S-nitrosoglutathione inhibits α₁-adrenergic receptor-mediated vasoconstriction and ligand binding in pulmonary artery

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S-nitrosoglutathione inhibits α₁-adrenergic receptor-mediated vasoconstriction and ligand binding in pulmonary artery. Am J Physiol Lung Cell Mol Physiol 290: L136–L143, 2006. First published August 26, 2005; doi:10.1152/ajplung.00230.2005.—Endogenous nitric oxide donor compounds (S-nitrosothiols) contribute to low vascular tone by both cGMP-dependent and -independent pathways. We have reported that S-nitrosoglutathione (GSNO) inhibits S-hydroxytryptamine (5-HT)-mediated pulmonary vasoconstriction via a cGMP-independent mechanism likely involving S-nitrosylation of its G protein-coupled receptor (GPCR) system. Because catecholamines, like 5-HT, constrict lung vessels via a GPCR coupled to Gq, we hypothesized that S-nitrosothiols modify the α₁-adrenergic GPCR system to inhibit pulmonary vasoconstriction by receptor agonists, e.g., phenylephrine (PE). Rat pulmonary artery rings were pretreated for 30 min with and without an S-nitrosothiol, either GSNO or S-nitrosocysteine (CSNO), and constricted with sequential concentrations of PE (10⁻⁸–10⁻⁴ M). Effective cGMP-dependence was tested in rings pretreated with soluble guanylate cyclase inhibitors [either 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or LY-83583] or G kinase inhibitor (KT-5823), and a thiol reductant [dithiothreitol (DTT)] was used to test reversibility of S-nitrosylation. Both S-nitrosothiols attenuated the PE dose response. The GSNO effect was not prevented by LY-83583, ODQ, or KT-5823, indicating cGMP independence. GSNO inhibition was reversed by DTT, consistent with S-nitrosylation or other GSNO-mediated cysteine modifications. In CSNO-treated lung protein, the α₁-adrenergic receptor was shown to undergo S-nitrosylation in vitro using a biotin switch assay. Studies of α₁-adrenergic receptor subtype expression and receptor density by saturation binding with [¹²⁵I]HEAT showed that GSNO decreased α₁-adrenergic receptor density but did not alter affinity for antagonist or agonist. These data demonstrate a novel cGMP-independent mechanism of reversible α₁-adrenergic receptor inhibition by S-nitrosothiols.

nitric oxide; S-nitrosylation; G protein-coupled receptor; guanosine 3’,5’-cyclic monophosphate

The low-resistance pulmonary vascular circuit is regulated by multiple neural, humoral, and endocrine factors among which the endothelium-derived relaxing factor, nitric oxide (NO), plays a fundamental role. NO bioactivity in the pulmonary circulation is present in the form of NO donor compounds (S-nitrosothiols) that maintain low vascular tone by both cGMP-dependent and -independent pathways (13). NO released from endothelial cells activates guanylate cyclase in vascular smooth muscle cells to increase cGMP levels, which in turn relaxes vascular smooth muscle. NO also exerts cGMP-independent effects on the smooth muscle including (but not limited to) stimulating Na⁺-K⁺-ATPase activity, modifying K⁺ and Ca²⁺ channels, and decreasing the sensitivity to, or release of, vasoconstrictors (1, 6, 12, 17, 24).

We previously demonstrated that pulmonary vasoconstriction elicited by serotonin (5-hydroxytryptamine, 5-HT) is reversibly inhibited by the NO donor S-nitrosoglutathione (GSNO) (23). In addition, use of nitric oxide synthase (NOS) inhibitors or removal of the endothelium to decrease NO release has been shown to augment phenylephrine (PE)-mediated vasoconstriction in isolated rat pulmonary artery (28). Administration of an NO donor blocks both PE and norepinephrine (NE)-mediated vasoconstriction in rat aorta (17). Persistent inhibition of NE vasoconstriction is a characteristic of S-nitrosothiols, including GSNO, not found in other classes of NO donors such as 3-morpholinosydnonimine (SIN-1) or 2-(N,N-diethylamino)-diazenoic acid (DEA-NO) (3, 4). Furthermore, S-nitrosothiol inhibition of PE-mediated vasoconstriction is independent of the actions of NO on cGMP (17). 5-HT and catecholamines effect intracellular signaling via activation of G protein-coupled receptors (GPCR). The mechanism(s) by which NO inhibits these GPCR responses is not well understood.

GPCR activity and expression are regulated by multiple mechanisms and are involved in numerous physiological processes. Several studies have reported effects of NO on signaling through the β₂-adrenergic receptors, as well as bradykinin, M₂ muscarinic, thromboxane, and AT1 GPCRs. (2, 5, 22, 31, 32). The effects of NO on these GPCR-mediated responses have both cGMP-dependent and -independent components that, together with our data on the 5-HT GPCR system (23), raise the issue of cGMP-independent regulation of GPCRs by S-nitrosothiols as a general physiological mechanism for the regulation of vascular signal transduction. Thus we hypothesized that S-nitrosothiols inhibit catecholamine-induced vasoconstriction by thiol modification of the α₁-adrenergic receptor in the pulmonary vasculature. In this study, we demonstrated that GSNO inhibits PE-mediated vasoconstriction in isolated rat pulmonary arteries (PA) through a reversible, cGMP-independent pathway. We further demonstrate that GSNO decreases α₁-adrenergic receptor-ligand binding, thereby localizing the target of NO to the receptor-G protein complex.

METHODS

Reagents and pharmaceuticals. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. GSNO and nitric oxide donor compounds (S-nitrosothiols) contribute to low vascular tone by both cGMP-dependent and -independent pathways. We have reported that S-nitrosoglutathione (GSNO) inhibits S-hydroxytryptamine (5-HT)-mediated pulmonary vasoconstriction via a cGMP-independent mechanism likely involving S-nitrosylation of its G protein-coupled receptor (GPCR) system. Because catecholamines, like 5-HT, constrict lung vessels via a GPCR coupled to Gq, we hypothesized that S-nitrosothiols modify the α₁-adrenergic GPCR system to inhibit pulmonary vasoconstriction by receptor agonists, e.g., phenylephrine (PE). Rat pulmonary artery rings were pretreated for 30 min with and without an S-nitrosothiol, either GSNO or S-nitrosocysteine (CSNO), and constricted with sequential concentrations of PE (10⁻⁸–10⁻⁴ M). Effective cGMP-dependence was tested in rings pretreated with soluble guanylate cyclase inhibitors [either 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or LY-83583] or G kinase inhibitor (KT-5823), and a thiol reductant [dithiothreitol (DTT)] was used to test reversibility of S-nitrosylation. Both S-nitrosothiols attenuated the PE dose response. The GSNO effect was not prevented by LY-83583, ODQ, or KT-5823, indicating cGMP independence. GSNO inhibition was reversed by DTT, consistent with S-nitrosylation or other GSNO-mediated cysteine modifications. In CSNO-treated lung protein, the α₁-adrenergic receptor was shown to undergo S-nitrosylation in vitro using a biotin switch assay. Studies of α₁-adrenergic receptor subtype expression and receptor density by saturation binding with [¹²⁵I]HEAT showed that GSNO decreased α₁-adrenergic receptor density but did not alter affinity for antagonist or agonist. These data demonstrate a novel cGMP-independent mechanism of reversible α₁-adrenergic receptor inhibition by S-nitrosothiols.
S-nitrosocysteine (CSNO) were prepared immediately before use by reaction of reduced glutathione or cysteine (Alexis, San Diego, CA) dissolved in 0.5 N HCl with equimolar sodium nitrite in water.

**PA ring preparation.** Animal studies were approved by the Duke University Institutional Animal Use and Care Committee. Rat PA rings (2 mm) from the first branch PA were removed immediately from 300-g male Sprague-Dawley rats killed by halothane overdose and aortic transaction. The rings were mounted in 15-ml tissue baths containing Krebs-Henseleit buffer and bubbled with 21% O2/5% CO2/balance N2 at 37°C. Krebs-Henseleit contained sodium chloride (82.8 mM), potassium chloride (4.7 mM), monobasic potassium phosphate (2.4 mM), sodium bicarbonate (25 mM), magnesium sulfate (1.2 mM), calcium chloride (2.7 mM), and dextrose (11.1 mM) at pH 7.4. All rings were suspended at baseline tension levels of ~1 g, and isometric tension was monitored continuously with a calibrated force transducer. In all rings, a dose-response curve using cumulative and isometric tension was monitored continuously with a calibrated maximum 10–6 g. Percent changes in ring tension from the initial maximum PE dose of 10–6 M were expressed as the percent change in ring tension from the initial maximum tension induced by PE (10–6 M). Control experiments were performed with reduced GSH and oxidized GSNO (250 μM) before the PE dose-response curve.

To determine whether NO inhibited the PE dose-response curve through cGMP-independent mechanism(s), we repeated PE dose-response curves (10–8–10–6 M) in the presence or absence of 250 μM GSNO after administering either a guanylate cyclase inhibitor or a protein kinase G (PKG) inhibitor to block the cGMP signaling pathway. The ring baths were rinsed with fresh buffer to remove the inhibitor and GSNO before administration of PE. Two guanylate cyclase inhibitors, 6-anilinoquinoline-5,8-quinone (LY-83583, 10 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 and 1 μM), were tested. LY-83583 blocks cGMP-dependent signaling by several mechanisms including inhibition of endothelial NO release and production of superoxide, both of which decrease guanylate cyclase activity. ODQ irreversibly inhibits guanylate cyclase as well as other heme-containing proteins (10). We confirmed the ability of LY-83583 and ODQ to block cGMP production by GSNO by measuring cGMP levels with a commercially available radioimmunooassay kit (Amersham Biosciences, Piscataway, NJ). PA were incubated for 30 min at 37°C in the oxygenated bioassay chambers in buffer containing 300 μM IBMX to prevent degradation of cGMP, and cGMP levels were measured according to the kit’s instructions. Ring responses were also tested in the presence of the PKG inhibitor KT-5823 (C5H5N2O5, 10 μM).

The thiol reducing agent dithiothreitol (DTT, 10 mM) was administered to GSNO-treated rings to determine whether PE constriction could be restored. In one series, DTT (10 mM) was added alone or with GSNO (250 μM) to the ring bath 30 min before PE. We removed DTT and GSNO by rinsing the baths, and the sequential concentrations of PE were tested. The responses were expressed as percent change in isometric tension from the initial maximum PE dose of 10–6 M. In the next series, DTT (10 mM) was added to the bath after the administration of PE (10–6 M) in both control rings and GSNO-treated rings. The responses in the presence of PE (10–6 M) and DTT were expressed as the percent change in ring tension from the initial maximum 10–6 M PE dose.

The dose-response curve to PE was also compared before and after treatment with α1-adrenergic receptor antagonists. Rings were treated for 30 min with RS-100329 (Tocris Cookson, Ballwin, MO), which has a high specificity for the α1A-adrenergic receptor subtype, or BMY-7738 (Tocris Cookson), which selectively targets α1D-adrenergic receptors at low concentrations (14). Three different concentrations of antagonists (10–8, 10–7, 10–6 M) were tested for each PE dose-response curve. Five concentrations of PE between 10–8 M and 10–6 M were studied to construct dose-response curves for statistical analysis of antagonist studies.

**Biotin switch assay.** S-nitrosylation of the α1-adrenergic receptor was determined by the biotin switch method (15). In brief, rat lung was treated with or without 100 μM freshly made CSNO or GSNO for 30 min at 4°C. The sample was treated with methyl methanethiosulfonate (20 mM) and 25% SDS at 50°C for 20 min, and protein was acetone-precipitated. The pellet was resuspended in buffer and treated for 1 h at room temperature with 400 μM N-[6-(biotinamido)hexyl]-1,3-(2-pyridyldithio) propionamide (biotin HPDP, Pierce) and 5 mM ascorbic acid. One aliquot of CSNO-treated protein was incubated with the biotin-HPDP without ascorbic acid as a negative control. Other negative controls were performed with DTT (10 mM) plus nitrosothiol to prevent S-nitrosylation of the receptor and nitrosothiol (CSNO) plus ODQ (10 μM) to confirm cGMP independence of S-nitrosylation. The samples were then aceton precipitated, resuspended in buffer, and incubated overnight with 30 μl of streptavidinagarose beads at 4°C. The beads were washed, and biotin-labeled protein was eluted with 45 μl of buffer containing 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-mercaptoethanol. Proteins were separated on gradient SDS-PAGE gels (Bio-Rad, Hercules, CA), transferred to polyvinylidene difluoro membranes, and blocked with 1% nonfat dried milk. The α1-adrenergic receptor was detected by Western blot analysis with a polyclonal rabbit anti-rat α1-adrenergic receptor antibody (1:400; Affinity Bioreagents, Golden, CO) followed by a secondary horseradish peroxidase-conjugated anti-rabbit antibody and developed with enhanced chemiluminescence.

**Radioligand binding assays.** Saturation binding isotherms were performed on rat lung membranes treated with and without GSNO (250 μM) at 37°C for 30 min. Membranes (5–15 μg protein) were incubated with 20–200 pM 125I-HEAT [2-[6-(4-hydroxyphenyl)-ethyl-aminomethyl] tetralone; New England Nuclear, Boston, MA}, a highly selective α1-adrenergic receptor antagonist, in a 0.25-ml final volume of assay buffer for 30 min at 25°C. Nonspecific binding was determined by incubation with 125I-HEAT in the presence of 10 μM phenolamine. The assay was terminated by rapid vacuum filtration on Whatman GF/C filters using a Brandel harvester, and each filter was counted for 2 min in a liquid scintillation counter (Beckman LS 1800). Filters were presoaked with 0.3% polyethyleneimine for 2 h to minimize nonspecific binding, which at the highest HEAT concentrations was roughly one-third of total binding. All assays were performed in triplicate. The α1-adrenergic receptor density (Bmax, fmol/mg protein) and affinity (Kd, nM) were calculated with Graphpad Prism (Graphpad, San Diego, CA). Bmax represents the membrane binding sites in the presence of saturating concentrations of 125I-HEAT, whereas Kd represents the concentration of ligand that half maximally occupies the receptor. A PE competition curve (10–8 – 10–4 M) was performed with 20 pM 125I-HEAT to calculate KD, the concentration of competitor (PE) that half-maximally displaces 125I-HEAT. Binding to 20 pM 125I-HEAT also was compared following 30-min incubations at each of three concentrations of GSNO (2.5, 25, and 250 μM).

**RT-PCR.** For RT-PCR, total RNA from rat pulmonary artery was isolated with TRIzol reagent (Life Technologies, Gaithersburg, MD). RNA was reverse-transcribed (M-MLV Reverse transcriptase, Life Technologies), and the resultant cDNA was amplified for up to 32 cycles using gene-specific primers for α1-adrenergic receptor subtypes as previously described (9). Control reactions were performed...
without reverse transcription. Adrenergic receptor amplicons were normalized to 18S ribosomal RNA as an external standard.

Statistics. PA ring data were analyzed by analysis of variance (ANOVA) followed by the Fisher protected least-square-difference test using commercially available software (Statview512+; Brain Power, Calabasas, CA). Data were expressed as means ± SE. Receptor binding studies were fit by nonlinear regression and compared by F-test (P<0.05; Graphpad, San Diego, CA). The B_max and K_d were also compared by t-test (Statview512+). The P values have been provided where statistical tests were performed.

RESULTS

PA responses to PE are inhibited by S-nitrosothiols. In isolated rat PA rings, the increase in ring tension to PE (10^{-8}–10^{-6} M) was similar for two consecutive PE dose-response curves. For each experiment, ring data were expressed as the percent change in isometric ring tension compared with the tension generated with the maximum PE dose (10^{-6} M) in the initial PE dose-response curve. Pretreatment with GSNO (250 μM, n = 7) for 30 min significantly blunted the dose-response curve to PE (10^{-8}–10^{-6} M) (n = 6, P < 0.05 vs. untreated control rings) despite removal of GSNO from the bath. Low concentrations of GSNO (25 and 2.5 μM), however, had no significant effect on the PE response (n = 3 each, P > 0.05) (Fig. 1A). Similar inhibition was produced by 250 μM of the nitrosothiol CSNO (Fig. 1B) with no significant effects of lower concentrations of CSNO (25 and 2.5 μM) on the PE response (n = 3 each, P > 0.1, data for lower concentrations not shown). The PE dose-response relationship was not altered by pretreatment with either 250 μM GSH (n = 3, P > 0.05) (Fig. 1C) or 250 μM GSSG (data not shown).

The effects of GSNO on PE constriction persisted in presence of guanylate cyclase and PKG inhibitors. Rings were pretreated with a guanylate cyclase inhibitor, either LY-83583 or ODQ, as well as PKG inhibitor KT-5823 to test whether a component of the GSNO inhibition on the PE-stimulated vasoconstriction was independent of cGMP. Control experiments confirmed that the doses of the inhibitors used in this study blocked the cGMP pathway. Both guanylate cyclase inhibitors, LY-83583 (10 μM) and ODQ (1 and 10 μM), blocked the increase in cGMP content produced by 250 μM GSNO (n = 3, P < 0.05 by ANOVA) (Fig. 2A). In addition, adding LY-83583 and KT-5823 to the ring bath without rinsing before adding PE produced an increase in PE-induced ring tension compared with controls (not shown). For Fig. 2A, GSNO was rinsed from the bath before PE dosing to eliminate effects of residual NO activity; therefore, in experiments with LY-83583, ODQ, or KT-5823, these compounds were also rinsed from the bath before PE dosing. In this design, the PE dose response tended to increase in rings pretreated with LY-83583 (10 μM) but was not statistically different from control rings (n = 3, P = not significant). However, PE vasoconstriction was still inhibited by GSNO in LY-83583-treated rings with low measured cGMP levels (n = 5) (Fig. 2B). In contrast, PE vasoconstriction was significantly increased by ODQ in control rings even after the agent was rinsed from the bath. ODQ-induced dose-dependent increases in PE constriction were independent of its action as a guanylate cyclase inhibitor because 10 μM ODQ pretreatment produced a further increase in PE ring tension compared with 1 μM ODQ (Fig. 2C) with no further change in cGMP content. The PE dose-response curve in rings treated with 1 μM ODQ and GSNO (n = 3) was similar to control rings, but the PE response in GSNO + ODQ-treated rings was significantly weaker than that in rings exposed to ODQ alone. (P < 0.05) (Fig. 2D). This same pattern was observed in rings pretreated with 10 μM ODQ with GSNO + 10 μM ODQ

Fig. 1. Nitrosothiols S-nitrosothiols (GSNO) and S-nitrosothiols (CSNO) inhibited responses to phenylephrine (PE) in rat pulmonary artery (PA) rings. Cumulative concentrations of PE (10^{-8}–10^{-6} M) were administered to PA rings before and after a 30-min treatment with GSNO or CSNO. The nitrosothiol was rinsed from the chamber before the 2nd PE dose-response curve. A: 3 concentrations of GSNO (2.5, 25, and 250 μM) were tested, and data are expressed as percent change in isometric ring tension compared with the initial maximum PE dose (10^{-6} M). Data were compared by ANOVA (n = 3 for 2.5 and 25 μM, n = 7 for 250 μM). *P < 0.05 vs. untreated control rings, n = 6. B: response to PE (10^{-8}–10^{-6} M) was measured after treatment with GSNO (250 μM) (n = 6) and compared with GSNO-treated and control rings. *P < 0.05 vs. untreated control rings, n = 6; ANOVA. C: the PE dose response was not decreased by pretreatment with 250 μM GSH (n = 3, *P < 0.05 for control rings).
significantly <10 μM ODQ controls (Fig. 2E). Because ODQ can inactivate heme-containing proteins including NOS, which can in turn increase tone, we examined whether the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) would produce similar effects to ODQ. Pretreatment with L-NAME (100 μM) for 30 min increased PE dose response, similar to the pattern with ODQ treatment. The PE dose response was also attenuated in GSNO + L-NAME rings compared with L-NAME controls (66% of control response to 10^−6 M PE, n = 4, P < 0.05 by unpaired t-test). PE constrictor responses were increased by KT-5823 at doses selected to inhibit PKG activity, consistent with a pharmacologic effect and, when rinsed immediately before PE, still tended to increase the PE dose response. The inhibitory effects of GSNO on PE constriction were not blocked by KT-5823, similar to LY-83583- and ODQ-treated rings (Fig. 2F; n = 7, P < 0.05 vs. KT-treated rings without GSNO).

**DTT restores PE dose-response curve in GSNO-treated rings.** In one set of experiments, GSNO-treated rings received the thiol-reducing agent DTT before the PE dose-response curve. The PE dose-response curve in rings treated with both GSNO and DTT (n = 4) or DTT alone (n = 4) was similar to the PE dose-response in the control ring. (P > 0.05) (Fig. 3A). DTT (10 mM) administered after the maximum dose of PE (10^−6 M) restored the vascular response to control levels in rings pretreated with 250 μM GSNO (n = 4, P < 0.05 by ANOVA) (Fig. 3B). DTT did not change the response to PE in control rings. Overall tension after 10^−6 M PE in GSNO-treated rings increased 3.1-fold with DTT. To test whether the effect might be due to oxidation of cysteine sulfhydryl group(s), which would also be DTT reversible, we pretreated a series of rings with 300 μM H_2O_2. H_2O_2, unlike the S-nitrosothiols, did not inhibit the PE dose response (Fig. 3C).

**In vitro S-nitrosylation of the α1-adrenergic receptor.** S-nitrosylated proteins were separated from total lung protein by biotin switch and probed for 1-adrenergic receptor (Fig. 4). Endogenous S-nitrosylation of α1-adrenergic receptors was not detectable in protein from control rat lungs, but receptor S-nitrosylation was demonstrated by the biotin switch assay after a 30-min treatment with CSNO. CSNO was used for its...
rapid and robust effects compared with GSNO (100 μM), which nitrosylated the receptor weakly. However, the addition of reduced cysteine (10 μM) to GSNO produced a strong signal similar to CSNO. As a negative control, the α₁-adrenergic receptor from lung protein treated with CSNO but without ascorbic acid or with CSNO + DTT (10 mM), and bands were not detectable. S-nitrosylation by CNSO was not blocked by ODQ (10 μM).

reproduced in three to five separate experiments, and Fig. 4 shows an example of a representative blot.

GSNO decreases specific ligand binding to α₁-adrenergic receptors. Total and nonspecific HEAT saturation binding to α₁-adrenergic receptors was measured in rat lung membranes with and without GSNO treatment. Specific HEAT binding was determined as the difference between total and nonspecific binding (20–200 pM) in the presence of 10 μM phenolamine. GSNO (250 μM) decreased receptor-ligand binding (P < 0.01 by nonlinear regression analysis) (Fig. 5A). Bₘₐₓ and Kᵋd for
HEAT binding were determined from individual saturation binding curves of control (n = 9) and GSNO-treated (n = 8) membranes. α1-adrenergic receptor density (B_max, fmol/mg protein) was decreased in GSNO-treated membranes (200 ± 10 fmol/mg protein in control vs. 133 ± 10 fmol/mg protein in treated, P < 0.05), whereas affinity for the ligand (K_d, nM) was not altered (33 ± 6 PM in control vs. 24 ± 10 7 PM in treated, P > 0.05). An agonist competition curve with PE (10^-8–10^-4 M) indicated only the low-affinity binding state in lung membranes. GSNO-treated lungs demonstrated a small but definite increase in calculated K_i for HEAT binding (K_i = 6.1 × 10^-9 M in control membranes compared with K_i = 8.3 × 10^-9 M in GSNO-treated membranes, n = 3 each, P < 0.05) (Fig. 5B). The decrease in HEAT binding was similar after treatment with 2.5, 25, or 250 μM GSNO (n = 2, data not shown).

PA response to PE is mediated by multiple α1-adrenergic receptor subtypes. Rat PA expressed mRNA for all three subtypes of the α1-adrenergic receptor family, α1A, α1B, and α1D adrenergic receptor. mRNA expression was determined by RT-PCR using subtype-specific primers. The (-)RT controls showed no band, confirming that the signal did not represent genomic DNA (Fig. 6A). Two distinct subtype-selective antagonists showed concentration-dependent inhibition of the PE dose-response curve using five PE doses between 10^-8 and 10^-6 M. BMY-7378 (α1D-selective antagonist) and RS-100329 (α1A-selective antagonist) significantly decreased PE potency (n = 4 for each group, *P < 0.05 vs. control rings) (Fig. 6, B and C).

**DISCUSSION**

NO contributes to low pulmonary vascular tone by both cGMP-dependent and -independent mechanisms (17, 21, 24). Diverse effects of NO result from its direct reaction with a range of specific molecular targets, including transition metal centers and protein thiols (13). Thus NO may prevent vasoconstriction by chemically modifying vasoactive mediators, cell surface receptors, and/or the contractile elements of vascular smooth muscle. S-nitrosothiols are well-characterized NO donors present in plasma or airway fluid, and their concentrations are altered in several human diseases (11). In this study, we examined the effects of S-nitrosothiols on α1-adrenergic receptor agonist-induced vasoconstriction and α1-adrenergic receptor-ligand binding in pulmonary vessels and found novel evidence of cGMP-independent inhibition of α1-adrenergic receptor activity.

α1-adrenergic receptors contribute to pulmonary vascular regulation by mediating vasoconstriction via activation of G_protein linked to phospholipase C, which catalyzes the hydrolysis of polyphosphoinositide, liberating both diacylglycerol and inositol triphosphate. Three different α1-adrenergic receptor subtypes have been identified and cloned (α1A, α1B, and α1D), each with specific tissue distributions (8, 27). The lung expresses mRNA for all three α1-adrenergic receptor isoforms, and more than one α1-adrenergic receptor subtype mediates the contraction to PE in the pulmonary vasculature (14, 25). Our data confirm both the presence of all three α1-adrenergic receptor subtypes and the contribution of more than one α1-adrenergic receptor subtype in PE-induced constriction in rat PA.

In this study, PE-induced vasoconstriction in PA rings was inhibited by pretreatment with either of two S-nitrosothiols, GSNO and CSNO. This observation is consistent with earlier studies examining the role of NO in response to α1-adrenergic receptor agonists in different vascular beds. In rat PA rings, NOS inhibitors or removal of endothelium increased the constrictor response to PE, indicating that endogenous NO attenuates vasoconstriction by catecholamines (28). Furthermore, a nonselective NOS inhibitor, but not the relatively selective inhibitor of inducible NOS (NOS II) aminoguanidine, augmented PE constriction in PA rings, implicating other NOS isoforms (e.g., NOS III) as the source of NO responsible for this effect (26). Use of the NOS inhibitor, L-NAME, or removal of the endothelium also augmented α1-adrenergic vasoconstriction to NE in rabbit bronchial arteries (34). Pretreatment with S-nitrosylating agents including S-nitroso-N-acetylpeni-
cillamine and GSNO blocked vasoconstriction to 1-adrenergic receptor agonists rat aorta, similar to our findings with GSNO in rat PA (3, 4, 17); however, neither a role for cGMP nor DTT reversibility was determined in those studies.

To better define these effects, we examined whether inhibition of PE-induced vasoconstriction by S-nitrosothiol required cGMP generation. The guanylate cyclase antagonists LY-83583 and ODQ blocked cGMP production with GSNO but did not prevent GSNO-mediated inhibition of PE vasoconstriction. The PKG antagonist KT-5823, which blocks downstream effects of cGMP, produced similar results, further supporting a cGMP-independent mechanism of action by GSNO. The interpretation of the ODQ experiments is complicated by dose-dependent effects of ODQ on PE vasoconstriction without concomitant changes in cGMP content. In addition to its effects on guanylate cyclase, ODQ can alter vascular tone by inhibiting other heme-containing enzymes like NOS (10). This effect could account for the leftward shift in the PE response curve similar to that of inhibiting endogenous NO with l-NAME treatment. The decreased PE response with low-dose ODQ + GSNO-treated rings compared with ODQ alone indicates a cGMP-independent component of GSNO activity and is consistent with the results of experiments with LY-83583 and KT-5823. Moreover, the effects of S-nitrosothiol on both PE vasoconstriction in thoracic artery and 5-HT vasoconstriction in PA persist after inhibition of guanylate cyclase (17, 23), further indicating cGMP-independence of vasoreactivity of (S)NO. Thus, in addition to the established NO-mediated vasorelaxation via the cGMP/PKG pathway, we observed that GSNO, by inhibiting PE vasoconstriction, can maintain low vascular tone through a cGMP-independent mechanism.

Several important lines of evidence indicate that S-nitrosothiol modify PE responses through thiol nitrosylation of the 1-adrenergic receptor system. S-nitrosothiol, including GSNO and CSNO, can facilitate transnitrosylation of other protein thiols (13, 20). The strong physiological effect of GSNO is not duplicated by the corresponding reduced thiol, GSH or its oxidized form, GSSG, indicating that the effect was specific to the S-nitrosothiol. The inhibitory effects of GSNO on PE vasoconstriction were fully reversed by the thiol reductant DTT, which can reverse disulfides, S-thiolation, and S-nitrosylation. The detection of S-nitrosylated 1-adrenergic receptor after S-nitrosothiol treatment supports the conclusion that the cysteine modification is S-nitrosylation. That GSNO-mediated S-nitrosylation was facilitated by reduced cysteine supports the idea that transnitrosylation is important for NO modification of proteins, and the ability of CSNO to enter the cell via specific amino acid transporters enhances its ability to react intracellularly (20). It is unlikely that GSNO S-thiolsates directly or leads to thiol oxidation since GSH, GSSG, or H2O2 did not affect vascular responses. The reactive nitrogen species peroxynitrite also promotes S-glutathionylation of the sarco/endoplasmic reticulum calcium ATPase in the presence of GSH to mediate aortic relaxation (1). Recent data also indicate that glutathione sulfone, a GSNO decomposition product, rather than GSNO, can promote mixed disulfide formation (29). We did not exclude the possibility that these other cysteine modifications may also occur in the pulmonary circulation (11, 16).

We have shown 1-adrenergic receptor S-nitrosylation but not yet the specific S-nitrosylation sites responsible for the S-nitrosylation effect because rat 1-adrenergic receptors contain as many as 14 cysteine residues that are potential targets of S-nitrosylation (27). Several cysteines are highly conserved in the 1-adrenergic receptor and are located in extracellular and intracellular regions of the 1-adrenergic receptor responsible for receptor-ligand binding and receptor activation (National Center for Biotechnology Information Entrez Protein, ClustalW Multiple Sequence Alignment).

S-nitrosylation covalently modifies cysteine residues to regulate function of many proteins including hemoglobin, caspase-3, GAPDH, calcium release channel/ryanodine receptor, and the N-methyl-d-aspartate receptor (13). Aortic rings exposed to S-nitrosylating agents including GSNO, but not DEA-NO, contain a higher NO content and increased cysteine-NO immunostaining in association with sustained effects on ring tension (3). A monomeric G protein, p21ras, changes both protein conformation and activity when S-nitrosylated (19). S-nitrosylation can be reversed to restore enzyme activity of caspase-3 and tissue transglutaminase (18). The ability to regulate vascular tone through S-nitrosylation and denitrosylation provides further evidence of an important physiological function for this NO mechanism. Whereas other posttranslational protein modifications including phosphorylation are well-accepted mechanisms regulating GPCRs, the potential role of S-nitrosylation in the regulation of GPCR signaling has not been established.

We further tested the effects of GSNO on 1-adrenergic receptor by comparing 125I-HEAT saturation binding isotherms in lung membranes in the presence and absence of GSNO. GSNO treatment decreased total 1-adrenergic receptor binding sites (Bmax) in the lung, while it had no significant effect on binding affinity (KD). This is the first direct evidence that nitrosothiol alters 1-adrenergic receptor binding. The ready explanation for the decrease in 1-adrenergic receptor binding sites with GSNO is that ligand binding is disrupted by NO modification of the receptor. Binding sites can also be decreased if the receptor is internalized or downregulated, but this is unlikely to occur after the relatively short 30-min incubation period with GSNO. It is also unlikely that GSNO treatment damaged the receptor sufficiently to disrupt binding sites. The effects of GSNO were reversed by DTT, restoring constrictor effects of PE and indicating receptor integrity after GSNO treatment. The competition curve with PE did not reveal the high-affinity state of the receptor, which was likely due to experimental limitations of using actual lung tissue. Therefore, it was not possible to determine the effects of GSNO on G protein coupling in this system. Cell culture studies have demonstrated effects of NO on G protein uncoupling from other receptors. For example, SIN-1, which releases NO and superoxide simultaneously, promoted uncoupling of Gi from 2-adrenergic receptor expressed in HEK-293 cells. (2). S-nitrosothiols also decrease coupling of M2 muscarinic, bradykinin, and AT1 GPCR to their respective G proteins (5, 7) (22). NO does have other effects on GPCR signaling, including desensitization. NO inhibition for example, promotes tachyphylaxis to the vasodilator effects of the β-adrenergic receptor agonist, isoproterenol, and pituitary adenylate cyclase activating polypeptide receptor agonist, PACAP-27 (30, 32, 33).

In conclusion, GSNO inhibits PE vasoconstrictor response in PA rings through a reversible cGMP-independent mechanism that decreases 1-adrenergic receptor binding sites. This
represents a novel mechanism of NO regulation of pulmonary vascular tone in which S-nitrosylation of the α1-adrenergic receptor decreases the vasoconstrictor response to agonists. Future studies will locate specific molecular target(s) of NO within the α1-adrenergic receptor-G protein complex and determine the associations with altered SNO levels and vascular disease.

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