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Airway nitric oxide release is reduced after PBS inhalation in asthma

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Shin H-W, Shelley DA, Henderson EM, Fitzpatrick A, Gaston B, George SC. Airway nitric oxide release is reduced after PBS inhalation in asthma. *J Appl Physiol* 102: 1028–1033, 2007. First published November 16, 2006; doi:10.1152/japplphysiol.01012.2006.—Exhaled nitric oxide (NO) is elevated in asthma, but the underlying mechanisms remain poorly understood. Recent results in subjects with asthma have reported a decrease in exhaled breath pH and ammonia, as well as altered expression and activity of glutaminase in both alveolar and airway epithelial cells. This suggests that pH-dependent nitrite conversion to NO may be a source of exhaled NO in the asthmatic airway epithelium. However, the anatomic location (i.e., airway or alveolar region) of this pH-dependent NO release has not been investigated and could impact potential therapeutic strategies. We quantified airway (proximal) and alveolar (peripheral) contributions to exhaled NO at baseline and then after PBS inhalation in stable (mild-intermittent to severe) asthmatic subjects (20–44 yr old; $n = 9$) and healthy controls (22–41 yr old; $n = 6$). The mean (SD) maximum airway wall flux (pl/s) and alveolar concentration (ppb) at baseline in asthma subjects and healthy controls was 2,530 (2,572) and 5.42 (7.31) and 1,703 (1,567) and 1.88 (1.29), respectively. Compared with baseline, there is a significant decrease in the airway wall flux of NO in asthma as early as 15 min and continuing for up to 60 min (maximum −28% at 45 min) after PBS inhalation without alteration of alveolar concentration. Healthy control subjects did not display any changes in exhaled NO. We conclude that elevated airway NO at baseline in asthma is reduced by inhaled PBS. Thus airway NO may be, in part, due to nitrite conversion to NO and is consistent with airway pH dysregulation in asthma.

pH; inflammation

NITRIC OXIDE (NO) appears in the exhaled breath (1, 9) and has been proposed to perform many functions in the lungs, such as smooth muscle relaxation, host defense, inhibition of platelet aggregation, and neurotransmission. Exhaled NO concentration is elevated in untreated asthma, reduced by corticosteroid therapy, and elevated during acute exacerbation of asthma relative to results in healthy controls (2, 14, 19, 24). The underlying mechanisms leading to increased NO release are not fully understood but likely involve increased expression of inducible NO synthase in the airway epithelium (8, 10) and nitrite conversion to NO at low pH (13, 18, 31, 32). Acute asthma impairs the ability of the lungs to buffer the breath condensate, resulting in a decrease in the pH of more than two log units (13). This observation may be due to altered expression and activity of glutaminase, which is expressed in both alveolar and airway epithelial cells (12). However, the anatomic location (i.e., airway or alveolar region) of this loss in buffering has not been investigated and could impact potential therapeutic strategies.

Our laboratory has previously described a two-compartment (airway and alveolar regions) model of NO exchange (26) and a single-breath technique (27) to characterize flow-independent NO exchange parameters that can partition exhaled NO concentration into proximal [maximum airway wall flux of NO ($J'aw_{NO}$)] and peripheral [steady-state alveolar concentration of NO ($C_{A_{NO}}$)] contributions. Previous work by our group and others has demonstrated that the increased level of exhaled NO observed in asthma is due primarily to alterations in airway NO (i.e., increased $J'aw_{NO}$) (11, 20, 24) but may also include an increase in the alveolar component (3, 7, 17, 29). Alterations in the buffering capacity of asthma patients may account, in part, for the observed alterations in airway and alveolar NO release. Thus we hypothesized that inhalation of neutral pH PBS would increase the pH of the airway and alveolar lining fluid in stable asthma patients and would decrease the concentration of NO in the exhaled breath by slowing the conversion of nitrite to NO (13, 18, 31, 32).

To test our hypothesis, we administered PBS (pH 7) by inhaled aerosol to both healthy controls and stable asthma patients and then monitored exhaled NO exchange dynamics using our single-breath maneuver and two-compartment model. Our results indicate that inhaled PBS significantly decreases $J'aw_{NO}$ but not $C_{A_{NO}}$ in asthma subjects, providing indirect evidence that the pH of the airway lining fluid can influence the release of NO into the exhaled breath.

METHODS

Subjects. Nine asthmatic adults (21–44 yr old), and six healthy adult controls (22–41 yr old) participated in this study. Inclusion criteria for asthma were 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 history of smoking, pulmonary diseases other than asthma, and cardiovascular or neurological disease. For the healthy adult group, inclusion criteria included no history of heart disease, lung disease, or smoking and normal standard spirometry [forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) > 80% predicted].

Protocol. Each subject performed baseline exhaled NO measurements (see below) in triplicate and spirometry. Spirometry included FVC, FEV_1, forced expiratory flow after exhalation of 25–75%, and FEV_1/FVC measured in triplicate (Vmax229; Sensormedics, Yorba Linda, CA). Each subject then completed a 10-min inhalation of 10

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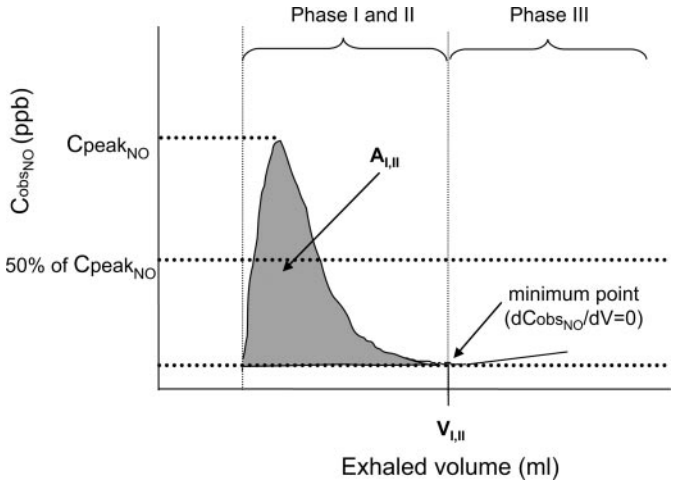

Fig. 1. Schematic of the exhaled nitric oxide (NO) profile. $Cobs_{NO}$, exhaled NO concentration observed experimentally from the analytical instrument after the 20-s preexpiratory breathhold, followed by a decreasing flow exhalation; $Cpeak_{NO}$, peak NO concentration. The exhalation profile demonstrates an initial bolus of NO, representing the accumulation of NO in the airways during the breathhold. The total mass of NO in this bolus can be characterized by the area under the curve in phases I and II ($A_{I,II}$, shaded region). The distinction between phase I and II and phase III is the point of zero slope (minimum point) in the exhalation profile as previously described (27).

mM dibasic PBS (pH 7) prepared aseptically by the University of Virginia Research Pharmacy using a Micro Mist nebulizer (Hudson RCI, Temecula, CA), which delivered ~3 ml of 10 mM PBS for 10 min. Each subject then repeated the exhaled NO measurements in triplicate at 15, 30, 45, and 60 min post-PBS inhalation. Spirometry was not repeated after PBS inhalation because it can influence exhaled NO (6, 25), and changes in airway NO exchange occur in a pattern distinct from spirometry (22). The use of inhaled PBS was approved (Investigational New Drug exception status granted for this protocol) by the U.S. Food and Drug Administration. The protocol was approved by the University of Virginia Human Investigation Committee, and written informed consent from all subjects was obtained.

Exhaled NO measurement. A NIOX instrument from Aerocrine (Stockholm, Sweden) was used to record NO, pressure, and flow for three repetitions of a 20-s preexpiratory breathhold followed by a decreasing flow rate maneuver in each subject (27). The profiles were characterized initially by the total mass (area) of NO in phases I and II ($A_{I,II}$) of the expirogram (Fig. 1), which is independent of a model (21, 27). In addition, the profiles were then used to determine the flow-independent NO exchange parameters ($J'aw_{NO}$, and CA_{NO}) using our previously described two-compartment model (26–28). In brief, the two-compartment model partitions the lung into a flexible balloon representing the alveolar region attached to a rigid tube representing the airways. The alveolar region is characterized by a steady-state CA_{NO}. Upon exhalation, air at CA_{NO} is convected through the airway compartment, where additional NO is added by radial diffusion from the airway wall at a rate equal to $J'aw_{NO}$. Thus the exiting or exhaled concentration of NO has contributions from both the alveolar and airway regions.

Statistics. Data were analyzed with the use of repeated-measure ANOVA to detect differences between groups and within subjects over time. Paired-comparison *t*-tests were employed to evaluate the differences between subjects' baseline values and their values after PBS challenge over time or over normalized exhaled volume intervals. Unpaired-comparison *t*-tests were employed to evaluate the differences between asthma subjects and healthy controls. Statistical significance was considered at $P < 0.05$.

RESULTS

The physical characteristics of the subjects such as age, height, weight, and ideal body weight were not statistically different between healthy controls and subjects with asthma (Table 1). All subjects were able to complete the 10-min PBS challenge without complication. Baseline FVC, FEV_1, forced expiratory flow after exhalation of 25–75%, and FEV_1/FVC for healthy controls and subjects with asthma are presented in Table 2. Baseline FEV_1 and FEV_1/FVC were not significantly

Table 1. *Physical characteristics of subjects*

	Sex	Age, yr	Height, cm	Weight, lb	BMI, kg/m^2	Iwgt, lb	V_{air}, ml	Primary Therapy
				Healthy adults				
1	F	25	170	140	21.9	131	156	
2	F	35	169	148	23.5	135	170	
3	F	35	150	94	19.0	100	135	
4	F	41	178	211	30.2	170	211	
5	F	33	170	269	42.2	184	217	
6	M	22	185	163	21.6	168	190	
Mean		31.8	170	171	26.4	148	180	
SD		7.05	11.7	61.1	8.61	31.4	32.0	
				Adults with asthma				
1	M	42	168	215	34.6	179	221	ICS
2	M	44	178	180	25.8	170	214	ICS
3	F	39	168	200	32.1	156	195	IBA/Sin
4	M	22	168	304	48.9	224	246	ICS
5	F	39	163	119	20.3	119	158	IBA/Sin
6	M	20	183	157	21.3	163	183	IBA
7	F	40	172	240	36.8	176	216	ICS
8	F	21	163	132	22.5	121	142	IBA/Sin
9	M	34	180	211	29.5	188	222	IBA
Mean		33.4	171	196	30.2	166	200	
SD		9.72	7.32	57.1	9.17	32.5	33.4	

BMI, body mass index; Iwgt, ideal body weight; V_{air}, volume of the airway compartment estimated in ml as the sum of the subjects ideal body weight (lb) plus age (yr) (27); ICS, inhaled corticosteroid; IBA, inhaled β_2-agonist (e.g., albuterol); Sin, Singulair; F, female; M, male.

Table 2. *Spirometry at baseline*

	FVC, liter	FVC, %predicted	FEV_1, liter	FEV_1, %predicted	FEV_1/FVC, %predicted	FEV_1/FVC
			Healthy adults			
1	4.82	116	3.81	120	108	0.79
2	4.08	100	2.55	76	76	0.63
3	3.31	114	2.81	110	97	0.85
4	4.54	108	3.59	110	102	0.79
5	4.96	122	3.89	121	99	0.78
6	6.09	101	5.27	111	110	0.87
Mean	4.63	110	3.65	108	98.6	0.78
SD	0.93	8.73	0.96	16.5	12.2	0.09
			Adults with asthma			
1	4.52	101	3.44	99	98	0.76
2	4.35	87	3.26	87	100	0.75
3	3.34	88	2.51	85	97	0.75
4	5.71	124	5.35	132	107	0.94
5	3.68	103	2.77	99	97	0.75
6	5.18	96	3.88	83	70	0.75
7	3.84	96	3.1	95	97	0.81
8	3.57	90	3.07	89	100	0.86
9	5.26	79	4.29	57	72	0.55
Mean	4.38	96.0*	3.52	91.8	93.1	0.77
SD	0.85	12.9	0.87	19.7	12.9	0.10

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s. *Statistically different from healthy controls at baseline ($P<0.05$).

different between healthy controls and subjects with asthma. FVC (%predicted) was statistically lower in the asthma group compared with healthy controls ($P = 0.04$).

All the subjects were able to complete the preexpiratory 20-s breathhold followed by a decreasing flow rate maneuver at baseline and after PBS inhalation. PBS inhalation did not generate any respiratory distress in any of the subjects. Composite experimental NO exhalation profiles for subjects with asthma and healthy controls for baseline and 60 min post-PBS challenge are presented in Fig. 2 and provide a qualitative assessment of exhaled NO. The mean exhaled concentrations of all subjects in a given group and condition (i.e., baseline and 60 min after PBS challenge) are plotted at equivalent exhaled volume intervals normalized by FVC. When exhaled NO values at 0.05-s intervals of normalized exhaled volume are compared, PBS inhalation significantly reduced the amount of NO in the exhaled breath in the asthmatic subject group throughout the entire exhalation profile ($P = 0.0018$). This can be seen by the reduced peak height in phase I and II, smaller area under the curve ($A_{I,II}$), and lower NO concentrations in phase III of the exhalation profile (Fig. 2*A*). The PBS inhalation did not impact the exhalation NO profile for healthy controls (Fig. 2*B*).

The experimentally observed changes in exhaled NO concentration such as $A_{I,II}$ are reflected in quantifiable changes in the flow-independent NO exchange parameters such as $J'aw_{NO}$ and C_{ANO} for both asthma subjects and healthy controls. Mean (SD) values of $A_{I,II}$ at baseline and 60 min post-PBS challenge were 31.6 (30.8) ppb·l and 17.8 (14.7) ppb·l for asthma subjects and 15.4 (10.4) ppb·l and 15.3 (6.9) ppb·l for healthy controls, respectively. Mean (SD) values of $J'aw_{NO}$ at baseline and 60 min post-PBS challenge were 2,530 (2,570) pl/s and 1,700 (1,570) pl/s for asthma subjects and 1,720 (1,570) pl/s and 1,990 (1,810) pl/s for healthy controls, respectively. Mean (SD) values of C_{ANO} at baseline and 60 min post-PBS challenge were 5.42 (7.31) and 2.47 (2.17) ppb for asthma subjects and 1.88 (1.29) and 1.90 (1.61) ppb for healthy controls, respectively.

Figure 3 presents the dynamic changes in exhaled NO as a percent change from baseline in both healthy controls and asthmatic subjects after PBS inhalation. $A_{I,II}$ and $J'aw_{NO}$ progressively decrease after the PBS challenge relative to baseline only in asthmatic subjects (Fig. 3, *A* and *C*). The decrease is statistically significant at 30, 45, and 60 min for $A_{I,II}$ and at 15, 45, and 60 min for $J'aw_{NO}$. In contrast, C_{ANO} from asthma subjects was not statistically altered after PBS relative to baseline (Fig. 3*E*). $A_{I,II}$, $J'aw_{NO}$, and C_{ANO} results from healthy controls were not significantly altered after the PBS challenge relative to baseline (Fig. 3, *B*, *D*, and *F*).

DISCUSSION

This is the first study to determine the effect of PBS on proximal and peripheral NO release. We found that inhalation of PBS (pH 7) caused a significant decrease in $J'aw_{NO}$ only in subjects with asthma. In contrast, C_{ANO} was not altered in asthmatic subjects or healthy controls. We conclude that *1*) pH-dependent NO release may contribute to the increase in exhaled NO observed in asthma and *2*) the decrease in NO release in asthma after inhalation of neutral pH PBS occurs primarily in the airway region. These results are consistent with previous reports of altered pH regulation in asthma (12, 13, 15) and suggest that exhaled NO in asthma may be, in part, an indicator of airway buffering capacity.

The sensitivity of exhaled NO to inhaled PBS suggests strongly that NO release from the airways is pH sensitive and thus may represent conversion of micromolar nitrite present in the airway lining fluid (13, 18) via the following reaction:

$$2NO_2^- + 2H^+ \leftrightarrow NO + NO_2 + H_2O \qquad (1)$$

This reaction scheme involves nitrous acid (HNO_2), NOOH, and N_2O_3 as intermediates, and the kinetics have been previ-

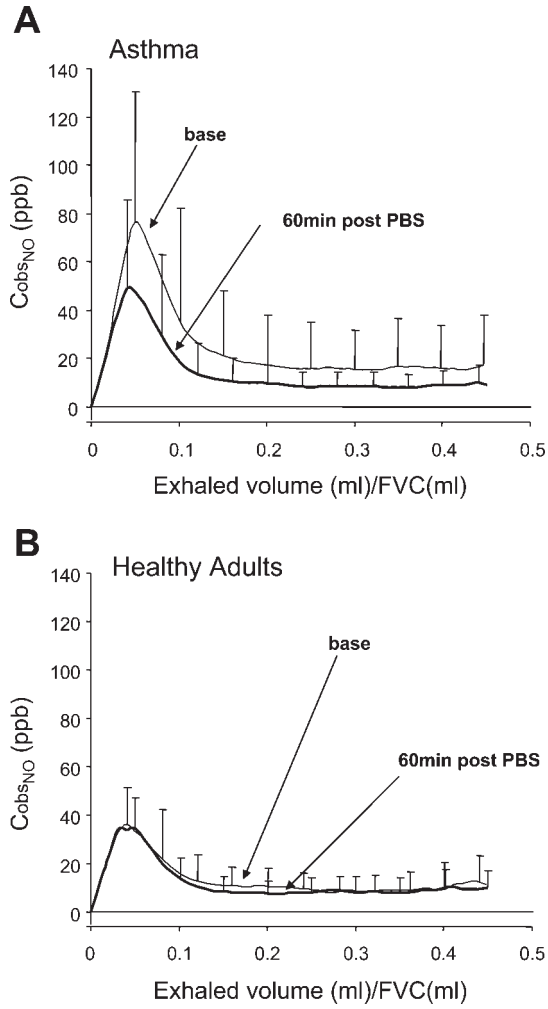

Fig. 2. Experimental composite NO exhalation profiles are presented for subjects with asthma ($n = 9$; *A*) and healthy control subjects ($n = 6$; *B*) at baseline and 60 min post-PBS inhalation. Briefly, the mean exhaled concentration of all subjects in a given group and condition (baseline and 60 min post-PBS inhalation) are plotted at equivalent normalized exhaled volume intervals [exhaled volume/forced vital capacity (FVC)]. SD results are presented at regular intervals for both healthy controls and asthma subjects.

ously described in detail (31) such that the rate of NO production (d[NO]/dt, M/s), can be written in the following simplified form:

$$\frac{d[NO]}{dt} = \frac{K_1}{K_\alpha K_\beta}[NO_2^-]_0^2[H^+] \quad (2)$$

where concentrations (in M) are denoted by brackets, $[NO_2^-]_0$ is the initial nitrite concentration, K_α is the dissociation constant of nitrous acid (pK = 3.4), and K_1 and K_β are constants previously determined from experimental data (31, 32) and equal to 9.3×10^{-4} s^{-1} and 1.6×10^{-4} M, respectively. It has been assumed that nitrous acid forms very rapidly, and the intermediates N_2O_3 and NOOH do not accumulate appreciably relative to nitrite. In addition, the form of *Eq. 2* has been derived by noting that $K_\alpha >> [H^+]$ for pH > 5 and $K_\beta >> [NO_2^-]_0$ for the physiological range (10–50 μM) (31).

It is evident from *Eq. 2* that, for an initial nitrite concentration of 10 μM, the rate of NO production from nitrite conversion is ~0.15 pM/s at a pH of 7, and this increases to 1.5 pM/s at a pH of 6. Both numbers are the same order of magnitude as that needed in the two-compartment model (0.55 pM/s) to generate exhaled NO levels present in the exhaled breath (26). Thus relatively modest changes in airway lining pH are likely to generate detectable changes in exhaled NO.

The dose of PBS inhaled by each subject in our study was 3 ml of a 10 mM sodium phosphate solution, or a total of 30 μmol of phosphate ($H_2PO_4^-$) was delivered to the lungs. In an airway lining fluid volume of ~1 ml/kg, this would provide an increase in dibasic phosphate buffer (pK_a of ~7.0) of ~100 μM in the airway based on ~20% deposition of total inhaled dose (i.e., 5–6 μmol of the 30-μmol inhalation), which is three logs higher than the pK_a of nitrous acid. This amount of dibasic phosphate was therefore predicted to decrease the number of free protons or hydronium ions, moving the pH toward 7.0. Our observation that only airway NO release was altered by PBS inhalation might be explained by a larger dose of PBS delivered to the airway surface but could also be explained by the possibility that only the airways exhibit altered pH regulation in asthma.

Although difficult to estimate the precise dose to the airway and alveolar regions, it is well established that aqueous particles with a diameter >5 μm deposit primarily in the airway tree, whereas particles with a diameter between 1 and 3 μm will deposit in the alveolar region (4, 5, 30). The mass median aerodynamic diameter of particles from the Micro Mist nebulizer is 3.6 μm, and thus a portion of the inhaled PBS dose should have been delivered to both regions of the lungs. Although PBS particles may shrink or grow (hygroscopic) depending on the relative humidity, the humidity of the inhaled aerosol mist should be close to 100%. Thus we do not anticipate any significant transfer of water to or from the aerosol particles once inhaled. However, other factors such as airway temperature, surface tension of airway lining fluid, or oxidative stress may affect the deposition pattern of the aerosol or local release of NO and thus impact the exhaled NO concentration after PBS challenge.

At baseline, our data (Fig. 3) demonstrate that $J'aw_{NO}$ is ~1.5-fold higher in asthmatic subjects than in healthy controls. This trend is similar to that previously reported in steroid-naive asthmatic subjects (20, 22, 24). Our group of asthmatic subjects included both steroid-treated and steroid-naive patients. Previous studies have reported a decrease in $J'aw_{NO}$ after steroid treatment (16, 20, 24), which may account for the fact that our result was not statistically different from healthy controls. However, as a mixed (i.e., both steroid treated and steroid naive) stable population, inhalation of PBS significantly reduced exhaled NO, particularly the contribution from the airway compartment, to levels nearly identical to healthy controls.

Figure 4 summarizes the proposed mechanisms of NO release into the gas phase based on the available literature and the data from the present study. NO is generated enzymatically in the epithelium from NO synthase isoforms where it can diffuse freely toward the mucus and enter the gas phase (23). NO can also be oxidized through reactions with oxygen and glutathione to form nitrite. Nitrite is present in the mucous layer in micromolar concentrations and can be reduced back to NO under acidic conditions.

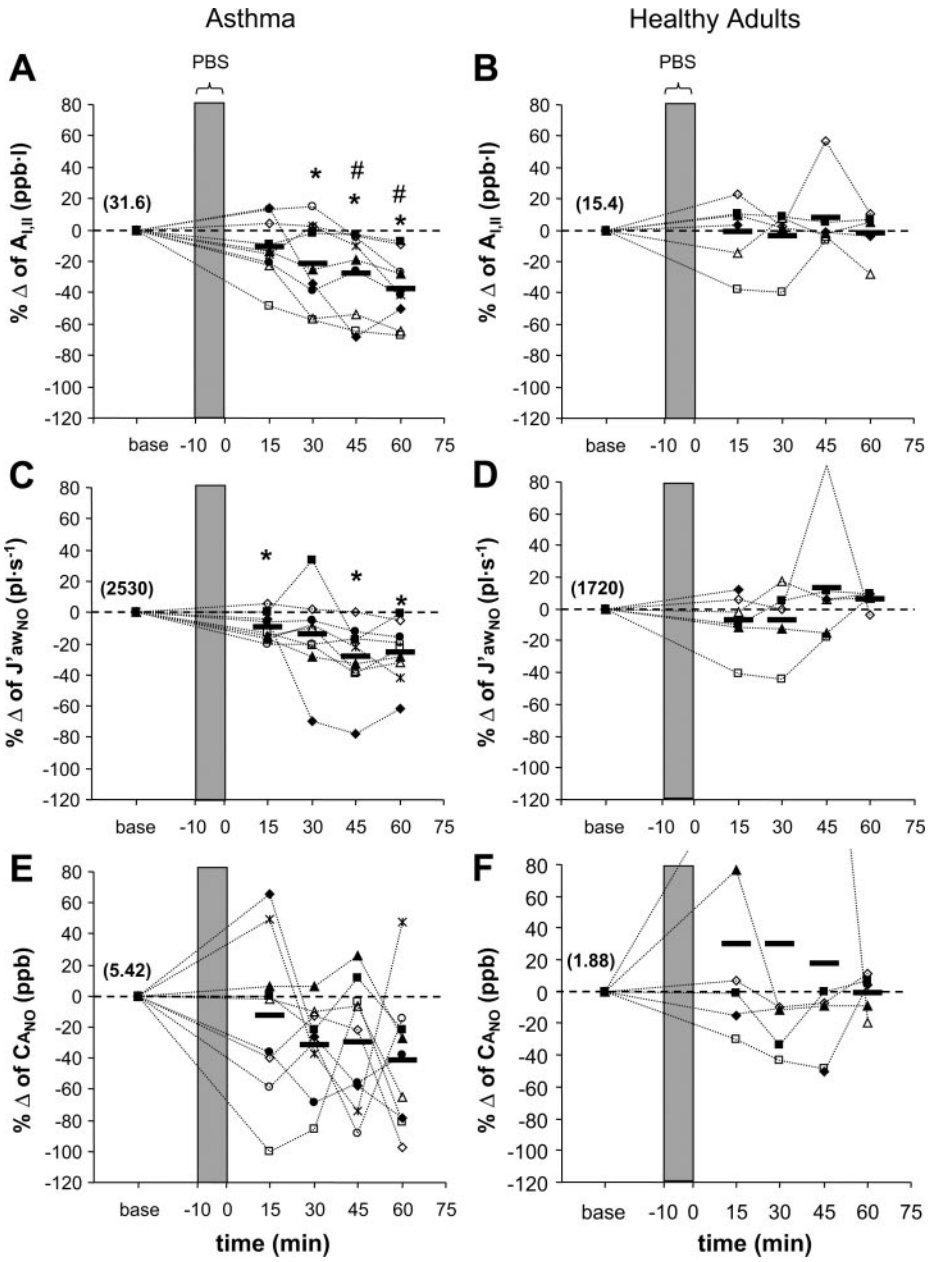

Fig. 3. Percent changes of $A_{I,II}$ (*A* and *B*), $J'aw_{NO}$ (*C* and *D*), and CA_{NO} (*E* and *F*) at post-PBS challenge relative to baseline are shown in 9 subjects with asthma (*A*, *C*, and *E*) and in 6 healthy controls (*B*, *D*, and *F*). Vertical bars indicate the window of time for the delivery of PBS. Dotted lines with open or closed symbols represent individual data. The mean value at each time point is shown by the horizontal solid rectangle. Baseline values are presented in parentheses, which were determined ~15 min before PBS challenge. The magnitudes of the *y*-axes of the healthy control subjects are set to the same scale as those of the asthma subjects; thus some of the off-scale values are not visualized in *F*. *Difference between post-PBS relative to baseline is statistically significant (paired *t*-test, $P < 0.05$). #Statistically different compared with healthy controls at the same time point (unpaired *t*-test, $P < 0.05$).

Inhalation of PBS can normalize the pH and thus reduce NO in the exhaled breath.

Finally, glutaminase-mediated conversion of glutamine to glutamate produces ammonia and serves as an important pH regulatory mechanism in the airway epithelium. Glutaminase activity in the airway epithelium is reduced in asthma, can be increased by corticosteroid therapy, and in vitro can buffer a decrease in medium pH even in the presence of excess bicarbonate (12). In addition, breath condensate pH and ammonia are both decreased in acute asthma, and this has been proposed as a mechanism to release NO into the gas phase from nitrite (12, 13, 15).

In conclusion, we have quantified airway and alveolar NO exchange in asthma after inhalation of PBS. An elevated maximum airway wall flux of NO at baseline is significantly decreased after inhalation of PBS without alteration of alveoli

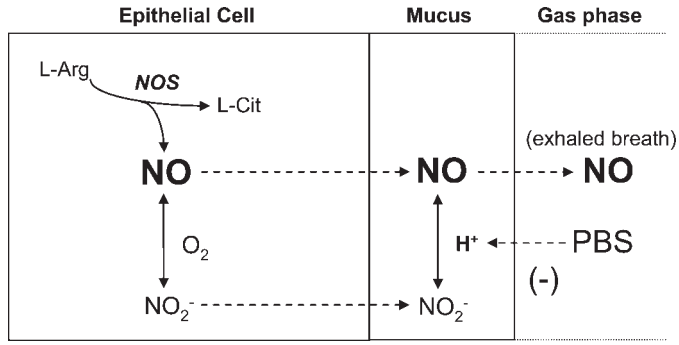

Fig. 4. Proposed mechanism for NO release to the gas phase. NO is produced enzymatically from nitric oxide synthase (NOS) isoforms in the epithelium, can diffuse freely to the mucus, and appear in the exhaled breath (23). A portion of intracellular NO may also be oxidized to the more stable form of nitrite. A low pH can convert NO_2^- to NO, which is released to the gas phase. Inhaled PBS can neutralize an acidic pH.

concentration in subjects with asthma. Thus a significant source of NO in the exhaled breath likely arises from nitrite conversion to NO at low pH. Our results suggest that increased exhaled NO in asthma may be, in part, an indicator of altered pH regulatory mechanisms and warrants further study to determine the precise cellular mechanisms.

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