, which is consistent with the nonlinear relationship between airway H^+ concentration and pH. Indeed, the *in vitro* model data show that the deaerated pH of the buffer and the change in headspace NO are not linearly related when the pH is titrated in the range 6 to 9. These data suggest the possibility that F_{ENO} testing after PBS challenge might be a more sensitive assay than EBC pH for subtle airway acidification in subjects with well-controlled asthma.

In contrast to the study by Ahmed et al,¹⁰ we did not find that buffer inhalation decreased airflow obstruction in asthma. The most likely explanation for this difference is that Ahmed et al studied patients during acute asthma exacerbations, whereas our patients were on stable outpatient treatment regimens. More profound airway acidification, and lower starting EBC pH values, would be expected in patients having an acute asthma exacerbation.^{1,2,4} In the setting of an acute asthma exacerbation, a simultaneous decrease in airway pH and increase in airway NO levels might be expected to worsen lung function because both NO and acid can contribute to airway narrowing associated with inflammation^{1,5,19}; indeed, concentrations of NO in the airways are too low to relax airway smooth muscle^{5,9} but can contribute to nitrosative stress, particularly in an acidic environment.^{1,7}

In conclusion, we report that phosphate buffer inhalation decreases F_{ENO} levels and that the magnitude of this change is greatest in subjects with persistent asthma. These data suggest that 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.

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