Intermittent hypoxia augments pulmonary vascular smooth muscle reactivity to NO: regulation by reactive oxygen species

Charles E. Norton, Nikki L. Jernigan, Nancy L. Kanagy, Benjimen R. Walker, and Thomas C. Resta
Vascular Physiology Group, Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Submitted 1 November 2010; accepted in final form 7 July 2011

Norton CE, Jernigan NL, Kanagy NL, Walker BR, Resta TC. Intermittent hypoxia augments pulmonary vascular smooth muscle reactivity to NO: regulation by reactive oxygen species. J Appl Physiol 111: 980–988, 2011. First published July 14, 2011; doi:10.1152/japplphysiol.01286.2010.—Intermittent hypoxia (IH) resulting from sleep apnea can lead to pulmonary hypertension. IH causes oxidative stress that may limit bioavailability of the endothelium-derived vasodilator nitric oxide (NO) and thus contribute to this hypertensive response. We therefore hypothesized that increased vascular superoxide anion (O$_2^-$) generation reduces NO-dependent pulmonary vasodilation following IH. To test this hypothesis, we examined effects of the O$_2^-$ scavenger tiron on vasodilatory responses to the endothelium-dependent vasodilator ionomycin and the NO donor S-nitroso-N-acetylpenicillamine in isolated lungs from hypocapnic-IH (H-IH; 3 min cycles of 5% O$_2$/air flush, 7 h/day, 4 wk), eucapnic-IH (E-IH; cycles of 5% O$_2$, 5% CO$_2$/air flush), and sham-treated (air/air) rats. Next, we assessed effects of endogenous O$_2^-$ on NO- and cGMP-dependent vasoreactivity and measured O$_2^-$ levels using the fluorescent indicator dihydroethidium (DHE) in isolated, endothelium-disrupted small pulmonary arteries from each group. Both E-IH and H-IH augmented NO-dependent vasodilation; however, enhanced vascular smooth muscle (VSM) reactivity to NO following H-IH was masked by an effect of endogenous O$_2^-$.

Further, H-IH and E-IH similarly increased VSM sensitivity to cGMP, but this response was independent of either O$_2^-$ generation or altered arterial protein kinase G expression. Finally, both H-IH and E-IH increased arterial O$_2^-$ levels, although this response was more pronounced following H-IH, and H-IH exposure resulted in greater protein tyrosine nitration indicative of increased NO scavenging by O$_2^-$. We conclude that IH increases pulmonary VSM sensitivity to NO and cGMP. Furthermore, endogenous O$_2^-$ limits NO-dependent vasodilation following H-IH through an apparent reduction in bioavailable NO.

sleep apnea; pulmonary hypertension; superoxide anion; endothelial nitric oxide synthase; protein kinase G

SLEEP APNEA (SA) affects ~20% of the adult population in the United States (47). In addition to causing systemic hypertension (32), intermittent hypoxia (IH) associated with SA exacerbates pulmonary hypertension (PH) in patients with chronic obstructive pulmonary disease and may be an independent risk factor for PH (14). Although the cardiovascular sequelae leading to sleep apnea-induced PH remain poorly characterized, recent studies using rodent models of the disorder have begun to elucidate these mechanisms of PH, which include vasoconstriction, arterial remodeling, and polycythemia (2, 6, 8, 40). Such models have employed chronic exposure to a variety of hypoxia-reoxygenation cycles (0.5–3 min, 7–12 h/day for 2–8 wk) to mimic the repetitive apneic episodes observed in patients with SA (6, 11, 27, 40). In addition, because IH alone leads to cyclical reductions in arterial PCO$_2$ and consequent alkalemia, other studies have introduced supplemental CO$_2$ into the cycling protocol to prevent hypocapnia and thus more closely approximate arterial blood gases in human SA (1, 10, 11, 40, 41).

Whereas the endogenous vasodilator and antimitogenic factor, nitric oxide (NO), may be protective in limiting the severity of IH-induced PH, little is known regarding effects of IH on NO-dependent vasoreactivity or NO signaling in pulmonary vascular smooth muscle (VSM). NO is a highly labile reactive nitrogen species that is produced by endothelial nitric oxide synthase (eNOS) in vascular endothelial cells. NO elicits pulmonary vasodilation through activation of the soluble guanylyl cyclase (sGC)-protein kinase G (PKG) signaling axis, leading to a reduced VSM intracellular calcium concentration ([Ca$^{2+}$]$_i$) and myofilament Ca$^{2+}$ desensitization (19). While a compensatory increase in eNOS expression occurs in some models of PH (22, 39, 42), NO-dependent pulmonary vasoreactivity is often compromised by reduced NO synthesis (26), inactivation of NO by reactive oxygen species (ROS) (13, 21, 27), or impaired NO signal transduction in VSM (17).

IH is associated with increased oxidative stress (24, 27, 41) that results from enhanced levels of ROS, including superoxide anion (O$_2^-$) and hydrogen peroxide. O$_2^-$ may limit the bioavailability of NO by the formation of peroxynitrite (ONOO$^-$) (21) or as a result of eNOS uncoupling (46). Additionally, ROS may directly interfere with NO signaling in VSM (45). We therefore hypothesized that increased vascular O$_2^-$ generation following IH reduces NO-dependent pulmonary vasodilatation. We tested our hypothesis by assessing the role of ROS in NO-dependent vasoreactivity in isolated lungs and small pulmonary arteries from hypocapnic IH, eucapnic IH, and sham-treated control rats. Our findings reveal a novel effect of IH to increase pulmonary VSM sensitivity to NO and cGMP. Furthermore, NO-dependent pulmonary vasodilatation is reduced by a nonendothelial source of O$_2^-$ following hypocapnic-IH, but not eucapnic-IH.

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). Male Wistar rats (body wt 250–350 g, age 3–4 mo; Harlan Industries) were used for all experiments. Animals were maintained on a 12:12-h light-dark cycle.

Experimental Groups

Rats were housed in Plexiglas chambers with free access to food and water and divided into three experimental groups. The first group...
was exposed to IH alone (3 min cycles of 5% O2-air flush), which leads to cyclical reductions in arterial P02 and Pco2, and consequent alkalemia (40), and is therefore termed hypoxic IH (H-IH). A second group was provided with supplemental CO2 during hypoxic cycling (3 min cycles of 5% O2, 5% CO2-air flush) to more closely approximate the arterial blood gas and pH changes that occur during apneic episodes in patients with sleep-disordered breathing (12). Previous studies from our laboratory indicate that this method of CO2 supplementation maintains normal arterial Pco2 and pH during hypoxic episodes (40), and is therefore termed eucapnic IH (E-IH). Both H-IH and E-IH animals were exposed to these conditions for 7 h/day for 4 wk. A third control group was exposed to sham conditions (3 min air-air cycles) for equal duration. For sham exposure, the inflow gas was always room air but the solenoid switches and inlets approximated the airflow and noise disturbances of the IH protocols. In addition to evidence of PH following H-IH and E-IH exposure (40), this model of E-IH causes systemic hypertension in rats (1, 20), consistent with known effects of SA in humans (47).

**Assessment of Right Ventricular Weight and Hematocrit**

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 IH-induced PH as described previously (17, 36).

**Isolated Lung Protocols**

Methods for lung isolation and perfusion have been previously reported (36). Lungs were perfused with a physiological salt solution and ventilated with normoxic gas (6% CO2 in room air) to eliminate possible complicating influences of differential hypoxic pulmonary vasoconstriction between groups on NO-dependent vasodilatory responses.

**Effects of IH on endothelium-dependent vasodilation.** To compare effects of H-IH and E-IH in isolated pulmonary artery preparations, IOEDNO-dependent pulmonary vasoreactivity, vasodilatory responses to the Ca2+ ionophore ionomycin (Calbiochem) were assessed in lungs from each group of rats. Experiments were conducted in the presence of vehicle (saline), the O2 scavenger SNAP (10-9–10-5 M, Sigma) were conducted in isolated pulmonary arteries from each group. Following equilibration, U-46619 (Cayman Chemical) was added in cumulative doses to the perfusate reservoir until a stable arterial pressor response of ~10 mmHg was achieved. The vasculature was then diluted with ionomycin (100 nM). Ionomycin was chosen as a non-receptor-mediated EDNO-dependent vasodilator in this study since interpretation of responses to receptor-mediated agonists could be complicated by possible changes in receptor number or affinity in response to sham, H-IH, or E-IH exposure. Furthermore, we have previously shown that ionomycin elicits vasodilatory responses in this preparation that are sensitive to NOS inhibition (35, 36). Ionomycin was dissolved in anhydrous dimethyl sulfoxide (Sigma) and stored at 4°C. Effects of IH on vasoreactivity to exogenous NO. Vasodilatory responses to the NO donor S-nitroso-N-acetylpenicillamine (SNAP; 1 μM, Sigma) were assessed in separate sets of U-46619-constricted lungs from rats from each group. Experiments were conducted in the presence of vehicle or tiron (10 mM) to further assess the influence of ROS on these responses. All protocols were performed in the presence of L-NNA (300 μM) to minimize potential complicating influences of endogenous NO on vascular reactivity.

**Isolated Vessel Protocols**

Effects of H-IH and E-IH on VSM reactivity to NO/cGMP were evaluated in isolated, cannulated small pulmonary arteries from each group of rats. Inner diameter (ID) and fura-2 340/380 emission ratios (F340/F380) were measured simultaneously to assess vasoreactivity and changes in VSM [Ca2+]i, as previously described (3, 19). All arteries were studied at a transmural pressure of 12 Torr under normoxic conditions (equilibrated with a 10% O2, 6% CO2, and balance N2 gas mixture) to eliminate possible complicating influences of differential hypoxic pulmonary vasoconstriction between groups on the subsequent vasodilatory responses to NO. Arteries were endothelium-disrupted to directly evaluate effects of H-IH and E-IH on VSM sensitivity to NO and cGMP independent of complicating influences of NO or ROS produced by the endothelium. The effectiveness of endothelial disruption was verified by the lack of a vasodilatory response to Ach (1 μmol/l) in UTP-constricted vessels.

**Effects of IH on NO- and cGMP-dependent vasodilation and changes in VSM [Ca2+]i.** Concentration-response curves to the NO donor SNAP (10-9–10-5 M, Sigma) were conducted in isolated pulmonary arteries from each group. Following equilibration and equilibration, as described above, vessels were preconstricted with UTP (5 μM) to ~40% of baseline ID. Vasodilatory responses and changes in VSM [Ca2+]i, were assessed in the absence of vehicle, tiron, or polyethylene glycol (PEG)-catalase (250 U/ml, Sigma) (7) to determine the effects of O2 and H2O2 on NO-dependent reactivity in these arteries.

**Effects of IH cGMP-mediated pulmonary vasodilation were assessed by performing cumulative concentration-response curves to the cell-permeable, phosphodiesterase-5-resistant cGMP analog 8-para-chlorophenylthio (8-pCPT)-cGMP (10-9–10-4 M, Sigma) (19), in UTP-constricted endothelium-disrupted arteries from each group. Vasodilatory and VSM [Ca2+]i responses were measured in the presence of vehicle or tiron to determine potential influences of endogenous O2 on reactivity to 8-pCPT-cGMP.

**Effects of PKG inhibition on NO-dependent reactivity.** The role of PKG in mediating NO-dependent vasodilation was determined by performing cumulative concentration-response curves to SNAP in the presence of the PKG inhibitor KT-5823 (10 μM, Sigma) (19) concentrations of SNAP were 3, 10, and 30 μM to assess potential influences of endogenous O2 on reactivity to SNAP.

**Effects of IH on arterial O2 generation.** Fluorescence detection of dihydroethidium (DHE, Molecular Probes) oxidation was used to compare effects of H-IH and E-IH treatment on basal levels of O2 in pressurized, endothelium-disrupted pulmonary arteries as previously reported (3).

**Western Blotting: Effects of IH on Arterial eNOS, PKG-1, and Nitrotyrosine Levels**

Pulmonary arterial eNOS and PKG-1 expression were compared between sham-treated, H-IH, and E-IH rats using previously pub-
lished methods (35). Nitrotyrosine levels were additionally determined by Western blotting as a measure of ONOO\textsuperscript{−} formation (30) in arteries from each group. Arteries used for nitrotyrosine blots were incubated for 30 min at 37\degree C, then treated with SNAP (10\textsuperscript{−6} M; 5 min), and snap-frozen in liquid nitrogen. A polyclonal anti-nitrotyrosine antibody (1:1,000, Millipore) was used to detect nitrotyrosine residues. Bands were compared between blots following normalization to an identical control sample. In addition, all bands of interest were normalized to those of beta-actin on the same blot.

Calculations and Statistics

For isolated lung experiments, total pulmonary vascular resistance was calculated as the difference between \( P_a \) and \( P_v \) divided by flow (30 ml min\textsuperscript{−1} kg body wt\textsuperscript{−1}). Vasodilatory responses were calculated as a percent reversal of U-46619-induced vasoconstriction. Vasodilatory responses in isolated arteries are expressed as a percent reversal of UTP-induced constriction. All data are presented as means ± SE, and \( n \) refers to the number of animals in each group. A one-way, two-way, or repeated-measures ANOVA was used to make comparisons when appropriate. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls test. A probability of \( P \leq 0.05 \) was considered significant for all comparisons.

RESULTS

IH-Induces Right Ventricular Hypertrophy and Polycythemia

Both H-IH and E-IH exposure produced right ventricular (RV) hypertrophy indicative of PH, as evidenced by greater RV-to-total ventricular weight (RV/T) and RV/body weight (BW) ratios compared with sham-treated controls (Table 1). However, these indexes of PH were significantly lower in E-IH compared with H-IH rats. No significant differences in left ventricle plus septum weight (LV + S)/BW were observed between groups, demonstrating a lack of LV hypertrophy in response to IH. Exposure to H-IH and E-IH resulted in lower BW compared with sham-treated rats, suggesting that IH attenuates weight gain independent of hypocapnic or eucapnic treatment. H-IH rats exhibited greater hematocrit vs. sham-treated rats, whereas no such response was observed in the E-IH group. We have previously reported similar effects of supplemental CO\textsubscript{2} to attenuate RV hypertrophy and polycythemia following 2 wk IH exposure (40).

IH Enhances EDNO-Dependent Vasodilation in Isolated Lungs: Role of O\textsubscript{2} \textsuperscript{−}

No differences in \( P_{O_2}, P_{CO_2}, \) or pH were observed in isolated lung perfusate effluent samples between groups. There were also no differences in baseline vascular resistance or U-46619-induced increases in resistance between groups in isolated lung protocols. Vasodilatory responses to the EDNO-dependent vasodilator ionomycin were greater in lungs from E-IH rats compared with those from sham-treated animals, whereas responses to ionomycin were unaltered following H-IH exposure (Fig. 1). However, the O\textsubscript{2} \textsuperscript{−} scavenger tiron augmented EDNO-dependent reactivity in the H-IH group only, revealing greater vasodilation compared with similarly treated sham lungs. NOS inhibition with L-NNA greatly reduced vasodilation in all three
groups, thus confirming the NO dependency of these responses.

Pulmonary Arterial eNOS Expression is Not Altered Following IH

To address whether greater EDNO-dependent pulmonary vasodilation following H-IH and E-IH (Fig. 1) is associated with increased eNOS expression, we measured eNOS protein levels in intrapulmonary arteries from each group by Western blotting. However, levels of pulmonary arterial eNOS were unaltered after either H-IH or E-IH exposure (Fig. 2).

IH Augments Vasodilation to Exogenous NO in Isolated Lungs: Regulation by O$_2$

Augmented EDNO-mediated vasodilation following IH could be a function of either increased NO synthesis (independent of a change in eNOS expression) or enhanced VSM sensitivity to NO. NO-dependent vasoreactivity was therefore examined by measuring vasodilatory responses to exogenous NO in lungs isolated from each group of rats. Similar to EDNO-mediated vasodilation (Fig. 1), vasodilatory responses to the NO donor SNAP were augmented in lungs from rats exposed to E-IH, but not H-IH (Fig. 3). Furthermore, tiron elevated NO-dependent vasoreactivity in the H-IH group only (Fig. 3). These findings suggest that both H-IH and E-IH increase pulmonary VSM sensitivity to NO, a response that is masked by endogenous O$_2$ following H-IH.

IH Increases VSM Reactivity to NO in Isolated Small Pulmonary Arteries: Role of O$_2$

Basal ID, VSM [Ca$^{2+}$], UTP-induced constriction, and VSM [Ca$^{2+}$] responses in isolated pulmonary arteries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Basal ID, μm</th>
<th>Basal [Ca$^{2+}$], %Constriction</th>
<th>Δ[Ca$^{2+}$], n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>189.5 ± 7.3</td>
<td>1.13 ± 0.20</td>
<td>34.3 ± 3.3, 9</td>
</tr>
<tr>
<td>H-IH</td>
<td>184.1 ± 6.9</td>
<td>1.14 ± 0.15</td>
<td>38.4 ± 2.5, 10</td>
</tr>
<tr>
<td>E-IH</td>
<td>185.3 ± 7.1</td>
<td>1.10 ± 0.11</td>
<td>37.8 ± 2.9, 10</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. Constriction was calculated as a percent change from basal inner diameter (ID). Basal vascular smooth muscle (VSM) intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) is expressed as 340/380-nm emission ratios. Changes in VSM [Ca$^{2+}$], (Δ[Ca$^{2+}$]) are calculated as the difference in 340/380-nm emission ratios from basal. There are no significant differences.

Fig. 4. Vasodilatory responses (A, C, E) and changes in vascular smooth muscle (VSM) intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) (B, D, F) to the NO donor SNAP in the presence of vehicle (A, B), tiron (C, D; 10 mM), or PEG-catalase (E, F; 250 U/ml) in isolated pulmonary arteries from sham-treated, H-IH, and E-IH rats. Values are means ± SE of n = 4–5 rats/group. *P < 0.05 vs. sham. #P < 0.05 vs. H-IH. tP < 0.05 vs. vehicle.
revealed greater vasodilation and \([\text{Ca}^{2+}]_{i}\) responses to SNAP in arteries from H-IH vs. sham-treated rats (Fig. 4, C and D), while having no significant effect on reactivity to NO in the E-IH group. In contrast, vasodilatory and VSM \([\text{Ca}^{2+}]_{i}\) responses to SNAP in the presence of the \(\text{H}_2\text{O}_2\) scavenger, PEG-catalase (Fig. 4, E and F), generally reflected those in the presence of vehicle (Fig. 4, A and B), with arteries from E-IH rats displaying significantly greater vasodilation and changes in VSM \([\text{Ca}^{2+}]_{i}\) vs. sham-treated arteries, whereas no differences were observed between sham-treated and H-IH arteries.

**IH Augments cGMP-Dependent Vasodilation Independently of \(\text{O}_2^\cdot\)** in Isolated Small Pulmonary Arteries

Increased vasodilation to NO following IH may be mediated by greater VSM sensitivity to the second messenger cGMP. To examine this possibility, we evaluated effects of both H-IH and E-IH exposure on responses to the cGMP analog 8-pCPT-cGMP in endothelium-disrupted pulmonary arteries. Dilation to 8-pCPT-cGMP was enhanced in arteries from both H-IH and E-IH rats compared with those of sham-treated controls (Fig. 5A). This increased vasoreactivity following H-IH and E-IH was associated with a greater fall in VSM \([\text{Ca}^{2+}]_{i}\) (Fig. 5B). The addition of tiron resulted in no significant changes in either vasodilatory or VSM \([\text{Ca}^{2+}]_{i}\) responses to 8-pCPT-cGMP in any of the groups (Fig. 5, C and D).

**PKG Inhibition Prevents NO-Dependent Responses in Isolated Small Pulmonary Arteries**

To determine whether NO-dependent vasodilation following IH and sham treatments is mediated by PKG, we evaluated responses to varying concentrations of SNAP in the presence of KT-5823, a PKG inhibitor. No significant vasodilation or change in VSM \([\text{Ca}^{2+}]_{i}\) to SNAP was observed in the presence of KT-5823 (Fig. 6, A and B) in arteries from any group.

**Pulmonary Arterial PKG Expression is not Altered by IH**

Western blots revealed no differences in pulmonary arterial PKG-1 levels between groups (Fig. 7), suggesting that increased VSM reactivity to NO and cGMP following IH is not a function of increased PKG-1 expression.

**IH Increases Pulmonary Arterial \(\text{O}_2^\cdot\) Production**

The effect of \(\text{O}_2^\cdot\) to selectively attenuate NO-mediated pulmonary vasodilation following H-IH may be due to greater vascular \(\text{O}_2^\cdot\) generation in that group. We therefore measured \(\text{O}_2^\cdot\) generation using the fluorescent \(\text{O}_2^\cdot\) indicator DHE in isolated pressurized arteries from each group. DHE fluorescence was greater in arteries from rats exposed to H-IH compared with sham-treated arteries (Fig. 8). While arteries from E-IH animals also demonstrated enhanced fluorescence vs. the sham-treated vessels, it was significantly less than in the H-IH group. The addition of tiron decreased \(\text{O}_2^\cdot\) levels in both H-IH and E-IH arteries to similar levels as expected, without altering fluorescence intensity in sham-treated arteries.

**Pulmonary Arterial Nitrotyrosine Levels are Increased Following H-IH**

Greater vascular \(\text{O}_2^\cdot\) generation following H-IH may limit NO-dependent vasodilation by reacting with NO to form nitrotyrosine (Fig. 9).

---

Fig. 5. Vasodilatory responses (A, C) and changes in VSM \([\text{Ca}^{2+}]_{i}\) (B, D) to the cell-permeable cGMP analog 8-pCPT-cGMP in the presence of vehicle (A, B) or tiron (C, D) in isolated pulmonary arteries from sham-treated, H-IH, and E-IH rats. Values are means ± SE of \(n = 4–5\) rats/group. *\(P < 0.05\) vs. sham.
ONOO$^-$, thus reducing NO bioavailability. Therefore, we measured levels of nitrotyrosine-positive proteins by Western blot as an index of ONOO$^-$ production (30) in intrapulmonary arteries from sham, H-IH, and E-IH rats. Consistent with increased ONOO$^-$ generation following H-IH, we observed greater protein tyrosine nitration in arteries from H-IH vs. sham-treated rats, whereas E-IH exposure had no significant effect on nitrotyrosine levels (Fig. 9).

**DISCUSSION**

The objective of this study was to evaluate mechanisms of altered NO-dependent pulmonary vasodilation in IH-induced PH. Our major findings are that 1) E-IH and H-IH augmented vasodilation to both EDNO and exogenous NO, but these effects were masked in H-IH by increased $O_2^-$; 2) greater reactivity to EDNO and exogenous NO following H-IH and E-IH was not associated with increased pulmonary arterial eNOS or PKG-1 expression; 3) H-IH and E-IH similarly increased VSM reactivity to cGMP; however, this response was independent of vascular $O_2^-$ generation; and 4) H-IH resulted in greater arterial protein tyrosine nitration indicative of increased NO scavenging by $O_2^-$. Together, these results demonstrate an effect of IH to increase pulmonary VSM reactivity to both NO and cGMP, and support a role for endogenous $O_2^-$ to limit NO-dependent pulmonary vasodilation following H-IH by reducing bioavailable NO.

Mechanisms of IH-induced PH are generally considered to be similar to those resulting from chronic sustained hypoxia (CH), including pulmonary vasoconstriction, arterial remodeling, and polycythemia (12). Whereas NO mediates an important protective influence to limit vasoconstriction and arterial remodeling in PH (9, 34), NO bioavailability is often reduced in both experimental and human forms of PH as a result of diminished NO synthesis (26) or increased scavenging of NO by $O_2^-$(13). Indeed, lung ROS levels are increased in IH mice (27), and ROS are implicated as contributing factors to both vasoconstrictor (3, 43) and vascular remodeling components of PH (28). However, IH effects on pulmonary vasoreactivity to increased VSM reactivity to cGMP; however, this response was independent of vascular $O_2^-$ generation; and 4) H-IH resulted in greater arterial protein tyrosine nitration indicative of increased NO scavenging by $O_2^-$. Together, these results demonstrate an effect of IH to increase pulmonary VSM reactivity to both NO and cGMP, and support a role for endogenous $O_2^-$ to limit NO-dependent pulmonary vasodilation following H-IH by reducing bioavailable NO.

Mechanisms of IH-induced PH are generally considered to be similar to those resulting from chronic sustained hypoxia (CH), including pulmonary vasoconstriction, arterial remodeling, and polycythemia (12). Whereas NO mediates an important protective influence to limit vasoconstriction and arterial remodeling in PH (9, 34), NO bioavailability is often reduced in both experimental and human forms of PH as a result of diminished NO synthesis (26) or increased scavenging of NO by $O_2^-$(13). Indeed, lung ROS levels are increased in IH mice (27), and ROS are implicated as contributing factors to both vasoconstrictor (3, 43) and vascular remodeling components of PH (28). However, IH effects on pulmonary vasoreactivity to
NO and regulation of this response by ROS have not previously been addressed, and were the focus of the present study.

**IH Augments EDNO-Dependent Pulmonary Vasodilation by Increasing VSM Sensitivity to NO and cGMP**

CH increases pulmonary arterial eNOS expression and enhances EDNO-dependent pulmonary vasodilation, responses that may mitigate the PH response (15, 31, 35, 36, 38). Although similar alterations in pulmonary vasoreactivity are not apparent following 2 wk IH exposure in rats (40), our present results demonstrate that 4 wk E-IH treatment augments vasodilation to the Ca$^{2+}$ ionophore, ionomycin. Interestingly, endogenous O$_2$ masked a similar increase in endothelium-dependent vasodilation in lungs from H-IH rats. Therefore, supplemental CO$_2$ during IH exposure appears to protect against the deleterious effects of ROS to limit endothelium-dependent vasoreactivity in the hypertensive pulmonary vasculature. Furthermore, enhanced EDNO-dependent reponsiveness following IH was not associated with increased arterial eNOS levels, suggesting that pulmonary eNOS expression is differentially regulated by IH and CH.

IH-mediated increases in EDNO-dependent vasodilation could result from greater VSM sensitivity to NO. In agreement with this possibility, vasoreactivity to the NO-donor SNAP was augmented following E-IH in both isolated lungs and endothelium-disrupted small pulmonary arteries. Furthermore, scavenging O$_2$ revealed a similar increase in reactivity to NO in lungs and arteries isolated from H-IH rats, consistent with effects of O$_2$ to limit EDNO-dependent dilation following H-IH. Because responses to SNAP were PKG-dependent, and since IH also augmented VSM reactivity to the PKG agonist, 8pCPT-cGMP, greater NO/cGMP-mediated vasodilation following IH appears to be mediated in large part by enhanced PKG signaling.

Whereas CH similarly augments pulmonary VSM sensitivity to NO and cGMP, distinct differences exist between CH and IH with respect to the mechanism of this response. For example, enhanced NO induced pulmonary vasodilation following CH is mediated by an increase in PKG-dependent myofilament Ca$^{2+}$ desensitization through inhibition of the RhoA/Rho kinase signaling pathway (19). Conversely, our present findings demonstrate that augmented VSM sensitivity to NO and 8pCPT-cGMP following IH is associated with a greater fall in VSM [Ca$^{2+}$], suggesting a Ca$^{2+}$ dependency of this response, although these data do not preclude a potential contribution of changes in Ca$^{2+}$ sensitivity to the observed responses. Furthermore, in contrast to effects of CH to upregulate PKG expression (18), arterial PKG levels were unaltered by IH exposure. Therefore, enhanced NO-dependent vasodilation following IH is likely a function of greater cGMP-mediated PKG activation or amplification of distal components of this signaling pathway. Our present observation that IH augments vasodilation to the PDE-resistant cGMP analog 8pCPT-cGMP (5) argues against a role of altered PDE activity in this response. Future studies, however, are needed to dissect the mechanism by which IH potentiates VSM PKG signal transduction to mediate this response, including possible increases in sarcolemmal Ca$^{2+}$ extrusion, greater sarcoplasmic reticulum Ca$^{2+}$ sequestration, or reduced Ca$^{2+}$ entry.

**Enhanced Pulmonary VSM Reactivity to NO following Hypocapnic-IH is Masked by O$_2$**

Candidate ROS species that limit pulmonary vasodilation to both endogenous and exogenous NO following H-IH include O$_2$, H$_2$O$_2$, both of which have been implicated in the regulation of pulmonary vascular tone (4). However, a primary role for O$_2$ in this response is indicated by the effect of the O$_2$ spin trap tiron to selectively restore NO-dependent vasoreactivity in both isolated lungs and arteries from H-IH rats, whereas responses to NO were unaltered by H$_2$O$_2$ scavenging with PEG-catalase. This effect of O$_2$ to attenuate NO-mediated vasodilation may result from increased vascular O$_2$ generation following H-IH exposure as suggested by greater DHE fluorescence in arteries from this group compared with both E-IH and sham-treated vessels. Although it is not clear why tiron was without effect on NO-mediated vasoreactivity following E-IH despite significantly elevated O$_2$ generation in the hypertensive pulmonary circulation to mediate this response, including possible increases in sarcolemmal Ca$^{2+}$ extrusion, greater sarcoplasmic reticulum Ca$^{2+}$ sequestration, or reduced Ca$^{2+}$ entry.

Based on our findings that endogenous O$_2$ does not alter cGMP-mediated dilation in arteries from H-IH rats, we postulate that O$_2$ limits NO-dependent responses proximal to PKG activation, possibly through oxidation of sGC (17) or direct scavenging of NO to generate ONOO$^-$ (13). In agreement with the latter possibility, we found that H-IH increases arterial nitrotyrosine levels, suggesting that production of ONOO$^-$ is elevated in response to H-IH. Consistent with this observation, Nisbet and colleagues (27) reported that increases in lung O$_2$ generation following long-term IH exposure in mice are associated with a reduction in bioavailable NO.

Although the vascular cell type responsible for IH-induced ROS generation has yet to be identified, our present finding that O$_2$ inhibits NO-mediated vasodilation in endothelium-disrupted arteries from H-IH rats supports a role for a nonendothelial source of O$_2$ in this response. Therefore, the likely sources of O$_2$ following H-IH are either the VSM (44) or adventitial fibroblasts (23, 37), both of which are reported to be sites of O$_2$ generation in the hypertensive pulmonary circula-
tion (23, 27). The enzyme responsible for increased ROS generation following IH remains unknown. However, NADPH oxidase isoforms (23, 25, 27) and xanthine oxidase (16) are the major sources of ROS implicated in the development of PH, and are candidates for mediating elevated ROS production in response to IH. Indeed, NADPH oxidase-generated ROS play an important role in the development of IH-induced PH and vascular remodeling in mice (27). Alternatively, elevated O₂ levels in arteries from H-IH rats could result from decreased superoxide dismutase expression or activity, as previously demonstrated in PH resulting from CH (29). Elucidating the cellular and enzymatic sources of ROS that limit NO-dependent pulmonary vasodilation following H-IH are important areas for future investigation.

Administration of inhaled CO₂ has previously been demonstrated to interfere with the development of RV hypertrophy, arterial remodeling, polycythemia, and PH resulting from CH (21, 33) and IH in rats (40). In agreement with these findings, we present our observations that CO₂ supplementation attenuates both RV hypertrophic and polycythemic responses to 4 wk IH treatment. Although the mechanism responsible for this protective effect of CO₂ is currently unknown, it is possible that CO₂ reduces lung oxidant stress to attenuate hypoxic PH, as previously demonstrated in response to 10% CO₂ exposure in CH neonatal rats (21). Consistent with this possibility, we found that pulmonary arterial O₂ levels were lower in arteries from E-IH compared with H-IH rats, and further, that NO-dependent vasodilatory responsiveness was unaltered by O₂ scavenging in isolated lungs and arteries from E-IH rats. However, whether supplemental CO₂ attenuates the development of IH-induced PH by limiting oxidative stress to increase NO bioavailability remains to be established.

In conclusion, the present study demonstrates a novel effect of IH to augment NO-dependent pulmonary vasodilation by increasing VSM sensitivity to NO and cGMP. However, enhanced pulmonary vasoreactivity to NO following H-IH was masked by increased O₂ scavenging in isolated lungs and arteries from E-IH rats. Furthermore, whether supplemental CO₂ attenuates the development of IH-induced PH by limiting oxidative stress to increase NO bioavailability remains to be established.

ACKNOWLEDGMENTS

We thank Minerva Murphy and Dr. Jessica Snow for technical assistance.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-92598 (N. L. Jernigan), HL-82799 (N. L. Kanagy); HL-58124, HL-95640, and HL-07736 (B. R. Walker); HL-88192 and HL-77876 (T. C. Resta).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


J Appl Physiol • VOL 111 • OCTOBER 2011 • www.jap.org
NO-DEPENDENT VASODILATION FOLLOWING INTERMITTENT HYPOXIA


