

# Acute Effects of Aerosolized S-Nitrosoglutathione in Cystic Fibrosis

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S-Nitrosoglutathione (GSNO), a naturally occurring constituent of airway lining fluid, enhances ciliary motility, relaxes airway smooth muscle, inhibits airway epithelial amiloride-sensitive sodium transport, and prevents pathogen replication. Remarkably, airway levels of GSNO are low in patients with cystic fibrosis (CF). We hypothesized that replacement of airway GSNO would improve gas exchange in CF. In a double-blind, placebo controlled study, we administered 0.05 ml/kg of 10 mM GSNO or phosphate buffered saline by aerosol to patients with CF and followed oxygen saturation, spirometry, respiratory rate, blood pressure, heart rate, and expired nitric oxide (NO). Nine patients received GSNO and 11 placebo. GSNO inhalation was associated with a modest but sustained increase in oxygen saturation at all time points. Expired NO increased in the low ppb range with GSNO treatment, peaking at 5 minutes but remaining above baseline at 30 minutes. There were no adverse effects. We conclude that GSNO is well tolerated in patients with CF and improves oxygenation through a mechanism that may be independent of free NO. Further, GSNO breakdown increases expired NO. We suggest that therapy aimed at restoring endogenous GSNO levels in the CF airway may merit study.

**Keywords:** S-nitrosoglutathione; cystic fibrosis; nitric oxide; glutathione

S-Nitrosoglutathione (GSNO) is an endogenous nitrogen oxide present in high concentrations in the extracellular fluids of the lung and brain (1, 2). It has a bioactivity profile that is well suited for maintaining airway homeostasis and, in many cases, distinct from that of its breakdown product, nitric oxide (NO) (3–5). In particular, GSNO in concentrations present in the airways relaxes airway smooth muscle (1), improves airway ciliary motility (6), inhibits airway epithelial amiloride-sensitive sodium transport while activating calcium-dependent epithelial chloride transport (7, 8), promotes neutrophilic apoptosis (9), and has antimicrobial effects (10–12). Additionally, it has recently been observed to increase cellular expression and maturation of the common mutant form of the cystic fibrosis transmembrane regulation protein (CFTR),  $\Delta F508$ , in physiologically relevant concentrations (13). There is increasing evidence that GSNO metabolism is regulated independently of NO and other S-nitrosothiols (SNOs) (5, 14, 15).

Levels of GSNO tend to be low in the cystic fibrosis (CF) airway (16). Depletion of GSNO may reflect the decreased levels of glutathione (17, 18), decreased airway epithelial NO

synthase (NOS) expression (19, 20) and/or accelerated catabolism of GSNO (5, 14) that may be features of the disease. GSNO depletion could contribute to the pathophysiology of CF lung disease by impairing mucus clearance, aggravating airflow obstruction, and predisposing to chronic airway neutrophilic inflammation. Here, we report that aerosolized replacement therapy with GSNO is well tolerated and results in a modest improvement in gas exchange in patients with CF.

## METHODS

### Subjects

Subjects 10–50 years old were recruited from CF clinic if they had a sweat chloride concentration greater than 60 mEq/L and chronic bronchiectasis with moderate obstructive disease. Subjects were excluded who had a room air oxygen saturation of less than 90%, who were hypotensive, or who had a history of gastrointestinal bleeding, massive hemoptysis, or any hemoptysis within the preceding month. The protocol was reviewed and approved by the institutional Human Investigation Committee.

### Experimental Protocol

After providing informed consent, subjects were sequentially assigned in a blinded fashion by the research pharmacist to receive either GSNO or placebo using a prerandomized code. GSNO (10 mM) was prepared by nitrosation of glutathione, assayed for purity as previously described (1, 21, 22) and brought to pH 7.0 in 10 mM phosphate buffered saline (PBS). Control subjects received 10 mM PBS alone. Both GSNO and PBS were filtered for sterilization (0.22  $\mu\text{m}$ ), cultured to confirm sterility in accordance with Food and Drug Administration guidelines (BioWhittaker, Walkersville, MD), and stored at  $-80^\circ\text{C}$  in foil-covered aliquots. After measurement of baseline oxygen saturation, heart rate, blood pressure, respiratory rate, spirometry, and expired nitric oxide concentration ( $\text{FE}_{\text{NO}}$ ), subjects received 0.05 ml/kg of GSNO or PBS, up to a maximum 3.0 ml, by nebulizer (Devilbis, Somerset, PA). This dose was calculated to increase the airway epithelial lining fluid concentration from undetectable (16) to a physiologically relevant and pharmacologically active concentration (1) (see online data supplement). Sputum was collected for 30 minutes before and 30 minutes after treatment. Vital signs, oxygen saturation and  $\text{FE}_{\text{NO}}$  were measured at 5, 10, 20, and 30 minutes after completion of the treatment. Oxygen saturation values accepted were those that were stable for 10 seconds. Spirometry was measured at 10 and 30 minutes after treatment (Sensorimedics, Yorba Linda, CA).

### Chemical Analysis

GSNO was analyzed for contaminants as previously described using mass spectrometry (22) and spectrophotometric analysis for ammonium ( $\text{NH}_4^+$ ) (23).

### Expired NO Measurement

Nitric oxide concentrations were measured by chemiluminescence (Sievers Instruments, Boulder, CO). Single breath vital capacity off-line collections were made using an 8.1-L Tedlar gas sampling bag (Fisher Scientific, Pittsburgh, PA) as previously described (24, 25). As noted in the American Thoracic Society guidelines for  $\text{FE}_{\text{NO}}$  measurement, this procedure allows for measurement at clinic sites remote from the chemiluminescence analyzer (26) and is highly reproducible (24).

(Received in original form May 11, 2001; accepted in final form December 10, 2001)

Supported by NIH (HL59337) (B.M.G.) and the University of Virginia Children's Medical Center.

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This article has an online data supplement, which is accessible from this issue's table of contents online at [www.atsjournals.org](http://www.atsjournals.org)

Am J Respir Crit Care Med Vol 165. pp 922–926, 2002

DOI: 10.1164/rccm.2105032

Internet address: [www.atsjournals.org](http://www.atsjournals.org)

TABLE 1. SUBJECT CHARACTERISTICS AT STUDY ENTRY

	GSNO Group	Placebo Group	p
Age, yr	16.1 ± 1.44	19.9 ± 3.45	0.34
Respiratory rate, minute <sup>-1</sup>	28.0 ± 5.22	24.4 ± 3.89	0.58
Heart rate, minute <sup>-1</sup>	99.0 ± 6.55	88.9 ± 6.18	0.28
Systolic blood pressure, mm Hg	105.8 ± 3.79	110.5 ± 3.06	0.34
Diastolic blood pressure, mm Hg	66.1 ± 3.68	70.4 ± 2.63	0.35
Oxygen saturation, %	94.9 ± 0.82	96.0 ± 0.73	0.33
Expired NO, ppb	6.74 ± 0.94	5.04 ± 0.57	0.12
FVC, L	2.59 ± 0.27	2.97 ± 0.39	0.45
FVC, %	71.5 ± 6.15	80.8 ± 6.98	0.35
FEV <sub>1</sub> , L	2.01 ± 0.26	1.92 ± 0.31	0.83
FEV <sub>1</sub> , %	60.5 ± 7.7	59.3 ± 8.02	0.916
FEF <sub>25-75</sub> , L/second	1.77 ± 0.46	1.35 ± 0.30	0.45

### Statistical Analysis

Means at multiple time points were compared by analysis of variance (ANOVA) and, where appropriate, by *t* test or—for nonparametrically distributed sets—by Rank Sum Testing. A value of *p* < 0.05 was considered significant. Data are presented as mean ± SEM.

### RESULTS

Nine patients were randomized to receive GSNO and 11 to receive PBS. They did not differ with respect to age, baseline vital capacity or forced expiratory volume at one second (FEV<sub>1</sub>) (as either absolute value or percent predicted), FEV<sub>1</sub>/forced vital capacity (FVC) ratio, vital signs, or oxygen saturation (Table 1).

The GSNO used in these experiments contained no NH<sub>4</sub><sup>+</sup>, peroxyxynitrite, nitrate, or oxidized glutathione (GSSG), that is, had not decomposed (Figure 1); solutions contained less than 10% of the reactant, GSH, which, given as an aerosol, would be a predicted increase in airway GSH concentration by 10 μM, or < 1% of that normally present in the airway (1).

Oxygen saturation increased at all time points in the GSNO-treated group. The change in saturation from baseline ranged from 0.89 ± 0.51 to 1.44 ± 0.58% for GSNO, greater at each time point than the change in saturation for the PBS group (range, -0.73 ± 0.3 to -0.273 ± 0.43); *p* < 0.01 by ANOVA (Figure 2). The difference between the GSNO-treated group and the PBS group remained robust at 30 minutes (change in saturation from baseline = 1.33 ± 0.53% for GSNO, versus -0.36 ± 0.36% for the PBS group; *p* < 0.05).

Oxygen saturation values did not change significantly from baseline following PBS inhalation alone (*p* = NS, ANOVA).

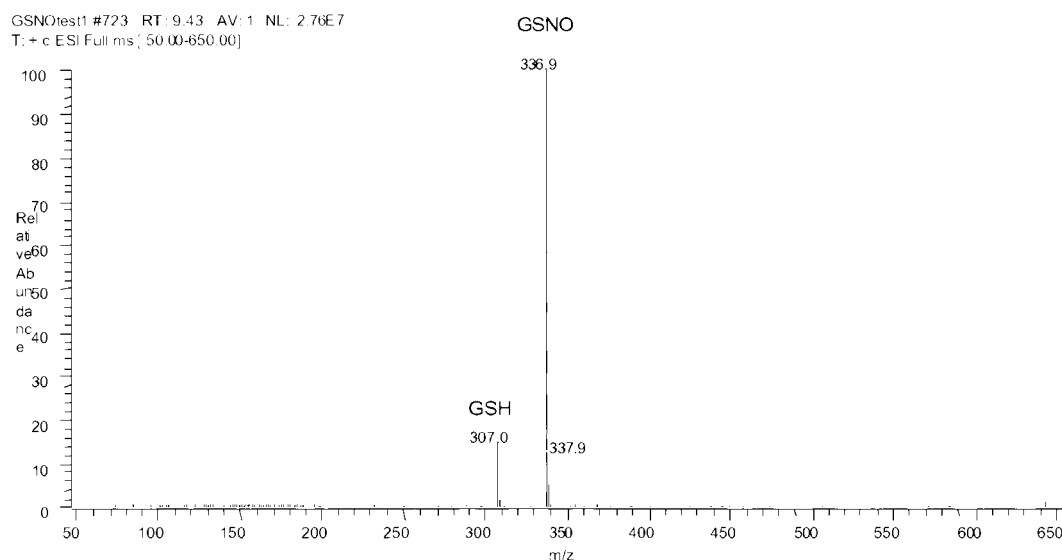
GSNO was well tolerated in all subjects. Spirometry did not change at any time point (Figure 3). Sputum production (*p* = NS) and vital signs also did not change (Figure 3).

Consistent with previous observations (25), baseline expired NO concentrations were somewhat low in CF compared with published normal data (24, 25). Inhaled GSNO treatment dramatically increased FE<sub>NO</sub> at five minutes (change in [NO] from baseline = 7.5 ± 3.6 ppb, versus -0.27 ± 0.48 ppb for PBS; *p* < 0.001), demonstrating directly that GSNO breakdown in the airway increases FE<sub>NO</sub>. Expired NO levels fell over the first 20 minutes, but were still higher than those in the PBS group at 30 minutes (change in [NO] from baseline = 1.69 ± 0.68 ppb versus 0 ± 0.37 ppb for PBS; *p* < 0.05) (Figure 4). The change in [NO] was unrelated to the change in oxygen saturation.

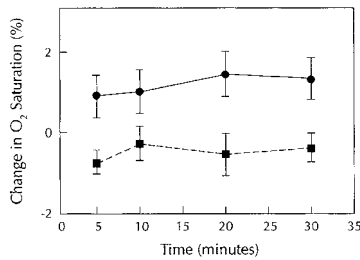
### DISCUSSION

The function-regulating activities of SNOs include a diversity of effects that oppose the major pathophysiologic features of airway disease in CF. Remarkably, levels of SNOs in general, and GSNO in particular, are relatively low in the bronchoalveolar lavage fluid of patients with CF (16), possibly as a result of decreased airway epithelial NOS expression (19, 20) and/or decreased glutathione levels (17, 18). It has also been shown that airway inflammation is associated with increased GSNO catabolism (14, 27), and thus accelerated breakdown may contribute to low SNO levels.

Both NO and GSH have actions that should promote lung health in patients with CF. However, long-term therapeutic administration of NO is impractical, and short-term use does not appear to be of benefit (28). This may be because of adverse effects of ppm NO concentrations—particularly in the oxidative environment of the CF airway—counterbalancing its potential salutary effects (29). Furthermore, although GSH inhalation may have beneficial biochemical effects in the CF airway, in part because of disorders of redox regulation and antioxidant genes may contribute to the pathophysiology of airway injury (30, 31), physiological benefit has not been demonstrated (32). Indeed, GSH given by nebulizer has been reported to cause bronchoconstriction (33), and long-term use of the cell permeable glutathione analog, N-acetylcysteine, by

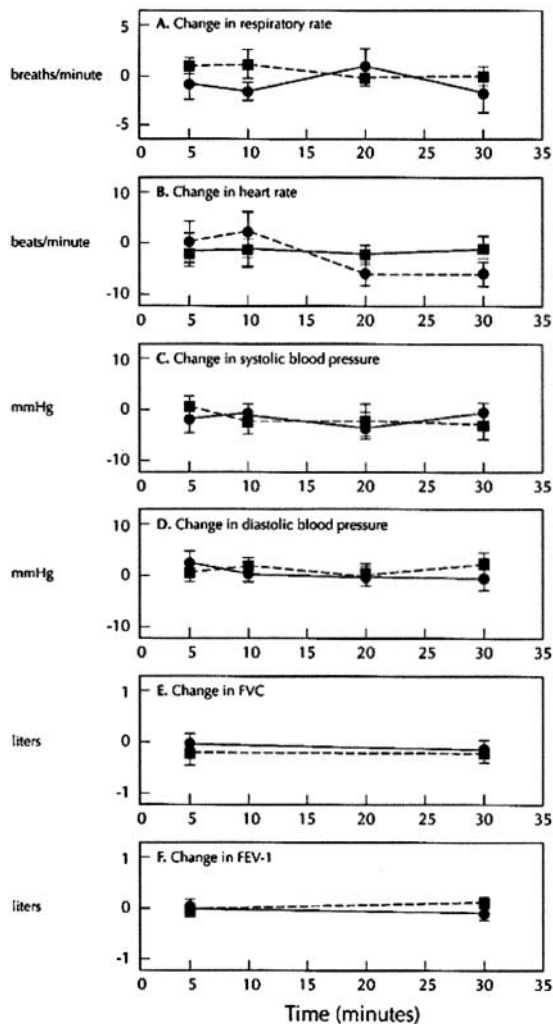


**Figure 1.** GSNO was assayed for purity using liquid chromatography–mass spectrometry. No impurities were present in the mass chromatogram except for glutathione (GSH). The spectrum of the major peak (9.42 minutes) is shown. Note that the instrument is not sensitive below a *m/z* of 50, but separate spectrophotometric analysis revealed no NH<sub>4</sub><sup>+</sup>.

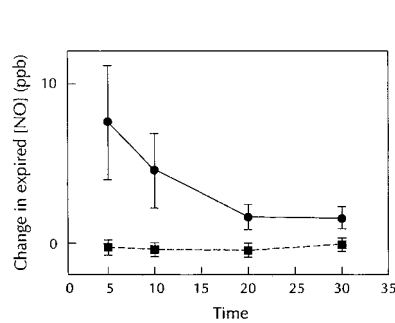


**Figure 2.** GSNO inhalation results in improved oxygenation in CF. Oxygen saturation improved at all time points in the GSNO-treated group (solid line;  $n = 9$ ) compared with the placebo-treated group (dashed line;  $n = 11$ ;  $p < 0.01$  by ANOVA). Data are presented as mean  $\pm$  SE.

nebulization has been disappointing (34). By contrast, GSNO is an attractive therapeutic candidate: (1) it is endogenously formed in high concentrations; (2) it is potentially more potent, less toxic, and longer lived than NO, and thus more practical to deliver; (3) it seems to produce only physiological (nM) concentrations of NO (Figure 4), potentially preventing toxicity; and (4) it is deficient in CF patients. Here, we demonstrate that aerosolized administration of GSNO to patients with CF is well tolerated and results in a modest improvement in oxy-



**Figure 3.** GSNO is well tolerated in CF. GSNO inhalation (circles;  $n = 9$ ) did not cause a significant change in (A) respiratory rate; (B) heart rate; (C) systolic blood pressure; (D) diastolic blood pressure; (E) forced vital capacity; or (F) forced expiratory volume at 1 second—at any time point after inhalation when compared with control (PBS inhalation) (squares;  $n = 11$ ;  $p = \text{NS}$  by ANOVA). Data are presented as mean  $\pm$  SE.



**Figure 4.** GSNO breakdown increases expired NO. Changes in expired NO in the subjects receiving GSNO (circles;  $n = 9$ ) were higher than those in the placebo group (squares;  $n = 11$ ) at each time point ( $p < 0.001$  by ANOVA). The  $\text{FE}_{\text{NO}}$  of GSNO-treated patients remained substantially increased relative to that in the PBS group for 30 minutes ( $p < 0.05$ ). Data are presented as mean  $\pm$  SE.

genation. These data suggest that GSNO may improve gas exchange by a mechanism independent of NO and GSH effects.

GSNO was originally described in the airways as an endogenous bronchodilator, two log orders more potent than theophylline and present in the normal airway in concentrations sufficient to relax human airway smooth muscle (1). Subsequently, SNOs have been shown to be well tolerated and cause bronchodilation in animal models (35), to increase airway ciliary beat frequency (6), and to have the potential to augment airway hydration by inhibiting amiloride sensitive sodium transport (7) and increasing chloride transport (8). GSNO also has antimicrobial effects, perhaps the most relevant of which is inhibition of viral replication through *S*-nitrosylation of cysteine proteases (10, 11).

Theoretical risks of administering GSNO include hypotension and bleeding. However, it should be noted that GSNO does not cause significant hypotension in rats or humans when given parenterally (36–38), and aerosolized GSNO had no systemic hemodynamic effects. None of our patients experienced bleeding or developed petechiae, although we were careful to exclude patients with a history of airway or gastrointestinal bleeding. It is also possible that GSNO metabolites such as NO and  $\text{NH}_4^+$  could have adverse effects on the CF airway (29, 39–41), though measured NO and predicted  $\text{NH}_4^+$  (low  $\mu\text{M}$ ) concentrations were log orders below those shown to be physiologically or pathologically relevant (39, 41, 42).

It had been widely held that expired NO is a marker of airway NOS activity. However, recent studies in patients with asthma have indicated that expired NO is at least partly derived from metabolism of airway lining constituents (14, 27, 43). These studies made several puzzling observations that may be reconciled by the proposal that GSNO is metabolized to NO within the airway (3, 14, 27). The increase in expired NO (hypernitrosopnea [24]) we have observed following GSNO administration demonstrates directly that airway GSNO breakdown can influence NO concentrations in expired breath.

The half-life of GSNO in solution will vary with redox state, nature of the redox cofactors, and the concentration of nucleophiles, but it is generally on the order of hours (14, 29, 32, 40, 44). Several enzymes have been reported to breakdown GSNO, as recently reviewed (5, 14, 15). Inhibition of such metabolic activity might represent a new therapeutic approach in CF. Of note, NO itself is generally regarded as a minor product of organic and inorganic GSNO catabolism (5, 14, 15, 29, 40).

GSNO did not break down before administration. There were no major catabolic products,  $\text{NH}_4^+$  and GSSG (15, 40), or minor products such as nitrate or peroxyxynitrite, in the preparation. Small ( $\mu\text{M}$ ) quantities of reactant (GSH) could be detected, but these would raise endogenous ELF concentrations of GSH less than 1% (1).

Improvements in oxygenation were unrelated to changes in expired NO gas concentrations. Further, the maximum FE<sub>NO</sub> did not exceed that seen in asthma (on the order of 15–20 ppb) (24, 25), a condition commonly associated with ventilation/perfusion mismatch and hypoxemia. NO concentrations a 1,000-fold higher, which may have local and systemic toxicity, are required to achieve a meaningful degree of pulmonary vascular smooth muscle relaxation in the therapeutic setting (3, 42, 45). Of note, even these higher concentrations of NO gas delivered to the CF airway do not improve oxygenation (28). Taken together, these observations suggest that GSNO does not improve oxygenation simply through release of NO. Support for this contention can be found in (1) the differing propensities of NO and GSNO to S-nitrosylate ion channels such as those that regulate airway smooth muscle tone and the viscosity of secretions (44, 46, 47); (2) the immunohistochemical (48) and chemical (1, 45) data establishing that S-nitrosylation reactions occur in airways; and (3) a recent study showing that inhaled ethyl nitrite, an S-nitrosylating agent that releases little NO, causes more potent and sustained improvement in oxygenation following lung injury than does NO (45). Of note, SNOs may increase both peripheral oxygen delivery (49) and the ventilatory response to hypoxia (22) through erythrocyte-mediated mechanisms—in addition to improving oxygen uptake in the lungs—as recently reviewed (50). These observations may form the theoretical basis for understanding improvements in oxygenation associated with GSNO inhalation.

In summary, we report that replacement of endogenous GSNO in patients with CF is well tolerated acutely and results in a modest improvement in oxygenation. Additionally, we demonstrate that GSNO breakdown directly affects concentrations of NO measured in expired air. Our data suggest that more in-depth studies of the pulmonary and nonpulmonary effects of GSNO or related compounds could possibly lead to therapies of benefit to patients with CF.

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