Contribution of oxygen radicals to altered NO-dependent pulmonary vasodilation in acute and chronic hypoxia

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Jernigan, Nikki L., Thomas C. Resta, and Benjimen R. Walker. Contribution of oxygen radicals to altered NO-dependent pulmonary vasodilation in acute and chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 286: L947–L955, 2004. First published December 12, 2003; 10.1152/ajplung.00215.2003.—Chronic hypoxia (CH) increases pulmonary arterial endothelial nitric oxide (NO) synthase (NOS) expression and augments endothelium-derived nitric oxide (EDNO)-dependent vasodilation, whereas vasodilatory responses to exogenous NO are attenuated in CH rat lungs. We hypothesized that reactive oxygen species (ROS) inhibit NO-dependent pulmonary vasodilation following CH. To test this hypothesis, we examined responses to the EDNO-dependent vasodilator endothelin-1 (ET-1) and the NO donor S-nitroso-N-acetyl penicillamine (SNAP) in isolated lungs from control and CH rats in the presence or absence of ROS scavengers under normoxic or hypoxic ventilation. ROS was inhibited in lungs used for SNAP experiments to eliminate influences of endogenously produced NO. Additionally, dichlorofluorescein (DCF) fluorescence was measured as an index of ROS levels in isolated pressurized small pulmonary arteries from each group. We found that acute hypoxia increased DCF fluorescence and attenuated vasodilatory responses to ET-1 in lungs from control rats. The addition of ROS scavengers augmented ET-1-induced vasodilation in lungs from both groups during hypoxic ventilation. In contrast, upon NOS inhibition, DCF fluorescence was elevated and SNAP-induced vasodilation diminished in arteries from CH rats during normoxia, whereas acute hypoxia decreased DCF fluorescence, which correlated with augmented reactivity to SNAP in both groups. ROS scavengers enhanced SNAP-induced vasodilation in normoxia-ventilated lungs from CH rats similar to effects of hypoxic ventilation. We conclude that inhibition of NOS during normoxia leads to greater ROS generation in lungs from both control and CH rats. Furthermore, NOS inhibition reveals an effect of acute hypoxia to diminish ROS levels and augment NO-mediated pulmonary vasodilation.

superoxide dismutase; tiron; pulmonary hypertension; S-nitroso-N-acetyl penicillamine; endothelin-1; dichlorofluorescein; nitric oxide (EDNO)-dependent vasodilators. However, following inhibition of NOS, vasodilatory responses to several different NO donors are attenuated in isolated lungs from CH rats (11). Although this could potentially be explained by a decrease in smooth muscle sensitivity to NO, this seems unlikely since we have recently shown that the ability to stimulate the NO-dependent vasodilatory signaling pathway via activation of cGMP (11) and PKG (12) is not diminished following CH, but rather, it appears to be enhanced. Although the mechanism(s) for this inconsistency between exogenous and endogenous NO-dependent vasodilatory responsiveness following CH is unclear, some investigators suggest the generation of reactive oxygen species (ROS) during hypoxia-reoxygenation impairs NO formation as well as directly inactivating NO (5, 8, 40). The present study examines the mechanisms responsible for the differential effects of acute and chronic hypoxic exposure on vasodilation to endogenous vs. exogenous NO and the role of ROS on NO-dependent vasoreactivity.

The observation that ROS inhibit NO-dependent vasodilation is not novel. Indeed, even before endothelium-derived relaxing factor (EDRF) was identified as NO, Rubanyi and Vanhoutte (34) demonstrated that EDRF could be inactivated by superoxide and stabilized by superoxide dismutase (SOD). More recent studies have confirmed the ability of antioxidants to improve vasodilatory responses following acute (5) and chronic hypoxia (40), suggesting that reoxygenation following hypoxia may play an important role in ROS production. However, it is equally probable that hypoxia, per se, elevates ROS production (13, 16, 17). Both endothelial cells and vascular smooth muscle cells generate superoxide anion (18, 35, 38), which has been shown to interact with NO producing the cytotoxic oxidant peroxynitrite, thereby preventing NO-induced vasodilation (1, 34); therefore, we hypothesized that increased levels of ROS following CH attenuate NO-dependent pulmonary vasodilation. To test this hypothesis, we assessed both EDNO- and exogenous NO-dependent vasodilatory responses in lungs from control and CH rats during both normoxic ventilation (NV) and hypoxic ventilation (HV). Parallel experiments were conducted in the presence of ROS scavengers to examine the effect of endogenous ROS on NO-dependent reactivity, and ROS levels were measured with the fluorescent indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) in small pulmonary arteries from control and CH rats. Our findings suggest that acute hypoxia increases ROS levels and diminishes EDNO-dependent pulmonary vasodilation in lungs from control rats. In addition, the attenuated vasodilatory response to...
exogenous NO following CH appears to be due to increased ROS production during NOS inhibition. Finally, NOS inhibition unmasks an acute hypoxia-induced decrease in ROS levels, a response that correlates with normalization of reactivity to exogenous NO between control and CH groups.

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 Sprague-Dawley rats (200–280 g, Harlan Industries) were divided into control and CH groups for each experiment. Animals designated for exposure to CH were housed in a hypobaric chamber with barometric pressure maintained at ~380 mmHg for 4 wk. The chamber was opened three times/wk 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% O₂-88% N₂ gas mixture to reproduce inspired P O₂ (70 mmHg) within the hypobaric chamber. Constant circulation of this gas mixture prevented accumulation of CO₂. 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 (11, 29, 30). Briefly, after each experiment, the atria and major vessels were removed from the ventricles. The right ventricle (RV) was dissected from the left ventricle (LV) and septum, and each was weighed. The degree of right ventricular hypertrophy is expressed as the ratio of RV to total ventricular weight (T). RV/T ratios were similar to previously obtained ratios from freshly isolated hearts (29), suggesting the current ratios were not compromised by ligation during experiments.

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 683) at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with a warmed and humidified gas mixture (6% CO₂-21% O₂-balance N₂, normoxia; or 6% CO₂-balance N₂, hypoxia). Inspiratory pressure was set at 9 cmH₂O, and positive end-expiratory pressure was set at 3 cmH₂O. 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 [PSS; (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose; all from Sigma] containing 4% (wt/vol) albumin (Sigma) and meclofenamate (30 µM, Sigma) at 0.8 ml/min with a Masterflex micropump drive (model 7524-10). Meclofenamate was added to minimize potential complicating influences of endogenous prostaglandins 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 (11).

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⁻¹·kg body wt (BW)⁻¹ 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 (Pv) was 3–4 mmHg. Pulmonary arterial pressure (P_a) and P_v were measured with Spectramed model 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, Dataq Instruments).

After a 30-min stabilization period, the thromboxane analog 9,11-dideoxy-9a,11R-epoxymethanoprostaglandin F₂α (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 (29, 30, 32), allowing assessment of subsequent vasodilatory responses as outlined in the following protocols.

Isolated Lung Experiments

Effect of NOS inhibition on endothelin-1-mediated vasodilation. To determine the contribution of NO to endothelin-1 (ET-1)-mediated vasodilation, we examined vasodilatory responses to ET-1 (1 nM) in U-46619-constricted lungs from control and CH rats in the presence or absence of the NOS inhibitor N⁴-nitro-l-arginine (l-NNA, 300 µM). This concentration of ET-1 was determined to result in EDNO-dependent vasodilation in previous experiments (32).

Effect of soluble guanylyl cyclase inhibition on ET-1-mediated vasodilation. The contribution of endogenous cGMP in mediating vasodilatory responses to ET-1 in each group of rats was assessed with a heme site-specific soluble guanylyl cyclase (sGC) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-c]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 our laboratory (11) to inhibit sGC in this preparation. Responses to ET-1 (1 nM) were then determined in control and CH lungs as described above.

Vasodilatory responses to ET-1 and S-nitroso-N-acetyl penicillamine during hypoxic ventilation. To determine the influence of acute HV on EDNO-dependent vasodilation, we isolated and ventilated lungs from control and CH rats with a normoxic gas mixture (6% CO₂, balance air). After a 15-min equilibration, the lungs were either maintained in normoxia for an additional 30 min before administration of U-46619 or switched to HV (6% CO₂, balance N₂) for the remainder of the experiment. Hypoxic vasoconstriction is transient in this preparation, and pulmonary vascular resistance was allowed to return to baseline before the administration of U-46619 (~30 min). Responses to ET-1 (1 nM) were then determined in U-46619-constricted lungs from control and CH rats.

A cumulative concentration-response relationship to the NO-donor S-nitroso-N-acetyl penicillamine (SNAP; 0.5, 1.0, 10 µM) was assessed in a separate set of U-46619-constricted lungs from both groups during NV and HV. For these and all other SNAP experiments, 300 µM l-NNA was added to minimize the potential complicating influences of endogenous NO on vascular reactivity (11). A stable vasodilatory response to each dose of SNAP was allowed to develop before administration of subsequent doses. Due to concerns with the longevity of stable U-46619 constriction, responses to only three concentrations of SNAP were assessed in each lung.

Vasodilatory responses to ET-1 and SNAP in the presence of ROS scavengers. The contribution of oxygen radicals in altering vasodilatory responses to NO in each group of rats was determined with SOD and catalase (a H₂O₂ reductase) or 4,5-dihydroxy-1,3-benzene-disulfonic acid (tiron). Catalase was used to reduce H₂O₂, since H₂O₂ has been shown to influence cGMP-mediated relaxation upon alterations.
We obtained either normoxic or hypoxic gas mixtures during the loading period. processed with MetaFluor 4.5 software (Universal Imaging). Normal-cooled, digital charge-coupled device camera (SenSys 1400) and CO₂, balance N₂). A cover was placed over the chamber so that the concentration of 5\textit{H}9262 (Molecular Probes). CM-H₂DCFDA was dissolved in anhydrous DMSO, and this mixture was diluted with PSS to yield a final concentration of 5 μM CM-H₂DCFDA and 0.05% pluronic acid. An excitation objective (numerical aperture 0.30). Vessels were superfused at 37°C for 30 min with PSS aerated with either normoxic (21% O₂, 6% CO₂, balance N₂) or hypoxic gas (6% O₂, 94% N₂). A cover was placed over the chamber so that the gas mixture also flowed over the top of the chamber bath. Samples of superfusate were not analyzed for measurement of PO₂, pcO₂, and pH with a blood-gas analyzer (Radiometer). After the 30-min equilibration, the pressurized pulmonary arteries were loaded with the cell-permeant ROS-sensitive fluorescent indicator CM-H₂DCFDA (Molecular Probes). CM-H₂DCFDA was dissolved in anhydrous DMSO at a concentration of 50 μg/ml. Immediately before loading, CM-H₂DCFDA was mixed with a 20% solution of pluronic acid in DMSO, and this mixture was diluted with PSS to yield a final concentration of 5 μM CM-H₂DCFDA and 0.05% pluronic acid. Vessels were incubated in this solution for 30 min at room temperature in the dark. The diluted CM-H₂DCFDA solution was aerated with either normoxic or hypoxic gas mixtures during the loading period. We obtained fluorescent images using a standard FITC filter before loading the vessel (for background subtraction) and after the 30-min incubation with CM-H₂DCFDA. Images were generated with a cooled, digital charge-coupled device camera (SenSys 1400) and processed with MetaFluor 4.5 software (Universal Imaging). Normalized fluorescence intensity is defined as average gray scale values for all pixels in the field above background. Because isolated lung experiments examining responses to exogenous NO were conducted in the presence of l-NNA, ROS production was measured in separate sets of vessels treated with l-NNA (300 μM).

To demonstrate changes in CM-H₂DCFDA fluorescence in response to agents known to produce or scavenge ROS, we prepared a separate set of normoxic control arteries as above and treated it with xanthine (2 mM) and xanthine oxidase (5 mM/ml) to stimulate ROS production. In separate experiments, vessels were treated with xanthine (10 mM) before the addition of xanthine/xanthine oxidase.

Calculations and Statistics

Pulmonary vascular resistance was calculated as the difference between Pp and Pw, divided by flow (30 ml/min⁻¹·kg BW⁻¹). Vasodilatory responses were calculated as percent reversal of baseline vascular resistance. All data are expressed as means ± SE, and values of n refer to the number of animals in each group. Data were analyzed with two-way ANOVA, and if differences were detected, 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 Right Ventricular Hypertrophy and Polycythemia

RV/T ratios were greater in CH rats (0.312 ± 0.003, n = 86) compared with control rats (0.208 ± 0.003, n = 84), thus demonstrating right ventricular hypertrophy indicative of pulmonary hypertension. Further, CH rats exhibited polycythemia as indicated by a significantly greater hematocrit in CH rats (60.8 ± 0.3%, n = 86) compared with control rats (42.6 ± 0.2%, n = 84).

Isolated Lung Experiments

Baseline vascular resistances and responses to U-46619. All baseline vascular resistances, regardless of treatment, were greater in lungs from CH rats compared with controls (Table 1). Because the pulmonary circulation exhibits no detectable tone in this preparation (31), these data provide functional evidence for CH-induced vascular remodeling. The concentration of U-46619 required to elicit comparable vasoconstriction between groups was significantly less in lungs from CH animals compared with controls (Table 1). Furthermore, in the presence of l-NNA, less U-46619 was required for similar vasoconstriction in both groups, suggesting that endogenous NO attenuates U-46619-induced vasoconstriction in both groups.

Effect of NOS inhibition on ET-1-mediated vasodilation. Vasodilatory responses to ET-1 (1 nM), in the absence of l-NNA, were augmented in lungs from CH rats compared with control rats (Fig. 1A) as previously reported (32). The presence of l-NNA abolished vasodilation in lungs from both control and CH rats (Fig. 1A), supporting a primary role for NO in ET-1-dependent pulmonary vasodilation.

Effect of GC inhibition on ET-1-mediated vasodilation. The sGC inhibitor ODQ effectively blocked vasodilatory responses to ET-1 (1 nM, Fig. 1B) in lungs from both CH and control rats. These data further suggest ET-1-dependent vasodilation is mediated through cGMP and are consistent with the previously reported inhibition of pulmonary vasodilation to exogenous NO by ODQ in this preparation (11).

Table 1. Baseline vascular resistance, concentration of U-46619 administered to induce an ~10 mmHg pressor response, and change in resistance with the administration of U-46619 in isolated lungs from control and CH rats in the presence or absence of l-NNA

<table>
<thead>
<tr>
<th>l-NNA Absent</th>
<th>l-NNA Present</th>
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<tbody>
<tr>
<td>Control</td>
<td>CH</td>
</tr>
<tr>
<td>(n = 62)</td>
<td>(n = 64)</td>
</tr>
<tr>
<td>Baseline resistance [U-46619], nM</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>∆ Resistance with U-46619</td>
<td>197 ± 6</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE, *P ≤ 0.05 vs. control; †P ≤ 0.05 vs. l-NNA absent. Resistances are in mmHg/ml⁻¹·min·kg. CH, chronic hypoxia; l-NNA, N⁵-nitro-arginine.
perfusion gases between control and CH groups within NV and HV treatments. We observed transient hypoxic vasoconstriction during the first 20 min of HV; however, this constriction was allowed to return to baseline before the administration of U-46619 (Table 3). The change in pulmonary resistance during the peak hypoxic pressor responses was not different between control and CH groups.

**Vasodilatory responses to ET-1 in the presence of ROS scavengers.** Whereas vasodilatory responses to ET-1 in both control and CH groups were unaffected by administration of either SOD/catalase or tiron during NV (Fig. 3A), oxygen radical scavengers greatly augmented ET-1-mediated vasodilatation in controls ventilated with hypoxic gas (Fig. 3B). A similar effect of oxygen radical scavengers to enhance reactivity to ET-1 was observed for CH lungs during HV, although significance was achieved only for SOD/catalase (Fig. 3B).

**Vasodilatory responses to SNAP during HV.** In contrast to ET-1 responses, vasodilatory responses to 0.5 and 1.0 μM SNAP in lungs from CH rats were significantly attenuated compared with those of control rats during HV (Fig. 4), although diminished reactivity was not observed at the highest concentration of SNAP (10 μM). Interestingly, HV augmented vasodilatory responses to 0.5 and 1.0 μM SNAP in lungs from both control and CH rats and normalized reactivity between these groups. Vasodilatory responses to 10 μM SNAP were significantly greater in lungs from CH rats compared with controls during HV (Fig. 4).

**Vasodilatory responses to SNAP in the presence of ROS scavengers.** Similar to effects of HV, administration of SOD/catalase or tiron significantly augmented vasodilatory responses to 0.5 and 1.0 μM SNAP in lungs from CH rats during

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**Table 1.** Saline perfusate pH, PCO₂, and PO₂ in isolated lungs from control and CH rats during normoxic and hypoxic ventilation.

<table>
<thead>
<tr>
<th></th>
<th>Normoxic Ventilation</th>
<th>Hypoxic Ventilation</th>
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<tbody>
<tr>
<td></td>
<td>Control (n = 63)</td>
<td>CH (n = 64)</td>
</tr>
<tr>
<td></td>
<td>Control (n = 63)</td>
<td>CH (n = 64)</td>
</tr>
<tr>
<td>pH</td>
<td>7.33±0.04</td>
<td>7.31±0.08</td>
</tr>
<tr>
<td>PCO₂, mmHg</td>
<td>27±3</td>
<td>29±2</td>
</tr>
<tr>
<td>PO₂, mmHg</td>
<td>137±1</td>
<td>138±2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P ≤ 0.05 vs. normoxic ventilation.

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**Table 2.** Saline perfusate pH, PCO₂, and PO₂ in isolated lungs from control and CH rats during normoxic and hypoxic ventilation.

<table>
<thead>
<tr>
<th></th>
<th>Normoxic Ventilation</th>
<th>Hypoxic Ventilation</th>
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<tbody>
<tr>
<td></td>
<td>Control (n = 63)</td>
<td>CH (n = 64)</td>
</tr>
<tr>
<td></td>
<td>Control (n = 63)</td>
<td>CH (n = 64)</td>
</tr>
<tr>
<td>pH</td>
<td>7.33±0.04</td>
<td>7.31±0.08</td>
</tr>
<tr>
<td>PCO₂, mmHg</td>
<td>27±3</td>
<td>29±2</td>
</tr>
<tr>
<td>PO₂, mmHg</td>
<td>137±1</td>
<td>138±2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P ≤ 0.05 vs. corresponding control group.

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**Table 3.** Normoxic baseline resistance, peak change in resistance to hypoxia, and hypoxic baseline resistance in isolated lungs from control and CH rats in the presence or absence of L-NNA.

<table>
<thead>
<tr>
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<th>Resistance, mmHg/ml⁻¹/min/kg</th>
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<tbody>
<tr>
<td></td>
<td>Normoxic baseline Peak Δ with hypoxia Hypoxic baseline</td>
</tr>
<tr>
<td>L-NNA absent</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23</td>
</tr>
<tr>
<td>CH</td>
<td>21</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P ≤ 0.05 vs. corresponding control value.

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**Fig. 1.** Vasodilatory responses (% of U-46619-induced tone) in lungs from control and chronically hypoxic (CH) rats to endothelin-1 (ET-1) in the presence of N⁵-nitro-l-arginine (l-NNA, 300 μM, n = 5) or vehicle (saline, n = 7). A) and 1H-[1,2,4]oxadiazolo[4,3-c]quinoxalin-1-one (ODQ, 50 μM, n = 5) or vehicle (DMSO, n = 5). B). Experiments were conducted in the presence of meclofenamate (30 μM). Values are means ± SE. *P ≤ 0.05 vs. control vehicle group; #P ≤ 0.05 vs. corresponding vehicle-treated group.

**Fig. 2.** Vasodilatory responses in lungs from control and CH rats to ET-1 (1 nM, n = 5) during normoxic ventilation (NV; 6% CO₂, balance air) or hypoxic ventilation (HV; 6% CO₂, balance N₂). Experiments were conducted in the presence of meclofenamate (30 μM). Values are means ± SE. *P ≤ 0.05 vs. control group; #P ≤ 0.05 vs. control-NV group.
NV (Fig. 5, A and B). Although a similar tendency for ROS scavengers to augment SNAP-induced vasodilation was observed for control lungs, significance was achieved only for tiron at the 0.5 μM concentration of SNAP (Fig. 5B). No differences between groups were noted at the highest concentration of SNAP (1 and 10 μM).

In contrast to effects of ROS scavengers in NV lungs, there was little or no effect of either SOD/catalase or tiron on vasodilatory responses to SNAP in lungs from either group of rats when ventilated with a hypoxic gas mixture (Fig. 6, A and B), although a significantly greater vasodilation to 0.5 μM SNAP was observed in control lungs treated with tiron vs. vehicle (Fig. 6B). Furthermore, NO-dependent vasodilation was augmented in lungs from CH rats vs. controls at the 10 μM dose of SNAP.

**ROS Production in Isolated, Pressurized Small Pulmonary Arteries**

We validated the CM-H2DCFDA signal by monitoring fluorescence obtained from treatment with xanthine-xanthine oxidase in the presence of tiron in pulmonary vessels from control rats under normoxic conditions. Treatment with xanthine-xanthine oxidase in the absence of tiron resulted in greater DCF average fluorescent intensity (988 ± 1100 gray scale values) compared with xanthine-xanthine oxidase in the presence of tiron (234 ± 48 gray scale values), thus demonstrating our ability to measure ROS changes in small pulmonary arteries. In the absence of L-NNa, hypoxia increased DCF fluorescence in vessels from control animals but had no effect in vessels from CH animals (Fig. 7A). However, in the presence of L-NNa, hypoxia greatly attenuated DCF fluorescence in both groups (Fig. 7B). Furthermore, ROS production was...
significantly greater in vessels from CH rats compared with controls under normoxia (Fig. 7B). These data suggest that acute hypoxia may decrease ROS production in the absence of an intact NOS system; however, ROS levels are largely influenced by NOS activity. Superfusate gas data collected during normoxia and hypoxia are shown in Table 4.

**DISCUSSION**

The major findings from this study are 1) ET-1-induced pulmonary vasodilation is mediated by NO/cGMP; 2) compared with NV, HV attenuates ET-1-mediated vasodilation in lungs from control, but not CH, rats. This correlates with increased ROS production in pulmonary arteries from control rats during hypoxia. Additionally, ROS scavengers augment ET-1-dependent vasodilation in lungs from both groups under HV, and 3) HV augments vasodilatory responses to SNAP in L-NNA-treated lungs from both control and CH rats and normalizes NO-dependent vasoreactivity between groups. Similarly to HV, ROS scavengers augment SNAP-mediated vasodilation in NV lungs from CH rats but have little influence in lungs ventilated with hypoxic gas. Consistent with these observations, ROS production is greater in both groups under normoxic conditions compared with hypoxia in the presence of L-NNA; however, this augmented ROS generation is significantly enhanced in pulmonary arteries from CH rats compared with controls. We conclude that inhibition of NOS during normoxia leads to greater ROS generation in lungs from both control and CH rats. Furthermore, NOS inhibition reveals an effect of acute hypoxia to diminish ROS levels and augment NO-mediated pulmonary vasodilation. Finally, impaired NO-dependent pulmonary vasodilation following CH appears to be mediated at least in part by greater ROS production during NOS inhibition. A summary of these findings is provided in Fig. 8.

Consistent with previous studies from our laboratory (29, 30, 32), the current study demonstrates that CH augments ET-1-mediated pulmonary vasodilation (Fig. 8B). It is possible the upregulation of eNOS mRNA and protein levels (6, 14, 29, 30, 45) during chronic hypoxia may lead to increased NO production, which could explain the observed differences in vasodilation between control and CH rats. However, further studies are needed to determine the molecular mechanisms underlying these effects.

**Table 4.** Saline perfusate pH, PaCO₂, and PaO₂ in isolated small pulmonary arteries from control and CH rats during normoxic and hypoxic superfusion

<table>
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<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CH</td>
</tr>
<tr>
<td>pH</td>
<td>7.39±0.02</td>
<td>7.40±0.01</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>30±1</td>
<td>32±6</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>104±2</td>
<td>100±5</td>
</tr>
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</table>

Data are expressed as means ± SE. *P ≤ 0.05 vs. normoxia.
In contrast to an inhibitory effect of acute hypoxia on EDNO-dependent reactivity to ET-1, acute hypoxia enhanced vasodilatory responsiveness to exogenous NO in l-NAME-treated lungs (Fig. 4). Similar to these effects of HV, ROS scavengers augmented reactivity to SNAP during NV (Fig. 5). Furthermore, the combination of hypoxia and ROS scavengers did not appear to be additive and tended to normalize reactivity between groups (Fig. 6), suggesting that hypoxia and ROS scavengers are acting to augment reactivity through a common mechanism to reduce ROS. Because l-NAME was administered for those experiments examining SNAP-induced vasodilation to minimize complicating influences of endogenous NO on vascular reactivity, we additionally examined ROS production from arteries in the presence of l-NAME. Interestingly, we found that inhibiting NO synthesis dramatically increased ROS levels under normoxic conditions in both groups and further revealed an effect of acute hypoxia to diminish ROS production (Figs. 7B and 8A). Such decreased ROS generation during acute hypoxic exposure likely results in increased NO bioavailability and could explain our present observations of enhanced reactivity to SNAP during HV.

Although the mechanism by which NOS inhibition augments ROS generation in pulmonary arteries under normoxic conditions is not clear, these results are consistent with previous findings in human systemic vessels and porcine pulmonary arteries (9, 21). Exogenous NO has additionally been shown to reduce aortic ROS levels (26), implying NO plays a protective role to scavenge ROS. However, such an effect of NO to scavenge ROS does not likely explain the increased DCF fluorescence observed following NOS inhibition in our preparation, since DCF also detects peroxynitrite, the product of superoxide/NO interaction (22). Furthermore, inhibition of NOS enhanced ROS production only during normoxia but resulted in decreased ROS production with hypoxia when O2 availability was limited. Also unlikely is the possibility that elevated levels of ROS following administration of l-NAME result from uncoupling of eNOS activity, since ROS production from eNOS has been recently demonstrated to be inhibitable by the similar L-arginine analog Nω-nitro-L-arginine methyl ester (l-NAME) (25). Alternatively, NO could be having a direct effect to inhibit ROS-generating enzymes. Cote et al. (4) demonstrated that the activity of the ROS-generating enzyme xanthine oxidase was decreased by l-arginine and increased by l-NAME in pulmonary artery endothelial cells, suggesting that NO negatively regulates this enzyme. Furthermore, NO can inhibit NADPH oxidase and cytochrome P-450 oxidase activity (3, 27), thus reducing ROS formation by these enzymes. Such an effect of NO to inhibit the activity of ROS-generating enzymes could explain our current findings that NOS inhibition leads to an apparent elevation in ROS

Fig. 8. Summary of major findings. A: effects of acute hypoxia on reactive oxygen species (ROS) generation and endothelium-dependent nitric oxide (EDNO)-dependent vasodilation under the presence or absence of nitric oxide synthase (NOS) inhibition in lungs/vessels from control rats. Directional arrows reflect changes vs. normoxia. ROS, reactive oxygen species. B: effects of CH on ROS production and EDNO-dependent vasodilation following acute restoration of normoxia or maintained hypoxia in lungs/vessels with an intact NOS system. Directional arrows indicate changes vs. control animals. C: effects of CH on ROS production and NO-dependent vasodilation following acute restoration of normoxia or maintained hypoxia in NOS-inhibited lungs/vessels. Directional arrows reflect changes vs. control animals.

37, 39) following CH contributes to this increased dilation. Indeed, ET-1-mediated vasodilation appears to be entirely dependent on NO/cGMP in this preparation, given that vasodilation was abolished by pretreatment with either the NOS inhibitor l-NAME or the sGC inhibitor ODQ (Fig. 1). Moreover, several investigators have shown NO synthesis to be enhanced following CH (7, 10, 20, 39), although eNOS expression and NO production may be diminished in other species in response to CH (15).

The EDNO-dependent vasodilatory response to ET-1 was attenuated during HV in lungs from control, but not CH, animals (Fig. 2). Although this observation could be explained by diminished NO production resulting from decreased eNOS activity in response to acute hypoxia (15), our finding that acute hypoxia was without effect on ET-1-dependent vasodilation in lungs from CH animals is inconsistent with this possibility. An alternative explanation is that HV differentially alters ROS production and thus NO bioavailability between groups. In agreement with this hypothesis, we found that acute hypoxia resulted in increased ROS levels in control arteries but was without a significant effect on ROS levels in arteries from CH rats (Fig. 7A) with an intact NOS system. Consistent with these observations, several investigators have demonstrated an increase in ROS with acute exposure to hypoxia (13, 16, 17). The mechanism by which CH impairs acute hypoxic increases in ROS generation is not presently understood but could potentially be explained by a chronic change in the redox status of the cytosol to a more reduced state in lungs from CH rats as previously reported (28).
production during normoxia. Furthermore, the ability of each of these enzymes to produce ROS is highly dependent on the availability of O₂, which may therefore account for the observed reduction in ROS generation during exposure to acute hypoxia.

Previous work from our laboratory suggests that attenuated pulmonary vasodilation to exogenous NO following CH is mediated in part by increased degradation of cGMP by phosphodiesterases (11). However, the present study suggests that elevated vascular production of ROS in pulmonary arteries from CH rats following return to normoxia may additionally contribute to this altered vasoresponsiveness (Fig. 8C). Further support for a role for increased ROS in attenuating reactivity to NO following long-term hypoxia is provided by the observation that both ROS scavengers and HV tended to normalize vasodilatory responses to NO between groups (Fig. 8C). Such an effect of CH to stimulate ROS synthesis could result from increased activity of ROS-generating enzymes or rather compromised mechanisms of ROS scavenging. Consistent with this possibility, pulmonary hypertension in fetal lambs also leads to increased ROS production and diminished NO-dependent vasoreactivity, apparently resulting from a reduction in pulmonary arterial SOD activity as well as elevated NAPDH oxidase expression (2). Furthermore, Nakashima et al. (23) similarly demonstrated that lung SOD enzyme levels and activity are increased following chronic hypoxia. NOS inhibition unmasks an effect of acute hypoxia to increase ROS levels during normoxia in arteries from both control and CH. Finally, impaired NO-dependent pulmonary vasodilation following CH appears to be mediated at least in part by greater ROS production during NOS inhibition (Fig. 8C). Further investigation is needed to develop a better understanding of the cellular and enzymatic sources of ROS and how they are influenced by hypoxia and NOS inhibition to influence NO vasoreactivity.

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