Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways

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ABSTRACT Recent discoveries suggesting essential bioactivities of nitric oxide (NO) in the lung are difficult to reconcile with the established pulmonary cytotoxicity of this common air pollutant. These conflicting observations suggest that metabolic intermediaries may exist in the lung to modulate the bioactivity and toxicity of NO. We report that S-nitrosothiols (RS-NO), predominantly the adduct with glutathione, are present at nano- to micromolar concentrations in the airways of normal subjects and that their levels vary in different human pathophysiologic states. These endogenous RS-NO are long-lived, potent relaxants of human airways under physiological O₂ concentrations. Moreover, RS-NO form in high concentrations upon administration of NO gas. Nitrite (10–20 μM) is found in airway lining fluid in concentrations linearly proportional to leukocyte counts, suggestive of local NO metabolism. NO itself was not detected either free in solution or in complexes with transition metals. These observations may provide insight into the means by which NO is packaged in biological systems to preserve its bioactivity and limit its potential O₂-dependent toxicity and suggest an important role for NO in regulation of airway luminal homeostasis.

There is accumulating evidence that nitric oxide (NO) is produced in mammalian airways: constitutive and inducible forms of NO synthase have recently been identified in the lung (1, 2), oxides of nitrogen (NO_x) are produced by airway cells (3), and exhaled NO gas can be measured in nonsmoking volunteers (4). Potential sources of NO include the lung parenchyma itself [smooth muscle, mast cells, nerves, and epithelium (1–3, 5–8)] as well as the cellular constituents of airway lining fluid [macrophages and neutrophils (1, 2, 9–11)]. NO has well established vasodilator properties that may play a role in the physiological regulation of blood flow and pressure in the pulmonary circulation (12). Although more disputed (13), the smooth muscle relaxant effects of NO in airways suggest that it may have an additional role as a bronchodilator (14–16).

NO, a common air pollutant and component of cigarette smoke, has been viewed as highly toxic to the lung, in part as a consequence of the high ambient O₂ tension (17–22). The reactions of NO with ambient O₂ and superoxide (O₂^{•−}) yield more reactive NO_x, including nitrogen dioxide (NO₂) and peroxynitrite (OONO[−]), which are implicated in bronchiolitis and lung edema (17, 20, 21, 23, 24). There is additional concern that the oxidative metabolism of NO may lead to formation of carcinogenic nitrosoamines (25, 26). Notably, these oxidative reactions of NO also result in the rapid loss of its smooth muscle relaxant activity (27, 28).

An alternative metabolic pathway for NO_x of potential biological relevance involves the reaction with thiols yielding S-nitrosothiols (RS-NO) (28). According to recent observations, RS-NO possessing biological activity are abundant in plasma compared to reactive free NO (29). Moreover, these RS-NO adducts appear to be more stable than NO itself (30, 31). Given that endogenous thiol is prevalent in the lung in the form of glutathione (32), we hypothesized that RS-NO may serve the dual function in airways of stabilizing NO in bioactive form and modifying its cytotoxic potential. We also sought to identify other endogenous NO_x and iron–nitrosyl adducts present in human airways.

METHODS

Subjects. Airway fluid samples were obtained from 20 normal subjects, 16 subjects with pulmonary disease, and 2 subjects receiving inhaled NO gas for treatment for pulmonary hypertension. Normal subjects (13 ± 2.5 years old) were defined as nonsmokers with normal pulmonary examinations and no history of respiratory illness. Bronchoalveolar lavage fluid (BALF) (i.e., fluid from the distal airways and alveoli) was obtained from 6 healthy volunteers (25 ± 5.3 years old). Tracheal aspirates (TA) (i.e., upper airway secretions) were obtained in 5 of these subjects and from an additional 14 normal subjects undergoing elective surgery. Selected analyses were also made on several normal lavages, which had been previously stored at −20°C in the hospital tissue bank. BALF was also obtained in 9 lung transplant patients (29 ± 6.3 years old) and in 3 patients with pneumonia (44 ± 12 years old). Of the lung transplant patients, 2 were experiencing acute rejection. All 9 were receiving cyclosporine, and 5 were receiving glucocorticoids. Pneumonia patients had unremitting fever and radiographic evidence of pneumonia. The etiology of the pneumonia was bacterial in one and undetermined in two patients. TA were also obtained from four patients on extracorporeal membrane oxygenation (ECMO) (8 ± 4 days old). Samples were obtained in accordance with protocols approved by the respective institutional research review boards at Children's Hospital and the Brigham and Women's Hospital (Boston).

Chemical and Cellular Analysis. Samples were transported in airtight syringes at 4°C in the dark for immediate study. Cell count, differential, and pH were measured on the native sample, and the remainder was spun at 7000 × g for 10 min. The supernatant was aliquoted for further study, and the cell pellet was snap frozen (−70°C) for electron paramagnetic resonance (EPR) spectroscopy. RS-NO were assayed by two

Abbreviations: NO_x, oxide(s) of nitrogen; RS-NO, S-nitrosothiol(s); BALF, bronchoalveolar lavage fluid; TA, tracheal aspirate(s); ECMO, extracorporeal membrane oxygenation; EPR, electron paramagnetic resonance; GS-NO, S-nitrosoglutathione.

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established methods: (i) NO^* , liberated by photolytic cleavage of RS-NO, was measured by chemiluminescence as recently described (29); (ii) verification of RS-NO concentration was made by the method of Saville (33), in which the NO group is measured colorimetrically after its displacement by HgCl_2 . RS-NO levels were closely correlated by using the two assay methodologies ($r = 0.85$; $P = 0.00042$) within the limits of sensitivity of the Saville assay ($n = 11$; $>150 \text{ nM}$), confirming previous reports (29, 31). In additional cases in which analysis of BALF by Saville was limited by its excessive dilution (see below), the presence of RS-NO was confirmed by the addition of Saville reagents (in the presence and absence of HgCl_2) directly to the lavage samples, which were then subjected to concentration under vacuum (Speed-Vac; Savant). Controls were prepared in the presence of excess free thiol (glutathione and/or cysteine) and nitrite (NO_2^-) at concentrations exceeding those in the lavage to exclude artifactual formation of RS-NO. RS-NO were synthesized as standards [and for bioassays (see below)], using equivalent concentrations of thiol (in H_2O) and acidified NaNO_2 (1 M HCl). Details of this method have been published (34). NO_2^- was assayed by the Griess reaction, involving diazotization of sulfanilamide and subsequent coupling to *N*-(1-naphthyl)ethylenediamine (29, 31). NO^* was assayed by chemiluminescence measurements made without chemical reduction of samples or photolysis (29). Transition metal-NO complex formation was measured by EPR on the TA and BALF cell pellet and supernatant at 0°C (flat cell) and 77 K (fused silica EPR tube) in a Varian E-109 spectrometer. Exemplary iron-nitrosyl complexes (with cysteine, glutathione, and albumin) were synthesized by established methods (35, 36) and used as standards. Additional spectroscopic references for NO-heme protein complex formation in BALF extracts were obtained from Maples and coworkers (37). In selected studies, the possible contribution of iron-nitrosyl species to other NO determinations (see above) was excluded by the addition of $500 \mu\text{M}$ EDTA to lavage samples. Sulfhydryls were assayed with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (38). BALF values were corrected for the dilution effect of lavage by comparison of serum and BALF urea nitrogen levels (urease endpoint) (39). Reagents were purchased from Sigma.

Human Airway Bioassay. To investigate the bioactivity of RS-NO, human bronchi (outer diameter, 5–12 mm) were obtained from the uninvolved periphery of specimens from 13 patients undergoing operative lobectomy or pneumonectomy: mean age, $64 (\pm 2.5)$ years; preoperative forced vital capacity, 77% predicted ($\pm 1.7\%$); forced expiratory volume at 1 sec, 69% ($\pm 4.7\%$); arterial oxygen tension, $87 (\pm 3.1)$ mmHg; arterial CO_2 tension, $39 (\pm 1)$ mmHg. Eleven patients had non-small-cell carcinoma, one had metastatic melanoma, and one had mesothelioma. All provided informed consent for tissue donation. Specimens were cleaned of adherent connective tissue, cut into 3-mm-thick rings, and connected to transducers for recording changes in isometric force. Tension was measured (model FTO.3C transducers; Grass Instruments, Quincy, MA) on tissues bathed in modified Krebs-Henseleit buffer (118 mM NaCl/5.4 mM KCl/1.10 mM NaH_2PO_4 /11.1 mM glucose/25.0 mM NaHCO_3 /1.38 mM MgSO_4 /2.32 mM CaCl_2 , pH 7.4) (Fisher Scientific) bubbled with 95% O_2 /5% CO_2 . Epithelial integrity was confirmed histologically at the end of the experiment. Details of methods of tissue preparation and airway bioassay have been published (40).

Oxygen-Dependent Synthesis and Decomposition of RS-NO. To study possible mechanisms of RS-NO formation in airway lining fluid, purified NO^* gas was bubbled into physiological solutions (10 mM PO_4^{3-} , pH 7.4) containing glutathione. Oxygen tension was regulated with a Bird blender and NO^*

and NO_2^- concentrations were determined by on-line chemiluminescence (Thermoenvironmental Institute, Franklin, MA).

The toxicity of free NO^* has been related to its oxygen-dependent decomposition (17). The stability of endogenous RS-NO was therefore studied in bronchoalveolar lavage at different oxygen tensions. Time-dependent degradation of specific RS-NO was assessed by UV spectroscopy using their absorption maximum of $\approx 340 \text{ nm}$ (30, 31), accompanied by the measured accumulation of the end degradation product, NO_2^- by the method of Saville. *S*-Nitrosoglutathione (GS-NO) was synthesized as described above and used as a control.

Statistical Analysis. Differences between BALF concentrations and bioassay concentration-effect relationships were analyzed by two-way analysis of variance, followed, where appropriate, by unpaired *t* tests. TA concentration differences were compared by unpaired *t* tests. Correlations between measured BALF and TA parameters were assessed by linear regression analysis. Results are expressed as means $\pm$ SEM. *P* values of <0.05 were considered significant.

RESULTS

Chemical Studies. Nitrite and RS-NO were detected in all samples. The NO_2^- concentrations of BALF and TA in normal subjects were similar— 15 ± 4.5 and $19 \pm 2.8 \mu\text{M}$, respectively; RS-NO concentrations in these samples were 0.24 ± 0.075 and $0.53 \pm 0.11 \mu\text{M}$, respectively ($P = \text{not significant}$ comparing BALF to TA concentrations of both NO_2^- and RS-NO). Airway levels of RS-NO varied significantly in the patients studied: RS-NO concentrations were 3.5-fold higher in lung transplant ($0.83 \pm 0.063 \mu\text{M}$) than in normal BALF ($P < 0.05$) and 20-fold higher in pneumonia BALF ($4.43 \pm 1.4 \mu\text{M}$; $P < 0.05$) (Fig. 1). RS-NO were 10-fold lower in the TA of ECMO patients ($0.056 \pm 0.027 \mu\text{M}$) than in normal TA ($P < 0.05$) (Fig. 2); there was no relationship between RS-NO concentration and subject age in normal TA. Nitrite concentrations did not vary from normal with disease state ($P = \text{not significant}$): levels were $13 \pm 16 \mu\text{M}$ in transplant patients, $14 \pm 8 \mu\text{M}$ in pneumonia patients, and $10 \pm 9 \mu\text{M}$ in patients undergoing ECMO. NO^* and iron-nitrosyl species were not detected in any sample. The metabolic fate of NO^* was further assessed in two subjects administered 40 ppm of NO^* gas (in 80% O_2 /balance N_2) for pulmonary hypertension. Tracheal aspirates, obtained after 10 min of inhalation, revealed NO_2^- levels of $\approx 100 \mu\text{M}$ and RS-NO levels of $12.3 \mu\text{M}$ (12 and $12.7 \mu\text{M}$) (Fig. 2).

Relationship of NO_x and RS-NO Concentrations to the Chemical and Cellular Constituents of Airway Aspirate Fluid (Table 1). BALF NO_2^- concentrations increased linearly with macrophage counts ($P < 0.05$) and total leukocyte counts (P

FIG. 1. Distal airway RS-NO levels in different patient populations. Lung lining fluid RS-NO concentrations in lung transplantation ($n = 9$) and pneumonia patients ($n = 3$) are greater than in normal subjects ($n = 6$) (*, $P < 0.05$).

FIG. 2. Tracheal lining fluid RS-NO concentrations in patients on ECMO ($n = 4$) are less than in normal subjects ($n = 19$) (*, $P < 0.05$). Levels of RS-NO increase significantly in upper airway aspirates upon administration of inhaled NO[•] gas ($n = 2$) ($P < 0.05$).

< 0.005). A similar trend was also observed with neutrophils ($P = 0.09$), but not with epithelial cell or erythrocyte counts ($P =$ not significant). RS-NO concentrations were directly proportional to free thiol levels ($P < 0.05$) but did not correlate significantly with NO₂⁻ levels or airway cell counts ($P =$ not significant). BALF pH ranged from 6.12 to 6.83 (mean, 6.25 ± 0.12) in normal subjects and from 6.0 to 7.6 in patients with lung pathology. RS-NO levels increased with increasing pH (6.0–7.6) ($P < 0.01$), and concentrations in BALF with pH < 6.9 were lower than those in more alkaline fluid ($P < 0.005$). NO₂⁻ concentrations were independent of pH. Supporting these findings, RS-NO formed relatively rapidly on bubbling NO[•] gas (40–80 ppm) into physiologic solutions supplemented with 500 μM glutathione. Moreover, the levels of RS-NO and the stoichiometric ratio of RS-NO/NO_x over time were dependent on a variety of factors including NO[•] concentration, O₂ tension, thiol concentrations, pH, and the presence or absence of contaminant redox metals.

RS-NO Characterization. In normal subjects, RS-NO in BALF was found to be resistant to rapid precipitation by trichloroacetic acid (7%; 4°C), which precipitates protein RS-NO (29) but not amino acid or small peptide derivatives. Limited artifactual formation of RS-NO, incurred by the acidic conditions, was prevented by the addition of *N*-ethylmaleimide (>100 -fold excess over thiol for 20 min at 4°C) to alkylate free thiols and/or ammonium sulfamate, which removes excess nitrite (33). These findings were further

Table 1. Human airway NO concentrations vary with the cellular and chemical composition of BALF

	<i>r</i>	<i>P</i>
[NO ₂ ⁻] vs.		
Total leukocyte count	0.60	<0.005
Macrophage count	0.50	<0.05
Polymorphonuclear cell count	0.36	0.09
[RS-NO] vs.		
pH 6–7.6*	0.57	<0.01
[Thiol] [†]	0.50	<0.05
[NO ₂ ⁻]	0.0006	0.99

*pH of samples.

[†]Thiol concentrations in airway lining fluid were 450 ± 330 μM (mean $\pm$ SD).

FIG. 3. Stability of GS-NO in human BALF mimics that of endogenous RS-NO. Under identical conditions (pH 7.30; 21% O₂; 25°C), endogenous RS-NO in BALF from a patient with cystic fibrosis and pneumonia (Δ) has stability identical to that of exogenous GS-NO ($\bullet$). The decomposition of exogenous GS-NO added to normal BALF is accelerated, although not markedly, in the presence of 95% O₂ ($\circ$). Under similar conditions, free NO[•] is oxidized within seconds (41).

verified by acetone precipitation in two cases. Reverse-phase HPLC [Hypersil ODS (C18) 5 μm, Alltech Associates; mobil phase, 70% acetonitrile/1% trifluoroacetic acid/29% H₂O] established the presence of glutathione in the samples. Taken together, these observations are consistent with the marked predominance of glutathione over other forms of thiol in alveolar lining fluid (32) and strongly suggest that the thiol moiety (RS) in RS-NO is glutathione. In further support of this identity, the half-life of endogenous RS-NO in the lavage fluid is ≈ 3 hr and is essentially identical to that of exogenous GS-NO (Fig. 3); notably, the half-life of *S*-nitrosocysteine is < 3 min under these conditions (data not shown). The kinetics of decomposition of GS-NO in BALF are influenced by both O₂ tension and pH. In 95% O₂ the half-life of GS-NO is ≈ 60 min (Fig. 3). In keeping with the relationship between BALF pH and endogenous RS-NO concentrations (Table 1), the stability of GS-NO was determined to increase in proportion to pH over the range 6.0–7.4. The inherent stability of GS-NO contrasts strikingly with that of free NO[•], which has a half-life on the order of seconds under similar conditions (41). In the BALF of patients with disease, RS-NO were identified in protein form to varying degrees (range, 0–50%; mean, 21%). This finding is in accord with the change in protein constituency of airway lining fluid in different pathological conditions and the altered integrity of the alveolar capillary membrane; the predominant BALF protein under similar circumstances has been shown in one study to be albumin (42), and mucins (mucus proteins) are also unusually rich in cysteine residues.

Human Airway Bioassay. GS-NO induced dose-dependent relaxations of rings contracted with methacholine ($IC_{50} = 12 \pm 6.1$ μM) (Fig. 4). At physiological GS-NO concentrations (250–500 nM), tissues were 30% relaxed. By comparison with classical bronchodilators, GS-NO was 20 times more potent than theophylline ($IC_{50} = 243 \pm 21$ μM) and 150 times less potent than isoproterenol ($IC_{50} = 0.083 \pm 0.063$ μM). At physiological concentrations of NO₂⁻ (15 μM), airways were 10% relaxed ($IC_{50} = 278 \pm 57$ μM). The exemplary protein RS-NO, *S*-nitrosoalbumin, did not differ significantly from GS-NO in potency of airway relaxation (Table 2).

FIG. 4. GS-NO relaxes human airway smooth muscle. Comparison of the relaxation effect of GS-NO (●) to NO₂⁻ (■) and glutathione (○) in human bronchial rings contracted with methacholine (7 μM). Concentration-effect relationships reveal an order of potency: GS-NO > NO₂⁻ = glutathione (*, $P < 0.05$; †, $P < 0.0005$).

DISCUSSION

Our findings provide indirect evidence for local NO_x metabolism in human airways and further establish that: (i) RS-NO are present in the airways of normal subjects; (ii) these NO adducts are relatively resistant to inactivation at physiological O₂ concentrations as compared to NO[•] itself; (iii) RS-NO possess potent bronchodilator activity and are present in concentrations potentially sufficient to influence airway tone; and (iv) levels of RS-NO vary significantly in different pathological conditions and increase upon administration of NO[•] gas. Taken together, these observations suggest that RS-NO may play a role in modulation of airway luminal homeostasis.

The contributions of NO[•] to both physiological and pathological processes in the lung are poorly understood. The accumulating evidence for essential biological actions of NO[•] needs to be reconciled with an extensive literature implicating NO_x in lung inflammation, impairment of lung function, and induction of lung tumors (17). Accordingly, the observations of RS-NO (GS-NO) formation from both endogenous and exogenous sources of NO[•] may have important implications. It has been previously shown that the NO_x content of smoke correlates with its cytotoxicity (18), whereas glutathione protects against smoke-induced injury (43). Furthermore, uptake of exogenous NO_x in rat BALF is a glutathione-

dependent process (44), and glutathione inhibits leukocyte-mediated N-nitrosation reactions (45)—the latter of some concern for their carcinogenic potential (17, 25, 26). Glutathione has also been reported to scavenge OONO⁻, leading to formation of GS-NO (53). Thus, the formation of GS-NO may provide a means of limiting the toxicity of NO[•], attributable to its rapid oxidative metabolism, while simultaneously stabilizing NO[•] in bioactive form. Other findings of this study and previous observations (46, 47) suggest that the pH, thiol concentration, and general redox state of the airway will affect the rate of formation, identity, and stability of RS-NO. Hence, physiochemical parameters in the lung may influence the susceptibility to NO[•]-dependent injury and the formation of endogenous carcinogens.

Airway NO₂⁻ levels appear to represent an indicator of NO[•] production by macrophages. Rodent macrophages demonstrate high output, cytokine-induced NO_x generation that is important in host defense (48). However, controversy exists concerning the relevance of this observation to human pathophysiology. The detection of NO₂⁻ in the airways of all subjects in concentrations proportional to macrophage cell counts corroborates limited evidence that the human macrophage is a likely source of NO[•] synthesis (49). Lending further support to this possibility, we recently found that human alveolar macrophages immunostain for inducible NO[•] synthase in areas of chronic inflammation (2). The cytostatic activity of activated macrophages *in vitro* is associated with nitrosylation of iron-sulfur centers (27, 48). We were, however, unable to detect iron-nitrosyls in the airways of normal subjects or in inflammatory disease settings, indicating levels at least 1–2 orders of magnitude lower than RS-NO, and confirming a previous report (37).

RS-NO concentrations in airway secretions are predictably dependent on thiol levels, but their independence of airway lining fluid NO₂⁻ levels and cell counts suggests that their formation may be regulated for a specific biological purpose. In this regard, RS-NO have—in addition to the bronchodilator activity demonstrated in this study—been implicated in vasorelaxation (12), bacteriostasis (50), inhibition of ligand-gated receptor activity (51), and modulation of enzyme activity (28). Although the site and mechanism of RS-NO formation in the lungs are not known, the finding of increased airway RS-NO in inflamed (pneumonia) and transplanted lungs suggests that inflammation and/or immune activation may be important effectors of RS-NO generation. These conclusions, however, may be limited by the complex cellular effects of glucocorticoids, antimicrobials, and other immunosuppressant therapy in these patients. Furthermore, infants on ECMO have low airway RS-NO levels. Whether this reflects the pathophysiology of the newborn gas exchange unit or the effect of partially diverting blood flow from the pulmonary artery, it suggests that the pulmonary vasculature may influence airway RS-NO concentrations as well. That RS-NO form upon inhalation of NO[•] gas indicates that RS-NO can form in the extracellular airway environment, probably indirectly through reaction with O₂ (52), and provides a second mechanism (i.e., in addition to inactivation by alveolar capillary hemoglobin) for the relative pulmonary vasodilator selectivity of this therapeutic modality.

In summary, we have provided supporting evidence for local NO_x metabolism in human airways and identified a class of bronchodilator in the form of RS-NO. These observations may provide insight into the means by which NO[•] is packaged in biological systems to preserve its bioactivity and limit its potential O₂-dependent toxicity. As such, our findings may have implications for nontoxic therapeutic delivery of NO[•] in clinical settings. The discovery of an endogenous bronchodilator reservoir in human airways suggests that our current understanding of the regulation of airway luminal homeostasis in general, and airway smooth muscle tone in particular,

Table 2. Relaxation of human bronchial rings*

	n	IC ₅₀ , μM†
GS-NO	6	12 ± 6.1‡
S-Nitrosoalbumin§	6	39 ± 6.1
NO ₂ ⁻	4	278 ± 57
Glutathione	5	NA¶
Albumin	5	NA¶
Theophylline	7	243 ± 21
Isoproterenol	5	0.083 ± 0.063

*Airways were contracted with 7 μM (EC₈₀) of methacholine.

†Mean ± SEM.

‡Order of potency: isoproterenol > GS-NO ($P < 0.001$) ≥ S-nitrosoalbumin ($P =$ not significant) > theophylline ($P < 0.001$).

§S-Nitrosoalbumin is included as an exemplary protein RS-NO, albumin being the predominant thiol exudate in airways.

¶Not applicable; 50% relaxation was not achieved in all experiments at millimolar concentrations.

deserves reevaluation. These findings notwithstanding, the biological role of RS-NO in the airway and their relevance to the pathophysiology of human lung disease remains to be shown.

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