Pulmonary vasoconstriction by serotonin is inhibited by S-nitrosoglutathione

EVA NOZIK-GRAYCK, TIMOTHY J. MCMAHON, YUH-CHIN T. HUANG, CHRISTINE S. DIETERLE, JONATHAN S. STAMLER, AND CLAUDE A. PIANTADOSI
Departments of Pediatrics and Medicine, Duke University Medical Center, Durham, North Carolina 27710

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Am J Physiol Lung Cell Mol Physiol 282: L1057–L1065, 2002. First published November 30, 2001; 10.1152/ajplung.00081.2001.—Nitric oxide (NO) functions as an endothelium-derived relaxing factor by activating guanylate cyclase to increase cGMP levels. However, NO and related species may also regulate vascular tone by cGMP-independent mechanisms. We hypothesized that naturally occurring NO donors could decrease the pulmonary vascular response to serotonin (5-HT) in the intact lung through chemical interactions with 5-HT2 receptors. In isolated rabbit lung preparations and isolated pulmonary artery (PA) rings, 50–250 μM S-nitrosoglutathione (GSNO) inhibited the response to 0.01–10 μM 5-HT. The vasoconstrictor response to 5-HT was mediated by 5-HT2 receptors in the lung, since it could be blocked completely by the selective inhibitor ketanserin (10 μM). GSNO inhibited the response to 5-HT by 77% in intact lung and 82% in PA rings. In PA rings, inhibition by GSNO could be reversed by treatment with the thiol reductant dithiothreitol (10 mM). 3-Morpholinosydnonimine (100–500 μM), which releases NO and O2, simultaneously, also blocked the response to 5-HT. Its chemical effects, however, were distinct from those of GSNO, because 5-HT-mediated vasoconstriction was not restored in isolated rings by dithiothreitol. In the intact lung, neither NO donor altered the vascular response to endothelin, which activates the same second-messenger vasoconstrictor system as 5-HT. These findings, which did not depend on guanylate cyclase, are consistent with chemical modification by NO of the 5-HT2 G protein-coupled receptor system to inhibit vasoconstriction, possibly by S-nitrosylation of the receptor or a related protein. This study demonstrates that GSNO can regulate vascular tone in the intact lung by a reversible mechanism involving inhibition of the response to 5-HT.

There is substantial evidence that NO also relaxes vascular smooth muscle by cGMP-independent pathways. cGMP-independent effects of NO on vascular smooth muscle can be mediated by stimulating Na+–K+–ATPase activity, modifying K+ channels, or decreasing sensitivity to or release of vasoconstrictors.

The vasoconstrictor effect of serotonin [5-hydroxytryptamine (5-HT)] in the lung can be inhibited with NO. In the pulmonary vasculature, 5-HT binds to G protein-coupled receptors (GPCR), including 5-HT1B and 5-HT2A receptors, to produce vasoconstriction (23, 27, 28, 32, 45). NO inhibits the response to 5-HT in several vascular tissues, including human umbilical arteries (35, 42), bovine coronary arteries (11), and rat pulmonary artery (44). Although the vascular response to 5-HT is altered by NO, the mechanisms by which NO modifies the responses to 5-HT and their importance in the lung are not known.

The effects of NO on cellular function are mediated by its reactions with specific molecular targets. NO can exist in several oxidation-reduction states (e.g., NO, NO–, NO2), which promote its reactions with protein thiolis, transition metals such as iron in heme proteins, O2, and reactive oxygen species. The biochemical reactions of NO have been shown to activate and inactivate protein function and alter receptor activity (20, 37, 38).

GPCR are known targets of NO via cGMP-independent pathways. Studies of β2-adrenergic receptors in cultured cells have shown that NO donors reduce signaling by β2-adrenergic agonists and promote depalmitoylation of the receptor (1, 40, 43). In the M2 muscarinic GPCR and angiotensin (AT1) GPCR, treatment with NO inhibits receptor-ligand binding (2, 8). The role of NO in modifying the response to 5-HT receptor function has not been investigated.

We hypothesized that naturally occurring NO donors could decrease the pulmonary vascular response to 5-HT in the intact lung through chemical interactions involving the receptor pathway. In this study, we characterized the response to 5-HT in intact rabbit lungs and isolated pulmonary artery rings and determined...
that vasoconstriction in our model was mediated by the 5-HT2 receptor. We studied the effects of the NO donor S-nitroso glutathione (GSNO) on the 5-HT response and questioned the mechanism of 5-HT inhibition. Our findings are consistent with chemical modification by NO of the 5-HT2 GPCR system to inhibit vasoconstriction by a reversible thiol-based mechanism.

**MATERIALS AND METHODS**

**Reagents and Pharmaceuticals**

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified.

**Isolated Lung Preparation**

The experiments were performed in isolated buffer-perfused lungs (IPL) of rabbits, as previously described (34). The buffer was Krebs-Henseleit (KH) solution containing final concentrations of 82.8 mM sodium chloride, 4.7 mM potassium chloride, 2.4 mM monobasic potassium phosphate, 25 mM sodium bicarbonate, 1.2 mM magnesium sulfate, 2.7 mM calcium chloride, and 11.1 mM dextrose, pH 7.4. New Zealand White rabbits (May’s Farm, NC) weighing 2.5–3.5 kg were anticoagulated with sodium heparin (5,000 U) and anesthetized with pentobarbital sodium (25 mg/kg) by ear vein. An incision was made in the left chest wall, exposing the heart. The animal was bled via the left ventricle, and the thorax was excised by excising the rib cage. Stainless steel cannulas were placed in the trachea, main pulmonary artery, and left atrium. The aorta also was tied with the pulmonary artery to prevent loss of perfusate to systemic circulation. The lungs were inflated with 80 ml of air and ventilated with 21% O2-5% CO2-balance N2 with an animal respirator (Harvard Apparatus, S. Natick, MA) at a rate of 30 breaths/min. The tidal volume was adjusted to maintain a peak tracheal pressure of 8–10 Torr with a positive end-expiratory pressure of 2–3 Torr.

The perfusion circuit contained a reservoir suspended freely from a force transducer (model FT100, Grass Instrument, Quincy, MA) and a water heater set at 37°C. Perfusate was circulated by a roller pump (Sarns, Ann Arbor, MI) and passed through a bubble trap before entering the pulmonary artery. The perfusate returned to the left atrium and then to the reservoir, which was set at the lowest portion of the lung to provide a left atrial pressure of zero. Perfusion began slowly and was gradually increased to 100 ml/min. After the lungs were rinsed free of blood with 500 ml buffer, a recirculating system was established. The total volume of KH buffer in the circuit was ~250 ml. Mean pulmonary arterial pressure (Ppa) and tracheal pressure were measured using pressure transducers (model P231D, Gould Statham Instruments, Hato Rey, PR). The weight gain of the lung as an index of pulmonary edema formation was measured as the loss of perfusate from the reservoir connected to the force transducer. Ppa, tracheal pressure, and weight gain were continuously recorded on a four-channel recorder (model 2450S, Gould, Cleveland, OH). The preparation was considered successful if the Ppa was stable between 10 and 20 Torr and there was <0.15 g/min weight gain during a 10-min stabilization period.

**Pulmonary Artery Ring Preparation**

Pulmonary artery rings (3 mm) were harvested from New Zealand White rabbits and mounted in 25-ml tissue baths filled with KH buffer and bubbled with 21% O2-5% CO2-balance N2. Isometric tension was measured. All rings in the study were suspended with similar baseline levels of tension (~2 g). Tissue baths were thoroughly rinsed with fresh buffer between interventions.

**Synthesis of GSNO**

GSNO was synthesized as previously described (7). Briefly, GSNO was prepared by reacting reduced glutathione (GSH) dissolved in 0.5 N HCl with equimolar sodium nitrite dissolved in water. GSNO used in IPL experiments was precipitated with acetone, filtered, and washed sequentially with water, acetone, and ether. Solutions of GSNO in KH buffer were prepared daily from precipitated samples, and concentrations were confirmed by absorption spectroscopy at 335 nm using an extinction coefficient of 0.92 optical density units·mM·cm-1. In isolated ring experiments, GSNO was prepared from stock solutions of GSH and sodium nitrate immediately before use in tissue baths.

**Measurements of NOx Level**

Perfusate was assayed for NOx (nitrite and nitrate) using a catalytic method for reduction of NO oxidation products to NO gas. Perfusate samples were injected into a refluxing glass reaction chamber containing 0.1 M vanadium(III) chloride and carried in nitrogen gas to a chemiluminescence detector. Measurements of known concentrations of nitrite and nitrate were quantitatively linear between 25 and 500 pmol (22).

**Experimental Protocols**

**Dose-response curve for 5-HT.** A dose-response curve for 5-HT was performed to determine an optimal dose of 5-HT for later experiments. Perfused lungs were treated with three consecutive doses of 5-HT to provide final perfusate concentrations of 0.1, 1.0, and 10.0 μM. Lungs were monitored until the Ppa returned to baseline (typically <20 min) before administration of the next dose. A similar dose-response curve for 5-HT (0.01–10 μM) was established in the pulmonary artery ring bioassay.

**5-HT in isolated perfused lungs.** Experiments in isolated lungs were preceded by a 60-min perfusion period before administration of 1.0 μM 5-HT. After 5-HT, lungs were monitored for another 30 min and Ppa and weight gain were recorded continuously. One group of lungs was pretreated with the 5-HT2 receptor antagonist ketanserin to confirm that the 5-HT2 receptor was responsible for the vasoconstrictor response to 5-HT. These lungs were pretreated with 10 μM ketanserin infused via the pulmonary artery 5 min before 5-HT administration. This dose of ketanserin was chosen because it selectively inhibits the 5-HT2 receptor (27, 28).

In the next series of experiments, lungs were perfused for 60 min with or without an NO donor before administration of 5-HT. After 5-HT, Ppa and weight gain were monitored for an additional 30 min. NO-treated lungs received 50 μM GSNO or 100 μM 3-morpholinosydnonimine (SIN-1; Alexis, San Diego, CA), which simultaneously releases NO and superoxide (O2·-) (9, 17). The responses were compared with those of control lungs perfused for 1 h without administration of an NO donor. Experiments were also done in lungs treated with 25,000 U of Cu,Zn superoxide dismutase (SOD) or the intracellular superoxide scavenger T2E (gift of James D. Crapo) to determine whether scavenging O2·- could alter the effects of GSNO or SIN-1. The initial response to 1.0 μM 5-HT was compared with the second dose of 5-HT after treatment for 60 min with Cu,Zn SOD along with 50 μM GSNO or 100 μM...
SIN-1. The sequential response to 5-HT was also examined after a 60-min treatment with T2E and GSNO. To determine whether NO donors modified 5-HT directly, lungs treated with 120 μM 5-HT were pretreated for 1 h with 50 μM GSNO or 100 μM SIN-1. 5-HT (1.0 μM) pretreated with NO donor was administered to IPL, and Ppa and weight gain were monitored for 30 min. The final concentration of GSNO or SIN-1 in the perfusate in these experiments was 2 or 5 nM, respectively.

Uptake of 5-HT in pulmonary vascular smooth muscle cells by a 5-HT transporter (5-HTT) contributes to inactivation of 5-HT in the lung (12, 26). To assess the role of 5-HT uptake in the acute vasoconstrictor response to 5-HT, lungs were treated with 1.0 μM 5-HT and then incubated for 30 min with the 5-HTT inhibitor flunitrazepam (10 μM) and a second dose of 5-HT. The response to 5-HT in each lung after flunitrazepam was compared with its baseline 5-HT response. In other experiments, the 5-HT responses in control lungs were compared with responses after 10 μM flunitrazepam for 10 min followed by GSNO (50 μM) for another 30 min.

Endothelin in IPL. To evaluate whether the effects of GSNO on 5-HT responses were specific for this vasoconstrictor, lungs were treated with endothelin-1 (ET-1) alone or ET-1 after pretreatment with GSNO or SIN-1. ET-1 was selected because it produces vasoconstriction by the intracellular signaling pathway used by 5-HT, in which the GPCR in smooth muscle is linked to phospholipase C (PLC), inositol trisphosphate (IP₃), and diacylglycerol (DAG) (23). Lungs were pretreated with 50 μM GSNO or 100 μM SIN-1 for 60 min before administration of 10 nM ET-1. Ppa and weight gain were monitored for 30 min after ET-1.

5-HT and ET-1 in pulmonary artery rings. A dose-response curve for 0.01–10 μM 5-HT and 0.1–100 nM ET-1 in isolated rabbit pulmonary artery rings was compared before and after treatment for 60 min with 250 μM GSNO. In subsequent experiments, rings were treated with a single dose of 1.0 μM 5-HT before and after each intervention. The vasoconstrictor response to 1 μM 5-HT in the pulmonary artery bioassay was studied before and after treatment for 60 min with 250 μM GSNO or 500 μM SIN-1. The baths were flushed three times thoroughly with buffer to remove residual GSNO and SIN-1 before second doses of 5-HT. Isometric tension was measured, and the response was expressed as the percent change in tension after the second dose of 5-HT compared with the initial 5-HT response. Control experiments for GSNO were performed with 100 μM GSH or oxidized glutathione (GSSG). GSH or GSSG was added to the baths for 60 min before the second 5-HT dose. The dose-response curve to 0.01–1 μM 5-HT was tested in the presence of the guanylate cyclase inhibitor LY-83583 (10 μM; Cayman Chemical, Ann Arbor, MI) with and without 250 μM GSNO.

The thiol reducing agent dithiothreitol (DTT, 10 mM) was administered in some experiments 5 min before the second 5-HT dose to determine whether the 5-HT response could be restored. Experiments were performed in rings incubated with buffer alone, 250 μM GSNO, or 500 μM SIN-1. Data are expressed as the percent change in tension after the second dose of 5-HT after DTT compared with the initial 5-HT response.

Statistical Analysis

Data were analyzed by ANOVA with repeated measures for experiments in isolated lungs and dose-response curves in rings. All other data were analyzed by ANOVA followed by Fisher’s protected least-square difference test using a commercially available software program (Statview 512+, Brain Power, Calabasas, CA). Values are means ± SE. P values are provided where statistical tests were performed.

RESULTS

Dose-Dependent Response to 5-HT in IPL and Pulmonary Artery Rings

5-HT transiently increased Ppa in rabbit IPL to a maximum at 5 min, and Ppa returned to baseline by 20 min. Dose-dependent vasoconstriction occurred with 0.1 and 1.0 μM 5-HT, but 10.0 μM 5-HT did not produce significant further vasoconstriction compared with 1.0 μM (Fig. 1A). Lungs treated with 5-HT showed no evidence of pulmonary weight gain throughout the experiments. On the basis of the results of the dose-response studies, the remaining IPL experiments were performed with 1.0 μM 5-HT. A dose-dependent response was also observed for 0.01–1 μM 5-HT in a pulmonary artery ring bioassay with no further in-

Fig. 1. Dose response curves to serotonin [5-hydroxytryptamine (5-HT)]. A: in isolated perfused lungs (IPL), 0.1 μM 5-HT (n = 3) produced minimal vascular [pulmonary arterial pressure (Ppa)] response, while 1.0 μM 5-HT (n = 6) produced transient vasoconstriction, which was maximal at 5 min. 5-HT at 10 μM (n = 3) did not produce any additional vasoconstriction compared with 1.0 μM 5-HT. Lungs did not develop edema with any dose of 5-HT. B: in isolated pulmonary artery rings, 0.01–1.0 μM 5-HT produced a dose-dependent increase in ring tension, which did not increase further with 10 μM 5-HT (n = 4, P < 0.05 by ANOVA).
crease in ring tension with 10 μM 5-HT (n = 4, P < 0.05; Fig. 1B).

Effects of the 5-HT2 Receptor Antagonist Ketanserin on the Response to 5-HT

Ketanserin (10 μM) virtually abolished the vasoconstrictor response to 1 μM 5-HT in the rabbit IPL (Fig. 2). The change in Ppa with 5-HT at 5 min was 0.5 ± 0.2 mmHg for lungs pretreated with ketanserin compared with 11.5 ± 3.9 mmHg in control lungs (P < 0.05). These results indicate that the constrictor response to 5-HT is mediated by the 5-HT2A receptor, which is present in the pulmonary vasculature (27).

Effects of NO Donors on the Response to 5-HT in IPL

Pretreatment with GSNO markedly inhibited the vasoconstrictor response to 5-HT in IPL (Fig. 3). A qualitatively similar result was found using SIN-1. The change in Ppa with 5-HT after 5 min was 2.6 ± 1.1 mmHg for lungs pretreated with 50 μM GSNO (23% of control 5-HT response) and 1.6 ± 0.7 mmHg for lungs pretreated with 100 μM SIN-1 (14% of control 5-HT response, P < 0.05 for each NO donor vs. control). There was no statistical difference in baseline Ppa in IPL pretreated with GSNO or SIN-1. NOx levels in the perfusate measured by chemiluminescence were higher in both NO donor groups compared with control but similar to each other (Table 1). Treatment with 25,000 U of Cu,Zn SOD did not prevent the inhibitory effects of GSNO or SIN-1 on the vasoconstrictor response to 5-HT (Fig. 4; change in Ppa at 5 min = 1.0 ± 1.0 and 0.3 ± 0.2 mmHg in GSNO- and SIN-1-treated lungs, respectively), indicating that the effect was not due to O2 generation. This result was confirmed by pretreatment of the lungs with 1.3 mg of T2E to scavenge extra- and intracellular O2. T2E did not prevent the inhibitory effects of GSNO on 5-HT. In SIN-1-treated lungs, Cu,Zn SOD prevented even the small increase in Ppa with 5-HT compared with that produced by SIN-1 alone. Blocking 5-HT uptake with 10 μM fluoxetine had no effect on the peak vasoconstrictor response to 5-HT, but it delayed the return of Ppa to baseline (n = 2, data not shown). In addition, GSNO-mediated inhibition of the constrictor response to 5-HT was not prevented by fluoxetine (change in Ppa at 5 min with 5-HT = 3.3 ± 1.4 mmHg, n = 3).

Effects of In Vitro Treatment of 5-HT With NO Donors in IPL

5-HT was treated in vitro with 50 μM GSNO or 100 μM SIN-1 to evaluate whether NO functionally modified the 5-HT peptide directly. The vasoconstrictor activity of NO donor-treated 5-HT (1 μM) was similar to that of untreated 5-HT (Fig. 5). With GSNO-treated 5-HT (n = 3), the change in Ppa at 5 min was 7.5 ± 1.3 mmHg, and with SIN-1-treated 5-HT (n = 4), the change in Ppa was 7.0 ± 0.5 mmHg (P = 0.30 vs. control 5-HT).

Effects of GSNO on the Response to ET-1 in IPL and Pulmonary Artery Rings

GSNO-pretreated or untreated lungs were treated with ET-1 to determine whether GSNO attenuated the response to ET-1. At the same concentrations that inhibited responses to 5-HT, GSNO failed to inhibit

Table 1. NOx levels in perfusate before administration of 5-HT increased to similar levels after 50 min of treatment with GSNO or SIN-1

<table>
<thead>
<tr>
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<th>Control (n = 4)</th>
<th>GSNO (n = 5)</th>
<th>SIN-1 (n = 5)</th>
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<tr>
<td>NOx, μM</td>
<td>3.5 ± 0.7</td>
<td>30.5 ± 5.0</td>
<td>24.6 ± 3.1</td>
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Values are means ± SE; n, number of observations. NOx, nitrite + nitrate; GSNO, S-nitrosothioglutathione (50 μM); SIN-1, 3-morpholinosydnonimine (100 μM); 5-HT, serotonin.
vasoconstriction to ET-1 (Fig. 6). A similar result was obtained using SIN-1. The change in Ppa 30 min after ET-1 was 33.0 ± 5.8 mmHg in control lungs, 29.4 ± 7.0 mmHg in GSNO-treated lungs, and 30.0 ± 3.8 mmHg in SIN-1-treated lungs. In pulmonary artery rings, the dose-response curve for 0.1–100 nM ET-1 was not shifted to the right by 250 μM GSNO. There was no increase in ring tension with 0.1–10 nM ET-1, while 100 nM ET-1 produced an increase of 0.85 ± 0.02 g in control rings and 0.63 ± 0.04 g in GSNO-treated rings (n = 4).

Effects of NO Donors on the Response to 5-HT in Pulmonary Artery Rings

In rabbit pulmonary artery rings, the increase in ring tension was the same with two sequential doses of 1 μM 5-HT. Similar to IPL, the response to 5-HT in rings was nearly eliminated by 10 μM ketanserin, a 5-HT₂ receptor antagonist (data not shown). As in the intact lung, the response to 5-HT also was significantly reduced after pretreatment with 250 μM GSNO (18 ± 3% of initial 5-HT response, n = 5, P < 0.05 vs. control rings) or 500 μM SIN-1 (27 ± 5% of initial 5-HT response, n = 8, P < 0.05 vs. control rings; Fig. 7A). The dose-response curve to 0.01–10 μM 5-HT for control rings was significantly attenuated in GSNO-treated rings (n = 4, P < 0.05; Fig. 7B). GSH or GSSG at the equivalent concentrations (250 and 125 μM, respectively) had no effect on the response to 5-HT (data not shown). The dose-response curve to 0.01–1 μM 5-HT was shifted to the left in pulmonary artery rings treated with the guanylate cyclase inhibitor LY-83583 (10 μM). GSNO (250 μM) prevented 90% of the response to 0.1 μM 5-HT and 40% of the response to 1.0 μM 5-HT in rings treated with LY-83583, indicating an effect of GSNO that was independent of guanylate cyclase activity (n = 3, P < 0.05; Fig. 8).

Fig. 4. Cu,Zn superoxide dismutase (SOD) did not prevent the effects of GSNO or SIN-1 on the response to 5-HT in IPL. Treatment with 25,000 U of Cu,Zn SOD did not prevent the inhibitory effects of 50 μM GSNO (n = 3) or 100 μM SIN-1 (n = 6) on the vasoconstrictor response to 5-HT. A: change in Ppa at 5 min in response to 1.0 μM 5-HT after pretreatment with GSNO or SIN-1 with or without Cu,Zn SOD. B: change in Ppa over 30 min in control lungs treated with 1.0 μM 5-HT (□), lungs pretreated with SIN-1 (●), or lungs pretreated with Cu,Zn SOD and SIN-1 (●). *P < 0.05 vs. control; †P < 0.05 vs. SIN-1-treated lung.

Fig. 5. Effects of in vitro treatment of 5-HT with NO donors. Vasoconstrictor activity of 5-HT treated in vitro with 50 μM GSNO (●, n = 3) or 100 μM SIN-1 (○, n = 4) was similar to that of untreated 5-HT (○). P ≥ 0.3 vs. control (by ANOVA).

Fig. 6. Effects of NO donors on the response to endothelin-1 (ET-1). Pretreatment of IPL with 50 μM GSNO (●, n = 6) or 100 μM SIN-1 (○, n = 7) for 1 h did not block ET-1 (10 nM)-mediated vasoconstriction (□, n = 6). Doses of GSNO or SIN-1 were the same as those used for the 5-HT studies. P ≥ 0.3 vs. control (by ANOVA).
Ability of DTT to Reverse the Inhibitory Effects of GSNO on the 5-HT Response

The thiol reducing agent DTT (10 mM) partially restored the response to 1 μM 5-HT in isolated rings treated with 250 μM GSNO (38 ± 8% of initial 5-HT response, n = 7, P < 0.05 vs. GSNO-treated rings; Fig. 9). In SIN-1 (500 μM)-treated rings, there was a trend toward restoration of the response, but the effect was not statistically significant (32 ± 6% of initial 5-HT response, n = 9, P = 0.2). DTT did not change the response in control rings (95 ± 9% of initial 5-HT response, n = 6). Overall, the response to 5-HT after DTT in GSNO-treated rings increased 2.6-fold vs. a 0.5-fold increase after DTT in SIN-1-treated rings.

DISCUSSION

NO binds to thiol and metal centers in proteins to regulate protein function (37). NO may function as an endothelium-derived relaxing factor by activating guanylate cyclase in smooth muscle to decrease vascular tone or by chemically modifying vasoconstrictor agents, their receptor proteins, or contractile components in smooth muscle to prevent vasoconstriction. We examined the effects of NO donors on vasoconstriction induced by two potent pulmonary vasoconstrictors of physiological and pathophysiological relevance, 5-HT and ET-1, which bind to distinct receptors within the superfamily of GPCRs but operate in vascular smooth muscle using the same intracellular transduction mechanisms (23).

In this study, 5-HT produced vasoconstriction in isolated rabbit lungs and pulmonary artery rings. The effect of 5-HT was inhibited by ketanserin, a specific antagonist of the 5-HT2A receptor. These findings are consistent with earlier studies demonstrating that ketanserin-sensitive 5-HT2A receptors in pulmonary artery smooth muscle mediate vasoconstriction (28, 41). The lung also contains 5-HT1B receptors, which have an important role in pulmonary artery vasoconstriction under physiological conditions, as well as receptors, including 5-HT1 receptor subtypes, implicated in vasodilation (27, 31, 33). Our observation that constriction does not increase further in rabbit lungs with 10 μM 5-HT may indicate that 5-HT2 receptors are saturated or that higher doses preferentially bind to vasodilating 5-HT1 receptors to counterbalance vasoconstric-
Vasoconstriction by 5-HT was inhibited in isolated lungs and pulmonary artery rings by pretreatment with NO donors. The doses of GSNO and SIN-1 administered to isolated rabbit lungs did not measurably decrease Ppa. Because the pulmonary artery vessels were not fully dilated in our perfused lung preparations, this finding suggested that cGMP-mediated vasodilation by NO could have only partially counteracted 5-HT-mediated constriction. In GSNO-treated vascular rings, an inhibitor of guanylate cyclase, it may also affect relaxation in the lung. Although LY-83583 inhibits guanylate cyclase, it may also affect S-nitrosylation and cAMP level; therefore, these data may underestimate the cGMP-independent contribution of NO donor on the 5-HT response (15). Other investigators have also shown that tissue cGMP levels do not always correlate well with pulmonary artery relaxation. Pulmonary artery relaxation by GSNO has been inhibited only partially when cGMP production is completely blocked by guanylate cyclase inhibitors (6, 18, 19). The 5-HT response inhibition by GSNO or SIN-1 in isolated lungs was reproducible in rings in which the chamber was replaced with fresh buffer to remove unreacted donor. This suggested that the NO donors had chemically modified a vascular target. These data are consistent with other studies where NO donors were found to inhibit the response to 5-HT. In rat pulmonary artery smooth muscle cells, NO-containing solutions inhibited the rise in intracellular calcium after 5-HT and inhibited vasoconstriction by 5-HT in pulmonary artery rings (44). In human umbilical artery rings, endogenous NO decreased the response to 5-HT (35). In bovine coronary artery rings, exogenous NO suppressed 5-HT-mediated contractions (11). These findings also indicate that NO modifies the pulmonary vasoconstrictor response to 5-HT but do not provide a molecular mechanism or assess reversibility.

SIN-1 releases NO and also O$_2^-$, which reacts rapidly with NO to form the strong nitrating species peroxynitrite (ONOOC$^-$. To examine possible involvement of ONOOC$^-$ in the 5-HT response inhibition by NO donors, lungs were pretreated with Cu,Zn SOD or an SOD mimetic, T2E, before the NO donors. Neither Cu,Zn SOD nor T2E restored the 5-HT response in lungs pretreated with GSNO. These findings suggest that GSNO acted as an S-NO donor and did not produce its effect by reacting with O$_2^-$ to form ONOOOC$. These data differ from studies in coronary arteries in which scavenging O$_2^-$ blocked the response to NO, implicating ONOOOC$. Cu,Zn SOD also did not restore the 5-HT response after pretreatment with SIN-1. In fact, lungs had a smaller response to 5-HT when pretreated with SIN-1 and Cu,Zn SOD than with SIN-1 alone. This indicates that scavenging of O$_2^-$ by SOD increases the bioactivity of NO and that SIN-1 had acted primarily as an NO donor.

Signaling for the vascular responses to 5-HT includes potential targets for NO within the GPCR system, the 5-HT uptake system that inactivates 5-HT, or the vasoconstrictor itself. Treating 5-HT with GSNO or SIN-1 in vitro produced vasoconstriction in the lung similar to native 5-HT, which indicates that modification of the 5-HT molecule by NO donors was not important under these conditions (4).

Another potential target for NO is the 5-HTT, which is necessary for uptake and metabolism of 5-HT by the lung and is involved in pulmonary vascular smooth muscle cell proliferation (12, 13, 26). In our study, inhibition of the 5-HTT with fluoxetine did not restore 5-HT vasoconstriction after treatment with GSNO. Therefore, the inhibitory effects of the NO donors were not attributable to enhancement of 5-HT uptake by the lung.

The pulmonary vasoconstrictor effects of ET-1, which are also mediated through a GPCR linked to PLC, were not inhibited by GSNO or SIN-1. GSNO therefore inhibits vasconstriction by 5-HT by modifying the response at a target(s) proximal to PLC, most likely the 5-HT$_2$ receptor. Activation of PLC liberates two second messengers, DAG and IP$_3$, which stimulate calcium influx and mobilize intracellular calcium from the sarcoplasmic reticulum to produce vasoconstriction (23). In pulmonary artery smooth muscle cells, NO inhibited 5-HT-induced calcium release by IP$_3$ and DAG, supporting our conclusion that the site of action for NO in the 5-HT pathway is proximal to PLC (44).

The present data strongly suggest that NO modifies the 5-HT$_{2A}$ GPCR. NO may prevent ligand-receptor binding or coupling of the receptor to the G protein. Studies of other GPCR demonstrate that NO can modify receptor function (24, 25). For example, NO reduced signaling by $\beta_2$-adrenergic agonists in vascular models (1, 40, 43) and inhibited receptor-ligand binding in M$_2$ muscarinic GPCR and angiotensin type 1 receptors (2, 8). In aortic endothelial cells, GSNO, but not glutathione or nitrite, inhibited bradykinin type 2 receptor-G protein coupling (29). NO may also directly modify the G protein. NO inhibits G$_o$ protein in synaptosomes, which prevents pertussis toxin-catalyzed ADP-ribosylation of the G$_o$ protein (16). Future experiments are needed to discriminate effects of NO on 5-HT$_{2A}$ receptor-ligand binding, G protein coupling, or G protein activity.

In pulmonary artery rings, GSNO inhibited the constrictor response to 5-HT, and the inhibitory effect of GSNO was largely reversible by the thiol reductant DTT. Nitrosothiol compounds such as GSNO have an NO$^+$ character that promotes transnitrosylation of other protein thiols. Reversal by DTT indicates that GSNO probably modified the 5-HT response by thiol nitrosylation. Specific acidic and/or basic amino acid sequences surrounding a given Cys favor S-nitrosylation (3, 39), and the 5-HT$_2$ receptor contains several Cys predicted to be susceptible to S-nitrosylation (39).
In contrast to GSNO, the NO donor SIN-1 modified the 5-HT response by a DTT-resistant mechanism. SIN-1 produces chemical modifications different from GSNO, e.g., nitrification, rather than S-nitrosylation. Thus the fate of NO released by SIN-1 may better represent chemical reactions associated with increased oxidative stress and pathological ONOO− formation. We did not determine, however, whether a specific S-nitrosylation event is responsible for the effects of GSNO. An alternative chemical explanation, that S-thiolation, e.g., glutathiolation, was responsible for the more potent effects of GSNO than SIN-1, cannot be distinguished from S-nitrosylation in these studies. However, lack of effect of GSSG argues against S-thiolation.

In summary, GSNO reversibly inhibits the response to 5-HT in the intact lung. The experimental data narrow the target of NO to the level of thiol residues on the G protein-coupled 5-HT2 receptor. 5-HT2 receptor density is low in the lung and difficult to study. The chemical modification produced by GSNO is different from that produced by SIN-1, which may reflect the specific redox chemistry of NO and the chemical properties of the receptor. Further investigations are necessary to identify specific molecular targets in the receptor-G protein complex and determine whether NO regulates other receptors in this superfamily.

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