S-nitrosoglutathione breakdown prevents airway smooth muscle relaxation in the guinea pig

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Received 24 February 2000; accepted in final form 24 April 2000

Fang, Kezhong, Roger Johns, Timothy Macdonald, Michael Kinter, and Benjamin Gaston. S-nitrosoglutathione breakdown prevents airway smooth muscle relaxation in the guinea pig. Am J Physiol Lung Cell Mol Physiol 23: L716–L721, 2000.—Airway levels of the endogenous bronchodilator S-nitrosoglutathione (GSNO) are low in children with near-fatal asthma. We hypothesized that GSNO could be broken down in the lung and that this catabolism could inhibit airway smooth muscle relaxation. In our experiments, GSNO was broken down by guinea pig lung homogenates, particularly after ovalbumin sensitization (OS). Two lung protein fractions had catabolic activity. One was NADPH dependent and was more active after OS. The other was NADPH independent and was partially inhibited by aurothioglucose. Guinea pig lung tissue protein fractions with GSNO catabolic activity inhibited GSNO-mediated guinea pig tracheal ring relaxation. The relaxant effect of GSNO was partially restored by aurothioglucose. These observations suggest that catabolism of GSNO in the guinea pig 1) is mediated by lung proteins, 2) is partially upregulated after OS, and 3) may contribute to increased airway smooth muscle tone. We speculate that enzymatic breakdown of GSNO in the lung could contribute to asthma pathophysiology by inhibiting the beneficial effects of GSNO, including its effect on airway smooth muscle tone.

S-nitrosothiol; asthma; nitric oxide; gold

S-NITROSO Thiols (SNOs) both store and execute nitrogen oxide bioactivities in physiological systems. For example, reactions of S-nitrosohemoglobin deliver both nitric oxide (NO) and low-mass SNOs to dilate precapillary systemic arterioles (20, 29, 49), and S-nitroso-L-cysteine acts independently of homolytic cleavage to NO as a stereoselective neurotransmitter (42). In the airway, S-nitrosoglutathione (GSNO) is an endogenous bronchodilator two log orders more potent than the-nitrosoglutathione breakdown prevents airway smooth muscle relaxation in the guinea pig

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trypsin inhibitor, antipain, leupeptin, and pepstatin, pH 7.4, 4°C). The lungs were homogenized (Polytron) in PIB. In selected experiments, tracheal tissue was cut into 2-mm-thick coronal rings for bioassay as previously reported (27, 35); care was taken to leave the lungs intact. Lungs from additional animals were inflated through a catheter in the main stem bronchi with 10% neutral Formalin, cut into 5-μm sections, and stained with hematoxylin and eosin to confirm the presence or absence of inflammation.

**Protein purification.** Homogenized fractions underwent centrifugation (20,000 g for 15 min at 4°C) and filtration (pore size 20 μm). Initial separation of the supernatant proteins in the PIB was performed across a 1 × 30-cm diethylaminoethyl cellulose (Whatman) column eluted with a 0–250 mM linear gradient of NaCl (pH 7.4). Fractions were screened for biochemical activities (see Biochemical methods) and separated further based on the results of this screening. Certain active fractions were further purified in PIB by NADPH activity binding on a reactive red column after 12 h (25, 31, 54). The reactive red 120 type 3000 agarose (Sigma) column (1 × 30 cm) equilibrated in PIB, and elution was performed with a linear gradient of 0–250 mM NaCl in PIB at the flow rate of 2 ml/min.

**Biochemical methods.** GSNO was assayed by introducing samples into a reaction chamber (NOA 280, Sievers Instruments, Boulder, CO) containing 1 mM cysteine and 100 μM CuCl in a continuous helium stream (pH 7.0, 50°C) as previously described (12, 14). This assay is highly specific and linear to 5 mM (12). Additionally, GSNO was assayed (pH 7.4, 25°C) by spectrophotometry (335 nm; absorption coefficient 586 M⁻¹ cm⁻¹) (13, 16). Xanthine oxidase-mediated GSNO catabolic activity was measured as GSNO decomposition in PBS (25°C) in 21% oxygen in the presence of 150 μM hypoxanthine (52). Glutathione peroxidase (Gpx) was measured in 50 mM K₂HPO₄, pH 7.4, 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 U/ml of glutathione reductase, 1 mM glutathione, and 0.25 mM H₂O₂ (total volume of 1 ml); all ingredients except the protein fraction and H₂O₂ were combined at the beginning of each day. Protein (100 μl) was added to 800 μl of the above mixture and allowed to incubate for 5 min at 25°C before initiation of the reaction by the addition of 100 μl of H₂O₂ solution. Absorbance at 340 nm was recorded for 5 min, and the activity was calculated from the slope of these lines as micromoles of NADPH oxidation per minute (25, 31, 54). Thioredoxin reductase was assayed by the addition of 10 μg of active fraction protein to 500 μl of assay mixture containing 100 mM potassium phosphate, 10 mM EDTA, 0.2 mM NADPH, 0.2 mg/ml of BSA, 1% ethanol, and 5 mM dithio-bis-nitrobenzoic acid, pH 7.0 (25°C). Absorbance at 412 nm was recorded between the first and second minutes of the reaction, and activity was calculated as micromoles of 2-nitro-5-thio-benzoate generated per minute (41). Glutathione was measured with dithio-bis-nitrobenzoic acid (16) in the presence [oxidized (GSSG)] or absence [reduced (GSH)] of glutathione reductase (1 U/ml) and β-NADPH (10 μM); GSSG was taken as the difference between total glutathione concentration and GSH concentrations (11). Inhibition of protein fractions with GSNO breakdown activity was studied by incubation with 100 μM aurothioglucose (ATG) (25), 100 μM deferoxamine (53), or 30 μM bathocuproine disulfoxide (18) in PBS (25°C) with active fractions before the reaction with GSNO. Protein was assayed by the method of Lowry (16). Kinetic studies were performed by measuring d[GSNO]/dt in the linear range (10 min) at uniform protein concentration (and NADPH where applicable) and varying GSNO concentrations (21°C in PBS). Reagents were purchased from Sigma.

**Bioassay.** Tracheal rings were preserved in Krebs-Henseleit solution (KHS; in mM: 118 NaCl, 55.4 KCl, 1.10 NaH₂PO₄, 11.1 glucose, 25.0 NaHCO₃, 1.38 MgSO₄, and 2.32 CaCl₂, pH 7.40, at 4°C) in 95% O₂ balanced with CO₂ and suspended on force transducers connected to a calibrated chart recorder (Gould Instruments, Latrobe, PA) with 1 g of tension, a previously determined optimum (27). After equilibration and conditioning, rings were rinsed twice, observed to return to baseline tension, and then contracted with 5 μM methacholine (EC₅₀). Rings were then exposed to serially increasing concentrations of GSNO that had been preincubated with active and inactive protein fractions with and without ATG or with KHS alone for 30 min (37°C). Percent relaxation was taken as percent of the initial response to methacholine.

**Statistical analysis.** Multiple means were compared with the use of ANOVA. Nonparametrically distributed means were compared with the Mann-Whitney rank sum test. Data are presented as means ± SE. P < 0.01 was considered significant.

**RESULTS**

**Guinea pig airway soluble proteins accelerate GSNO breakdown.** Intact lung tissue slices accelerated the rate of GSNO breakdown sevenfold compared with PBS (0.64 ± 0.04 vs. 0.09 ± 0.01 μM/min; P < 0.001; n = 4 tests each). OS significantly accelerated breakdown to 0.88 ± 0.05 μM/min (P < 0.01; compared with unsensitized tissue; n = 5 samples each) with matched lung homogenate protein concentrations from each set of animals (unsensitized, 6.7 ± 0.37 mg protein vs. sensitized, 6.5 ± 0.34 mg protein). Because there are proposed GSNO catabolic proteins that require (25, 28, 32, 38) and do not require (18, 24, 30, 41, 52, 53) NADPH, fractions were screened in the presence and absence of this cofactor. Two anion exchange protein fractions from whole lung homogenate protein concentrations from each set of animals were compared with the Mann-Whitney rank sum test. Data are presented as means ± SE. P < 0.01 was considered significant.
Preliminary characterization of the soluble GSNO catabolic activity of guinea pig lungs. The NADPH-dependent fraction was further purified by reactive red chromatography. This fraction did not show activity as thioredoxin or GPx. Its apparent Michaelis-Menten coefficient ($K_m$) was $500 \text{ nM (100} \mu\text{M NADPH)}$. A later NADPH-independent fractionated protein, likely a separate enzyme, eluted in the 40 mM fraction during phosphocellulose chromatography. Its $K_m$ was $1.3 \mu\text{M (Fig. 2)}$. Of note, the NADPH-independent fraction produced GSSG stoichiometrically ($59 \pm 4.5 \mu\text{M GSSG from 100} \mu\text{M GSNO; 45 min at 21°C; } n = 3 \text{ assays})$. It was partially reversed (57.4%) after treatment with ATG but was not inhibited by deferoxamine or bathocuproine disulfonate (Table 1).

Inhibition of GSNO-mediated airway smooth muscle relaxation by airway catabolic proteins. Consistent with previous reports (27, 35), GSNO caused dose-dependent relaxation of guinea pig tracheal smooth muscle. The relaxation was inhibited by preincubation of GSNO with the catabolically active fraction from the guinea pig lung protein homogenate (independent fraction) but not with inactive fractions (25 $\mu$g protein/ml each, 30 min at $37^\circ\text{C}$) in KHS. Relaxation after expo-
sure to 50 μM GSNO (IC_{50} was not achieved after incubating with the active fraction) was 56 ± 4.4, 47 ± 2.3, and 24 ± 4.1%, respectively, for GSNO alone (n = 7 rings), GSNO with inactive fraction (n = 5), and GSNO with active fraction (n = 5 rings; P = 0.0002 by ANOVA; Fig. 3). As expected (27), we also demonstrated a brisk and robust relaxation (73–100%) to isoproterenol (1 μM) in the presence of the active fraction (n = 7 rings). Incubation of GSNO with the active fraction and with excess (100 μM) gold under the same conditions partially stabilized GSNO bioactivity (38 ± 4.2% at 50 μM GSNO; P < 0.05 compared with GSNO and the active fraction alone; n = 7 rings), consistent with partial ATG-mediated attenuation of the biochemical activity of the same fraction (Fig. 3). Of note, relaxation of airway rings from ovalbumin-sensitized animals by 10 μM GSNO was inhibited relative to that for control rings (13.7 ± 1.6 vs. 22.6 ± 3.7% relaxation in ovalbumin-sensitized and control animals; P < 0.05; n = 7 and 5 rings, respectively), consistent with previous observations (51) made in guinea pig whole lung preparations. Blinded analysis of airway sections confirmed the presence of mucosal thickening and eosinophilic and lymphoecytic airway inflammation in the airways of ovalbumin-sensitized but not of control guinea pigs (n = 9 sections from 3 animals).

**DISCUSSION**

SNOs are endogenous relaxants of airway smooth muscle (3, 16, 27, 35, 51). Severe asthma is associated with depletion of GSNO and other SNOs in the airway, suggesting that a SNO deficiency may contribute to airway narrowing in the clinical setting (17). Of note, OS and rechallenge dramatically inhibit the bronchodilator effect of GSNO (51). Here, we have shown in the guinea pig that 1) lung tissue degrades GSNO, 2) this degradation prevents airway smooth muscle relaxation, 3) one of the two soluble catabolic proteins is NADPH dependent, whereas the other is partially inhibited in the presence of ATG, and 4) lung tissue GSNO catabolism is increased after OS. These findings suggest that GSNO breakdown may be relevant to pulmonary pathophysiology.

SNO bioactivities may be distinct from those of NO because they often involve electrophilic reactivity toward organic sulfur, nitrogen, carbon, and transition metal moieties as opposed to free radical reactions (7, 29, 48, 49). In other words, SNOs may act independently of, and in fact be inhibited by (7), degradation to NO. Nitrosylation-mediated bioactivities that may be relevant in the lung include smooth muscle relaxation (3, 15, 16, 27, 34, 35), immune functions (9, 37, 43, 45), neurotransmission (10, 36, 42, 50), modulation of ion channel conductivity and airway hydration (6, 15), inhibition of oxidant stress enzymes (1, 5), and antimicrobial effects (22, 44).

SNOs are formed in the lung by organic and inorganic reactions. NOS, present in the normal airway epithelium, may produce GSNO under conditions like those in the airway (8, 16) in which reduced glutathione concentrations are high (47). Reactions between oxygen, superoxide, and NO to form nitrosating species may proceed at physiologically relevant concentrations (1, 5, 8, 19, 20, 22, 26, 31, 44, 50, 55) and are favored both in the relatively oxidative environment of the airway and in biological membranes. Formation of iron-nitrosyl intermediates has also been proposed to contribute to endogenous SNO formation (53), although these species do not appear to be present in the airway (16). The existence of additional cellular mechanisms for SNO synthesis has been proposed, but these processes are not fully understood (9, 18).

In addition to being directly bioactive, GSNO and other SNOs may act as reservoirs for nitrogen oxides in the airway (16, 17), brain (33), plasma (46), erythrocytes (29, 49), and neutrophils (9). Thus SNO catabolism in these storage pools may serve not only to attenuate nitrosylation-mediated activities but also to 1) upregulate the effects of NO, hydroxylamine, peroxynitrite, and other SNO breakdown products (25, 28, 41, 52) and 2) facilitate transmembrane NO_s signaling. In this sense, SNOs may be thought of as

**Table 1. Effect of metal chelators and aurothioglucose on GSNO catabolic protein activity**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>n</th>
<th>Inhibitory Effect, %</th>
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<tbody>
<tr>
<td>Bathocuproine disulfonate (30 μM)</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>Deferoxamin (100 μM)</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td>Aurothioglucose (100 μM)</td>
<td>7</td>
<td>57.4 ± 6.6†</td>
</tr>
<tr>
<td>Trypsin (0.25%; 60 min)</td>
<td>5</td>
<td>85 ± 9.4</td>
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Values are means ± SE; n, no. of samples. GSNO, S-nitroso glutathione. †Value for 40 mM NaCl NADPH-independent phosphocellulose fraction after DEAE chromatography. P < 0.001 compared with the activity in PBS.

![Fig. 3. Active catabolic fractions prevent GSNO-mediated guinea pig airway smooth muscle relaxation. Tracheal rings were harvested from control guinea pigs, transduced under tension, and contracted with methacholine. Rings were then exposed to GSNO after incubation for 0.5 h in Krebs-Henseleit solution (KHS; n = 7 rings; ●); KHS in the presence of pooled active protein fractions (n = 5 rings; ▲); or inactive protein fractions (n = 5 rings, ◆); or KHS in the presence of these active fractions and 100 μM aurothioglucose (n = 7 rings; ⨁) at 37°C. Active fractions inhibited GSNO-mediated catabolism more than inactive fractions (P < 0.0002), and this inhibition was partially reversed by aurothioglucose (P < 0.05).](http://ajplung.physiology.org/ by 10.22032/ajl.17.247)
capacitors for signals originating from NOS activation. In the airway, as in the brain, GSNO is the principal SNO (16, 33), probably because its formation is favored in transnitrosation equilibria under physiological conditions (39, 46).

Catabolic processes have been previously described for GSNO. These include inorganic copper- and iron-mediated reactions in vitro (2, 53) that may have limited relevance in vivo where free concentrations of these ions are generally extremely low. Our active soluble protein fractions were >10 kDa in size and were inhibited by trypsin, which suggested that they were lung proteins. Enzymatic processes have been proposed to exist in neutrophils, platelets, and bacteria (9, 18, 22), and catabolic activity has recently been demonstrated for specific enzymes. Neither of the activities we identified were likely to have been 1) γ-glutamyl transpeptidase, which is membrane bound (22); 2) Cu/Zn superoxide dismutase, which should be inhibited by bathocuproine disulfonate (24); or 3) xanthine oxidase, which should be augmented by hypoxanthine (52). The gold-inhibitable fraction may be a seleno protein (25), but it does not have GPx activity. The NADPH-dependent fraction could be a guinea pig-specific isoform of NADPH-dependent alcohol dehydrogenase (24). Nitrogen oxides other than NO may be produced in vitro (data not shown), which is consistent with this possibility. Work is ongoing to purify those proteins in quantities sufficient for sequencing and further characterization.

Endogenous SNOs are present in physiologically relevant concentrations. GSNO concentrations as high as 7 μM have been reported in normal brain tissue (22), and airway levels may be on this order in certain settings (16). Of note, the potency of GSNO in relaxing airway smooth muscle may be lower than that previously reported (27), probably reflecting some degree of inorganic breakdown in physiological (control) buffer incubated at neutral pH for 30 min (16). The mechanism by which SNOs relax airway smooth muscle is unclear and does not exclusively involve liberation of NO with subsequent activation of guanylate cyclase (3, 14). It is therefore possible that, in vivo, GSNO catabolism could activate or inactivate its smooth muscle relaxant effect depending on the product and location. Our data suggest the possibility that inactivation may be more physiologically relevant.

In summary, our findings suggest the presence of novel metabolic pathways with the potential to affect airway smooth muscle tone. Specifically, breakdown of the endogenous bronchodilator GSNO by lung proteins prevents guinea pig airway smooth muscle relaxation in vitro. We have identified two soluble protein fractions involved in this process, although other proteins, particularly membrane-associated γ-glutamyl transpeptidase, may also be relevant. SNO metabolism may be involved in several additional aspects of pulmonary biology including immune, antimicrobial, airway clearance, and neuronal functions in vivo. Recent evidence that humans with severe asthma have low airway SNO or GSNO levels suggests that SNO catabolism may be accelerated in certain patients with asthma. This accelerated catabolism may in turn contribute to bronchoconstriction and/or to other elements of asthma pathophysiology such as impaired inflammatory cell apoptosis. Taken together, these findings suggest that further efforts to define, localize, and characterize the effects of GSNO metabolic pathways in the lung may provide a novel direction for pulmonary research, with the potential for leading to new asthma therapies.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-58337.

REFERENCES