Chronic hypoxia attenuates cGMP-dependent pulmonary vasodilation

NIKKI L. JERNIGAN AND THOMAS C. RESTA
Vascular Physiology Group, Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131-5218

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Address for reprint requests and other correspondence: N. L. Jernigan, Dept. of Cell Biology and Physiology, Univ. of New Mexico Health Sciences Center, 915 Camino de Salud NE, Albuquerque, NM 87131-5218 (E-mail: njernigan@salud.unm.edu).

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Chronic hypoxia attenuates cGMP-dependent pulmonary vasodilation.

The corresponding functional alterations are responsive-ness to vasoactive factors. Studies from our laboratory (23, 24, 26) and others (12, 20, 22, 28, 29) have shown that CH augments pulmonary vasodilatory responses to endothelium-derived nitric oxide (EDNO)-dependent vasodilators. EDNO is synthesized in the vasculature by endothelial nitric oxide synthase (eNOS), and several studies have demonstrated that CH is associated with increased pulmonary eNOS levels, gene expression, and activity (12, 15, 20, 23, 24, 32, 39). Consistent with elevated eNOS levels, nitric oxide (NO) synthesis appears to be greater in lungs isolated from CH rats compared with normoxic controls (12, 20, 30, 36). Despite enhanced reactivity to EDNO-dependent vasodilators, some studies have demonstrated impaired responsiveness to exogenous NO following CH (7, 19, 24, 27, 38). However, it is unclear whether this attenuated reactivity results from acute effects of increased endogenous NO production or rather from decreased sensitivity of vascular smooth muscle (VSM) to NO. The present study investigates downstream effectors in the NO-dependent signal transduction pathway to determine the mechanism of decreased NO sensitivity in the pulmonary vasculature following CH.

Although studies suggest NO can act through cGMP-independent mechanisms (4), the most prominent target in VSM for NO is soluble guanylyl cyclase (sGC), where binding to the heme moiety activates the enzyme, increasing intracellular cGMP. Levels of cGMP are further regulated by the rate of degradation by cGMP-specific phosphodiesterase type 5 (PDE5), which plays a significant role in modulating pulmonary VSM tone (5, 6, 8, 13, 21, 40). Once formed, cGMP can mediate VSM relaxation through several mechanisms involving a decrease in intracellular calcium and/or decreased sensitivity of the contractile apparatus to calcium (17). Therefore, we hypothesized that attenuated NO-dependent pulmonary vasodilation following CH is mediated by downregulation of VSM sGC expression and/or activity, increased cGMP degradation by PDE5, or decreased sensitivity of VSM to cGMP. To study this, we examined vasodilatory responses to both NO donors and dissolved NO solutions in lungs iso-

CHRONIC EXPOSURE TO HYPOXIA results in structural as well as functional alterations in the pulmonary vasculature, leading to the development of pulmonary hypertension and right ventricular hypertrophy. The principal mediators of chronic hypoxia (CH)-induced pulmonary hypertension are polycythemia, pulmonary arterial remodeling, and arterial constriction. Among
lated from normoxic and CH rats. The NOS inhibitor N\textsuperscript{-}nitro-L-arginine (L-NNA) was used in some experiments to eliminate any acute influence of endogenously produced NO. Additional lungs from each group were isolated to determine effects of sGC inhibition and PDE5 inhibition on NO-dependent vasodilation. Expression and activity of pulmonary sGC were assessed by Western blot analysis and a cGMP radioimmunoassay (RIA), respectively. Furthermore, we assessed effects of CH on pulmonary VSM sensitivity to the membrane-permeable cGMP analog 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP).

**METHODS**

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM).

**Experimental Groups**

Female (200–250 g) Sprague-Dawley rats (Harlan Industries) were divided into two groups for each experiment. Animals designated for exposure to CH were housed in an hypobaric chamber with barometric pressure maintained at ~380 mmHg for 4 wk. The chamber was opened three times per week to provide animals with fresh food, water, and clean bedding. On the day of experimentation, rats were removed from the hypobaric chamber and immediately placed in a Plexiglas chamber continuously flushed with a 12% O\textsubscript{2}-88% N\textsubscript{2} gas mixture to reproduce inspired P O\textsubscript{2} (~70 mmHg) within the hypobaric chamber. Age-matched control animals were housed at ambient barometric pressure (~630 mmHg). All animals were maintained on a 12:12-h light-dark cycle.

**CH-Induced Right Ventricular Hypertrophy and Polycythemia**

Blood samples were obtained by direct cardiac puncture at the time of lung isolation for measurement of hematocrit. Right ventricular hypertrophy was assessed as an index of CH-induced pulmonary hypertension, as previously described (23, 24). Briefly, after isolation of the heart, the atria and major vessels were removed from the ventricles. The right ventricle (RV) was dissected from the left ventricle and septum, and each was weighed. The degree of right ventricular hypertrophy is expressed as the ratio of RV to total ventricle weight (T).

**Isolated Lung Preparation**

Rats from each group were anesthetized with pentobarbital sodium (52 mg ip). After the trachea was cannulated with a 17-gauge needle stub, the lungs were ventilated with a Harvard positive-pressure rodent ventilator (model 680) at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with a warmed and humidified gas mixture (6% CO\textsubscript{2} in room air). Inspiratory pressure was set at 9 cmH\textsubscript{2}O, and positive end-expiratory pressure was set at 3 cmH\textsubscript{2}O. After a median sternotomy, heparin (100 units) was injected directly into the RV, and the pulmonary artery was cannulated with a 13-gauge needle stub. The preparation was immediately perfused with a physiological saline solution (in mM: 129.8 NaCl, 5.4 KC\textsubscript{1}, 0.83 MgSO\textsubscript{4}, 19 NaHCO\textsubscript{3}, 1.8 CaCl\textsubscript{2}, and 5.5 glucose; all from Sigma) containing 4% (wt/vol) albumin (Sigma), meclofenamate (30 \mu M; Sigma), and L-NNA (300 \mu M; Sigma) at 0.8 ml/min with a Masterflex micropump drive (model 7524-10). Meclofenamate and L-NNA were added to acutely minimize the potential complicating influences of endogenous prostaglandins and NO on vascular reactivity. This dose of meclofenamate is approximately threefold higher than that previously shown to provide effective inhibition of prostaglandin synthesis in this preparation (9). We have previously demonstrated that the dose of L-NNA employed is effective in inhibiting EDNO-dependent pulmonary vasodilation in the isolated perfused rat lung (24, 26). Additional experiments were performed in the absence of L-NNA where noted.

The LV was cannulated with a plastic tube (4-mm outer diameter), and the heart and lungs were removed en bloc and suspended in a humidified chamber maintained at 38°C. The perfusion rate was gradually increased to 30 ml/min kg\textsuperscript{-1} body weight and maintained at this rate for the duration of the experiment. Twenty milliliters of perfusate were washed through the lungs and discarded before recirculation was initiated with 40 ml. Experiments were performed with lungs in zone 3 conditions, achieved by elevating the perfusate reservoir until pulmonary venous pressure (P\textsubscript{v}) was 3–4 mmHg. Pulmonary arterial pressure (P\textsubscript{a}) and P\textsubscript{v} were measured with Spectramed P23XL pressure transducers and recorded on a Gould RS 3400 chart recorder. Data were stored and processed with a computer-based data acquisition/analysis system (AT-CODAS, Dataga Instruments).

After a 30-min stabilization period, the thromboxane analog 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F\textsubscript{2α} (U-46619, Cayman Chemical) was added to the perfusate reservoir until a stable arterial pressor response of ~10 mmHg was achieved. U-46619 provides consistent and stable pressor responses in this preparation (23, 24, 26), allowing assessment of subsequent vasodilatory responses as outlined in the following protocols. A double occlusion technique was employed for all protocols to allow calculation of segmental vascular resistances as described previously (23–26).

**Isolated Lung Experiments**

**Vasodilatory responses to NO donors.** To examine the effect of CH on NO-dependent pulmonary vasodilation, we assessed responses to two mechanistically independent NO donors, S-nitroso-N-acetylpenicillamine (SNAP) and spermine NONOate. After attainment of a stable vasoconstrictor response to U-46619, a cumulative dose-response relationship to SNAP (1 and 10 \mu M; Sigma) was assessed in lungs from control and CH rats in the presence or absence of L-NNA. A stable vasodilatory response to the first dose of SNAP was allowed to develop before administration of the second dose. A similar dose-response relationship was generated for spermine NONOate (0.1 and 1 \mu M; Cayman Chemical) in separate sets of lungs from each group. The concentrations of each NO donor were determined to provide dose-dependent vasodilation in preliminary experiments. Parallel experiments were performed with N-acetylpenicillamine (1 and 10 \mu M; Sigma) and spermine NONOate (0.1 and 1 \mu M; Sigma) to test for possible nonspecific actions of SNAP and spermine NONOate, respectively.

**Vasodilatory responses to pinacidil.** To determine whether decreased responsiveness to NO following CH represents an attenuated reactivity to vasodilators in general, we examined responses to the ATP-sensitive K\textsuperscript{+} channel (K\textsubscript{ATP}) activator pinacidil. A cumulative dose-response relationship to pinacidil (10 and 100 \mu M; Sigma) was assessed in U-46619-constricted lungs from both groups. A stable vasodilatory response to the first dose of pinacidil was allowed to develop before administration of the second dose.
Effects of sGC inhibition on NO-mediated vasodilation. The contribution of endogenous cGMP in mediating vasodilatory responses to NO donors in each group of rats was assessed using a heme site-specific sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ; Sigma). ODQ (50 μM) or its vehicle (DMSO, 75 μl) was added to the recirculating reservoir (40 ml) immediately after lung isolation and was present throughout the experiment. This dose of ODQ has been previously employed by other investigators to inhibit sGC in this preparation (3). Responses to SNAP (1 and 10 μM) and spermine NONOate (1 μM) were then determined as described above. To demonstrate specificity of ODQ, we assessed vasodilatory responses to the β-adrenergic receptor agonist isoproterenol (10 nM) in the presence or absence of ODQ (50 μM) in separate sets of lungs from control animals.

Effect of PDE5 inhibition on NO-dependent vasodilation. cGMP is rapidly hydrolyzed to GMP by the PDE5 family of PDEs in pulmonary VSM (6, 18). To assess the possible role of increased PDE5 activity in the attenuated NO-dependent vasodilation following CH, we examined responses to SNAP (0.1, 0.5, and 1 μM) in lungs from each group after treatment with the selective PDE5 inhibitor, dipyridamole (10 μM), or its vehicle [ethanol (EtOH); 100 μl]. This dose of dipyridamole was determined to augment SNAP-induced pulmonary vasodilation in preliminary experiments.

Additional experiments examined effects of PDE5 inhibition on the amplitude and duration of vasodilatory responses to bolus administration of NO-containing solutions in lungs from each group of rats. After treatment with dipyridamole (10 μM) or vehicle (EtOH, 100 μl), lungs were administered successive arterial boluses of NO solution (100 μl of 10^4-, 10^5-, and 10^6-fold dilutions from a saturated stock solution). Vasodilatory responses to NO solutions were transient, and therefore full recovery of each response was allowed before administration of subsequent doses. Parallel experiments were conducted using the more selective PDE5 inhibitor, T-1032 (1 μM; Sigma) (14), or its vehicle (methanol, 100 μl). Saturated NO solution was diluted by first deoxygenating double distilled water (ddH2O) by bubbling with 100% N2 for 20 min. The deoxygenated ddH2O was then bubbled with 100% NO gas (Matheson Gas Products) for 20 min at 0°C. The saturated NO solution was diluted to working concentrations with deoxygenated ddH2O in airtight Vacutainers.

Vasodilatory responses to 8-Br-cGMP. Because attenuated NO-dependent pulmonary vasodilation following CH could potentially result from altered VSM reactivity to cGMP, additional experiments examined responses to the membrane-permeable cGMP analog 8-Br-cGMP (1 and 10 μM; Sigma) in U-46619-constricted lungs from each group in the presence or absence of 1-NNa. 8-Br-cGMP produced slowly developing and progressive vasodilatory responses in lungs from control rats; therefore, assessments were made 20 min after administration. In contrast, responses to 1 μM 8-Br-cGMP in lungs from CH rats were minimal and stable during this period. Consequently, responses to each dose were assessed in separate lungs from each group of rats. Although 8-Br-cGMP is reportedly a PDE-resistant molecule (41), we further assessed vasodilatory responses to 1 μM 8-Br-cGMP in the presence of dipyridamole or its vehicle in each group of animals to confirm PDE resistance in this preparation.

cGMP RIA

Whole lung cGMP levels were assessed to examine whether attenuated responsiveness to NO following CH is associated with decreased cGMP synthesis. Lungs from normoxic and CH rats were isolated and prepared for experimentation as described above and treated with dipyridamole (10 μM). Lungs were quickly frozen in liquid N2 at maximal dilution to a 10^4-fold dilution of NO stock solution. A separate set of normoxic control lungs was treated with ODQ (50 μM) before administration of NO. Lungs were coarsely ground with a mortar and pestle. Acid extract homogenization buffer (0.1 N HCl, 450 μM IBMX) was added to each tissue sample and allowed to incubate for 1 h at room temperature. Tissue samples were homogenized with a Dounce homogenizer and centrifuged at 6,000 g for 10 min. Supernatant was collected and lyophilized overnight. Whole lung cGMP content was assessed using a standard RIA kit (Amersham) and expressed as femtomoles per milligram of lung tissue.

sGC Western Blots

The enzyme sGC is a heterodimer consisting of α- and β-subunits (17). Western blots were performed to determine the level of sGC-α protein in the lung from control and CH rats. Lungs were snap-frozen in liquid N2 at the completion of isolated lung experiments and homogenized in 10 mM Tris-HCl homogenization buffer containing 255 mM sucrose, 2 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, 0.3 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 10,000 g for 10 min at 4°C to remove cellular debris. The supernatant was collected, and sample protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay). Control experiments were conducted using different concentrations of protein to ensure linearity of the densitometry curve. A molecular weight standard (Bio-Rad) was added to each gel, and the optimal concentration of sample protein (25 μg/lane) was separated by SDS-PAGE (7.5% Tris-HCl gels, Bio-Rad) and transferred to polyvinylidene difluoride membranes. Blots were blocked overnight at 4°C with 5% nonfat milk, 3% bovine serum albumin, 5% goat serum, 5% rabbit serum, and 0.05% Tween 20 (Bio-Rad) in Tris-buffered saline containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.5). The blots were then incubated for 1 h at room temperature with a rabbit polyclonal antibody for sGC (1:500; Cayman Chemical). Supplementary gels were run using a sGC-α blocking peptide (Cayman Chemical) incubated simultaneously with the primary antibody to confirm band identity. For immunochromelabeling, blots were incubated for 1 h at room temperature with goat anti-rabbit IgG horseradish peroxidase (1:5,000; Stressgen). After chemiluminescence labeling (Amersham), sGC bands were detected by exposing the blots to chemiluminescence-sensitive film (Kodak). Membranes were stained with Coomassie brilliant blue to confirm equal protein loading in all lanes. Quantification of the bands was accomplished by densitometric analysis of scanned images (Sigma-Gel software, SPSS). All reagents were purchased from Sigma unless otherwise noted.

Calculations and Statistics

Pulmonary vascular resistance in isolated, perfused lungs was calculated as the difference between Pp and Ph divided by flow (30 ml·min^−1·kg^-1·body wt). Vasodilatory responses were calculated as percent reversal of U-46619-induced vasconstriction. All data are expressed as means ± SE, and values of n refer to the number of animals in each group. Where appropriate, a t-test or two-way ANOVA was used to make comparisons. If differences were detected by ANOVA, individual groups were compared with the Student-Newman-Keuls test. A probability of P ≤ 0.05 was accepted as significant for all comparisons.
**RESULTS**

**CH-Induced RV Hypertrophy and Polycythemia**

RV:T ratios were greater in CH rats \( n = 88, 0.312 \pm 0.003 \) compared with normoxic control rats \( n = 94, 0.209 \pm 0.002 \), thus demonstrating RV hypertrophy indicative of pulmonary hypertension. Furthermore, CH rats exhibited polycythemia as indicated by a significantly greater hematocrit in CH rats \( n = 87, 61 \pm 0\)% compared with normoxic rats \( n = 98, 43 \pm 0\)%. 

**Isolated Lung Experiments**

Baseline vascular resistances and responses to U-46619. Consistent with previous reports \( 23, 24, 26 \), baseline vascular resistances were significantly greater in lungs from CH \( n = 62, 0.112 \pm 0.003 \) mmHg·ml\(^{-1}\)·min\(^{-1}\)·kg\(^{-1}\) rats compared with normoxic controls \( n = 68, 0.071 \pm 0.002 \) mmHg·ml\(^{-1}\)·min\(^{-1}\)·kg\(^{-1}\). Because the pulmonary circulation exhibits no detectable tone in this preparation \( 25 \), these data provide functional evidence for CH-induced vascular remodeling. U-46619 produced similar increases in resistance between CH \( 0.331 \pm 0.009 \) mmHg·ml\(^{-1}\)·min\(^{-1}\)·kg\(^{-1}\) and normoxic \( 0.356 \pm 0.012 \) mmHg·ml\(^{-1}\)·min\(^{-1}\)·kg\(^{-1}\) groups. Furthermore, we observed no differences in the concentration of U-46619 required to elicit comparable vasoconstriction between CH \( 124 \pm 7 \) nM and normoxic \( 110 \pm 27 \) nM groups.

**Vasodilatory responses to NO donors.** Vasodilatory responses to both 1 and 10 μM SNAP were attenuated in lungs from CH rats (Fig. 1A) compared with the normoxic control group. In addition, vasodilatory responses were reduced at both doses of spermine NONOate \( 1 \) and \( 10 \) μM after CH (Fig. 1B). N-acetylpenicillamine \( n = 2, 1 \) and \( 10 \) μM and spermine \( n = 2, 0.1 \) and \( 1 \) μM exhibited no apparent vasoactive properties in lungs from normoxic rats (data not shown). In the absence of L-NNA, vasodilatory responses to 1 μM SNAP were similarly attenuated in lungs from CH rats (Table 1). Because vasodilatory responses were similar for arterial and venous segments of the pulmonary vasculature as assessed by the double occlusion method, segmental responses are not shown for these and all subsequent protocols.

**Vasodilatory responses to pinacidil.** In contrast to NO-dependent responses, CH lungs demonstrated augmented vasodilatory responses to the K\(_{ATP}\) channel activator, pinacidil, compared with lungs from normoxic rats (Fig. 2).

**Effects of sGC inhibition on NO-mediated vasodilation.** The sGC inhibitor ODQ effectively blocked vasodilatory responses to SNAP (1 and 10 μM, Fig. 3A) and spermine NONOate (1 μM, Fig. 3B) in lungs from both CH and normoxic rats. There was no effect of ODQ on vasodilatory responses to the cGMP-dependent dilator isoproterenol (DMSO vehicle: 83.97 ± 1.82%, \( n = 4 \); ODQ: 78.52 ± 3.77%, \( n = 4 \)). Together, these data demonstrate the specificity of ODQ for sGC and suggest NO-dependent vasodilation is mediated through cGMP in this preparation.

**Effects of PDE5 inhibition on NO-dependent vasodilation.** The selective PDE5 inhibitor dipyridamole significantly augmented vasodilatory responses to SNAP (0.5 and 1 μM) in lungs from CH rats (Fig. 4). In contrast, dipyridamole augmented reactivity to SNAP in lungs from control animals only at the 1-μM concentration. Although decreased responsiveness to 1 μM SNAP persisted in lungs from CH vs. normoxic rats after treatment with dipyridamole, reactivity to 0.5 μM SNAP was significantly enhanced in lungs from CH rats.

**Table 1. Vasodilatory responses to SNAP and 8-Br-cGMP in lungs from normoxic and CH rats without pretreatment with L-NNA**

<table>
<thead>
<tr>
<th></th>
<th>% Vasodilatory Response</th>
<th>8-Br-cGMP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>Normoxic</td>
<td>30.6 ± 6.1</td>
<td>87.3 ± 2.9</td>
</tr>
<tr>
<td>CH</td>
<td>1.9 ± 7.2*</td>
<td>70.2 ± 11.6</td>
</tr>
</tbody>
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Values are expressed as means ± SE. *P < 0.05 vs. normoxic rats. SNAP, S-nitroso-N-acetylpenicillamine; 8-Br-cGMP, 8-bromo-guanosine 3',5'-cyclic monophosphate; L-NNA, N^o^-nitro-l-arginine; CH, chronic hypoxia.
SNAP was not different between groups after PDE5 inhibition. No significant differences in responses to 0.1 μM SNAP were observed between groups (Fig. 4).

Similar to effects of PDE5 inhibition on SNAP-induced vasodilation, dipyridamole significantly augmented vasodilatory responses to bolus NO injections (10^{-8} and 10^{-4}-fold dilutions) in lungs from CH rats (Fig. 5A). However, impaired reactivity to NO was maintained in lungs from CH vs. control rats in the presence of dipyridamole. In contrast, dipyridamole had no effect on the amplitude of vasodilatory responses to bolus NO injections in lungs from normoxic rats (Fig. 5A). Dipyridamole modestly increased the duration of the vasodilatory response in lungs from normoxic rats, although significance was achieved only at the lower doses of NO (10^{-8} and 10^{-4}-fold dilutions; Fig. 5B). However, the duration of NO-mediated responses was markedly increased by dipyridamole in lungs from CH rats. Similarly, T-1032 augmented the amplitude and duration of vasodilatory responses to bolus NO injections in lungs from CH rats (Fig. 6, A and B). The amplitude of the vasodilatory response was significantly greater only at the lowest NO dose (10^{-8}-fold dilution; Fig. 6A); however, the duration was augmented by T-1032 at each NO dose in CH lungs (Fig. 6B). The amplitude of vasodilatory responses to bolus NO injections in lungs from normoxic rats was significantly increased by T-1032 at the higher doses of NO (10^{-4} and 10^{-5}-fold dilutions; Fig. 6A); however, there were no significant differences in the duration of responses between T-1032 and vehicle groups (Fig. 6B).

Vasodilatory responses to 8-Br-cGMP. Similar to NO-dependent reactivity, lungs from CH rats exhibited significantly smaller vasodilatory responses to 1 μM 8-Br-cGMP compared with those of normoxic rats in the presence (Fig. 7A) or absence of L-NNA (Table 1). However, no differences in reactivity were observed between groups at the 10 μM dose of 8-Br-cGMP (Fig. 7A and Table 1). Dipyridamole had no effect on vasodilatory responses to 8-Br-cGMP in lungs from either group (Fig. 7B).

cGMP RIA

There were no differences in cGMP levels in response to a 10^{-4}-fold dilution of NO stock solution between
lungs from CH and normoxic rats in the presence of dipyridamole. However, lungs from normoxic rats treated with ODQ demonstrated significantly lower levels of cGMP compared with normoxic lungs pretreated with dipyridamole (Fig. 8A).

**sGC Western Blot**

Immunoreactive sGC-α was detected as a single band of ~80 kDa in lungs from normoxic and CH rats (Fig. 8B) that was eliminated by sGC-α blocking peptide. Although there was a tendency for sGC-α protein levels to be greater in lungs from CH rats (Fig. 8C), this did not reach statistical significance (P = 0.051).

**DISCUSSION**

The present study examined potential mechanisms by which CH attenuates NO-mediated pulmonary vasodilation. The major findings from this study are: 1) CH attenuates pulmonary vasodilation to exogenous NO; 2) vasodilatory responses to the K_{ATP} channel agonist pinacidil are augmented after CH; 3) pulmonary vasodilatory responses to NO are cGMP dependent; 4) although PDE5 inhibition produced a greater potentiation of NO-mediated vasodilation in lungs from CH vs. normoxic rats, CH-induced attenuation of NO-dependent responsiveness persisted after PDE5 inhibition; 5) decreased pulmonary vasoreactivity to NO after CH does not appear to be associated with reduced sGC expression or activity; and 6) CH similarly inhibited 8-Br-cGMP-mediated pulmonary vasodilation. These data suggest the attenuated pulmonary vasodilatory response to NO after CH is mediated by both increased PDE5 activity and decreased VSM sensitivity to cGMP.

Previous studies from our laboratory (23, 24, 26) and others (12, 20, 22, 28, 29) suggest that CH augments EDNO-dependent pulmonary vasodilation, a response associated with upregulation of pulmonary eNOS mRNA and protein levels (12, 15, 20, 23, 24, 32, 39) as well as increased synthesis of NO (12, 20, 36). Despite this enhanced reactivity to EDNO-mediated agonists, we have recently reported that CH attenuates vasodilatory responsiveness to exogenous NO in lungs isolated from female rats (24). However, considering this impaired responsiveness to NO after CH could potentially be explained by acute effects of increased endogenous NO synthesis and thus greater basal activity of NO-associated signal transduction pathways in VSM, the present study examined responses to both NO donors and authentic NO after nitric oxide synthase.
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CH-induced inhibition of NO-dependent vasodilation in lungs from ovarian-intact and ovariectomized rats (24). Furthermore, unpublished observations from our laboratory have demonstrated a similar inhibition of reactivity to both 1 and 10 μM SNAP after NOS inhibition in lungs from CH vs. normoxic male rats (n = 6/group), further suggesting this response to CH is not gender specific. In contrast to these effects of CH on NO-mediated responses, we observed augmented vasodilation to the K<sub>ATP</sub> channel agonist pinacidil after hypoxic exposure (Fig. 2), as previously reported in isolated pulmonary arteries from male rats (27, 38). The mechanism of this enhanced responsiveness to pinacidil after CH is not clear but may be secondary to CH-induced pulmonary VSM membrane depolarization (33, 34, 35) and, therefore, greater inhibition of calcium influx through voltage-dependent calcium

(NOS) inhibition with L-NNA in lungs isolated from normoxic and CH female rats. We found that CH-induced attenuation of reactivity to both SNAP and spermine NONOate (Fig. 1) as well as authentic NO (Figs. 5 and 6) persisted after NOS inhibition, suggesting this change in reactivity to NO after CH represents decreased VSM sensitivity to NO. However, the mechanisms responsible for this effect of CH on NO-dependent pulmonary vasoreactivity are not presently understood.

Consistent with our present findings are reports of impaired NO-dependent vasorelaxation in isolated conduit pulmonary arteries from CH male rats compared with normoxic controls (7, 19, 27, 38). However, these results are at odds with previously published observations from our laboratory and others demonstrating no significant effect of CH on vasodilatory responsiveness to NO-donors (26) or inhaled NO (25, 8) in isolated lungs from male rats. Furthermore, Isaacson et al. (12) reported that CH enhanced vasodilatory responses to arterial boluses of saturated NO solution in isolated lungs. The reasons for these discrepancies are not presently clear but could be consequences of different preparations employed, a gender difference, and/or the presence of NOS inhibition in the current study. However, this effect of CH appears to be independent of ovarian hormones, as evidenced by similar
channels after pinacidil-induced membrane hyperpolarization. Nonetheless, these data support the view that attenuated NO-dependent responsiveness after CH is not associated with a generalized decrease in reactivity to vasodilators but may be specific to the NO pathway.

Although NO appears to mediate VSM relaxation, at least in part, through cGMP-independent mechanisms in some vascular preparations (4), the primary target of NO in the pulmonary circulation is sGC (4, 31). Activation of sGC leads to cGMP synthesis, which results in VSM relaxation through various mechanisms involving a decrease in intracellular calcium and desensitization of the contractile apparatus to calcium (17). Consistent with a primary role of sGC activity in mediating NO-dependent pulmonary vasodilation, we found that the heme site-specific sGC inhibitor ODQ abolished vasodilatory responses to SNAP and spermine NONOate in lungs from both normoxic and CH rats (Fig. 3). However, ODQ did not alter vasodilatory responses to the β-adrenergic agonist isoproterenol, suggesting ODQ is selectively blocking sGC and not cAMP-dependent vasodilatory pathways.

cGMP hydrolysis is regulated primarily by cGMP-specific PDEs (PDE5) in pulmonary VSM (5, 6, 18). In addition, selective PDE5 inhibitors are potent vasodilators in the hypertensive pulmonary circulation, suggesting an important role for PDE5 in regulation of pulmonary vascular tone (5, 6, 8, 13, 21, 40). Interestingly, PDE5 expression (2) and activity (2, 18, 21) appear to be increased after pulmonary hypertension. Considering this, we hypothesized that increased PDE5 activity is mediating the impaired vasodilation to NO donors in lungs from CH rats. Although the selective PDE5 inhibitors dipyridamole and T-1032 largely augmented vasodilatory responses to SNAP and arterial boluses of NO solutions in lungs from CH rats, these inhibitors appeared to have minimal effects on NO-dependent responses in lungs from normoxic rats. Although it is possible that other PDE isofoms contribute to cGMP hydrolysis and diminished vasodilation to NO after CH, these results nevertheless suggest increased PDE5 activity is, at least in part, responsible for this effect of CH. However, the maintained attenuation of reactivity to NO in CH lungs after PDE5 inhibition (Figs. 4–6) suggests that other mechanisms may be involved, including decreased sGC expression/activity or reduced VSM sensitivity to cGMP.

Considering that impaired reactivity to NO after CH could result from decreased pulmonary sGC expression, we further examined relative expression levels of sGC-α in whole lung homogenates from each group of rats. However, Western blot analyses revealed a tendency for upregulation of sGC-α after CH, although this did not reach statistical significance (Fig. 8, B and C, P = 0.051). These findings are consistent with recent studies demonstrating elevated sGC expression and activity in pulmonary hypertensive lambs (2) as well as increases in lung sGC protein, mRNA levels, and increased sGC activity in whole lung tissue from male rats after 21 days of hypoxic exposure (16). Furthermore, this change in sGC expression appeared to be localized primarily to small pulmonary arteries as assessed by immunohistochemistry and in situ hybridization (16). In the current study, we also did not see differences in NO-stimulated cGMP levels between lungs from CH and normoxic rats after PDE5 inhibition (Fig. 8A), suggesting that the maintained attenuation of NO-mediated dilation after PDE5 inhibition in CH lungs is a function of decreased VSM reactivity to cGMP, as opposed to reduced cGMP synthesis.

Finally, we tested the hypothesis that a decrease in pulmonary VSM sensitivity to cGMP mediates CH-induced attenuation of NO-dependent reactivity. Consistent with this hypothesis, we observed decreased vasodilation to the relatively stable and PDE-resistant cGMP analog 8-Br-cGMP in lungs from CH rats compared with lungs from normoxic rats (Fig. 7A and Table 1). Dipyridamole had no effect on vasodilatory responses to 1 μM 8-Br-cGMP in lungs from normoxic and CH rats, suggesting that 8-Br-cGMP is PDE5 resistant in our preparation (Fig. 7B). These data are in agreement with other studies, which have shown an inhibitory effect of CH on responsiveness to 8-Br-cGMP in rat main pulmonary arteries and isolated lungs from neonatal pigs (1, 27). These findings suggest that the reduction in vasodilatory responsiveness to NO after CH involves a decrease in VSM sensitivity to cGMP.

CH could desensitize pulmonary VSM to cGMP through several mechanisms, including changes in the activity of downstream targets of cGMP. The most well recognized of these is protein kinase G, which mediates VSM relaxation through activation of multiple Ca2+-lowering mechanisms as well as Ca2+-desensitization of VSM (17). Other potential targets include cGMP-inhibitable cAMP PDEs (PDE3) (10, 37) and protein kinase A (PKA) (11, 31), although the relative contribution of the cAMP/PKA pathway to NO-dependent pulmonary responses is not well understood. Alternatively, impaired NO-mediated responsiveness after CH could be due to altered Ca2+ sequestration, influx or eflux mechanisms, or a change in sensitivity of the contractile machinery to Ca2+ (17). However, any such changes are likely specific to the NO/cGMP pathway, considering that CH augmented pulmonary vasodilation to the cGMP-independent agonist pinacidil (Fig. 2).

In summary, we have investigated the possibility that alterations in cGMP formation/degradation or reactivity to cGMP influence the attenuated NO-dependent pulmonary vasodilatory response observed after CH. Our findings suggest that whereas NO-dependent dilation is cGMP mediated, altered reactivity to NO after CH is not likely a function of decreased pulmonary sGC expression or activity. Rather, this effect of CH appears to result from increased cGMP degradation by PDE5 and decreased pulmonary VSM sensitivity to cGMP. These findings could have important clinical implications with respect to pulmonary vasoreactivity in patients with hypoxic pulmonary hypertension secondary to chronic lung disease. Further re-
search is necessary to determine whether diminished pulmonary vasodilation to NO after CH is mediated by changes in activity/ expression of intracellular cGMP targets.

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T. C. Resta is a Parker B. Francis Fellow in Pulmonary Research.

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