Unaltered vasoconstrictor responsiveness after iNOS inhibition in lungs from chronically hypoxic rats

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Resta, Thomas C., Theresa L. O’Donaughy, Scott Earley, Louis G. Chicoine, and Benjimen R. Walker. Unaltered vasoconstrictor responsiveness after iNOS inhibition in lungs from chronically hypoxic rats. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L122–L130, 1999.—Previous studies suggest that inducible (i) nitric oxide synthase (NOS) expression within the pulmonary vasculature is increased in rats with chronic hypoxia (CH)-induced pulmonary hypertension. We therefore hypothesized that enhanced iNOS expression associated with CH causes attenuated pulmonary vasoconstrictor responsiveness. To test this hypothesis, we examined the effect of selective iNOS blockade with L-N(ω-[(1-iminoethyl)lysine dihydrochloride (L-NIL)] and nonselective NOS inhibition with N-nitro-L-arginine (L-NNA) on vasoconstrictor responses to U-46619 in isolated saline-perfused lungs from both control and CH (4 wk at 380 mmHg) rats. We additionally measured pulmonary hemodynamic responses to L-NIL in conscious CH rats (fraction of inspired O2 = 0.12). Finally, iNOS mRNA levels were assessed in lungs from each group of rats using ribonuclease protection assays. Despite a significant increase in iNOS mRNA expression after exposure to CH, responses to U-46619 were unaltered by L-NIL but augmented by L-NNA in lungs from both control and CH rats. Pulmonary hemodynamics were similarly unaltered by L-NIL in conscious CH rats. We conclude that iNOS does not modulate pulmonary vasoconstrictor responsiveness after long-term hypoxic exposure.

isolated rat lungs; conscious rats; Western blotting; ribonuclease protection assay; gene expression; lipopolysaccharide; inducible nitric oxide synthase

PULMONARY ARTERIAL hypertension develops in response to generalized airway hypoxia after prolonged residence at high altitude and in patients with chronic obstructive pulmonary diseases. This chronic hypoxia (CH)-induced pulmonary hypertension places a greater afterload on the right ventricle, resulting initially in right ventricular hypertrophy, and may ultimately lead to complications associated with right heart failure in severe cases of lung disease (22, 26). Hypoxic pulmonary vasoconstriction, increased blood viscosity resulting from polycythemia, and pulmonary arterial remodeling are responsible for the pulmonary arterial hypertension that occurs with CH (6, 21). Associated with the development of vascular remodeling are morphological and functional changes in the pulmonary arterial endothelium, including cellular hypertrophy and hyperplasia, and increased synthesis and release of both endothelium-derived mitogenic and vasoactive factors (2, 11, 16, 19, 20, 25, 29, 31). However, the potential influence of these mediators on the development of CH-induced pulmonary hypertension is not entirely understood.

Nitric oxide (NO) is a potent pulmonary vasodilator produced by three isoforms of nitric oxide synthase (NOS) within the lung, namely endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS; see Refs. 11–13, 19, 20, 23, 25, 28, 29, 33, 34). Evidence that endogenously produced NO attenuates hypoxic pulmonary vasoconstriction (3) and exhibits antimitogenic properties in vascular smooth muscle (8) suggests a potential role for NO in modulating the vasoconstriction and arterial remodeling associated with CH. Consistent with this possibility, several recent studies suggested that both eNOS and iNOS are upregulated within the pulmonary vasculature after exposure to CH (12, 13, 20, 30, 34). Studies from our laboratory have further demonstrated that CH selectively augments endothelium-derived NO (EDNO)-dependent pulmonary arterial dilation (25), a response associated with upregulation of immunoreactive eNOS within the pulmonary arterial, but not venous, vasculature (23). However, whether the reported upregulation of pulmonary vascular iNOS (13, 20, 34) is similarly associated with altered vasoactivity is not clear. We therefore hypothesized that enhanced iNOS expression during CH causes diminished pulmonary vasoconstrictor responsiveness. To examine this possibility, we tested the effect of selective iNOS blockade on pulmonary vasoconstrictor reactivity in isolated lungs from control rats, CH rats, and rats treated with lipopolysaccharide (LPS) to induce pulmonary iNOS. We further examined pulmonary hemodynamic responses to iNOS inhibition in conscious CH rats and assessed pulmonary iNOS protein and mRNA levels in lungs from each group using Western blotting and ribonuclease protection assays (RPA), respectively.

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. Male Sprague-Dawley rats (250–400 g; Harlan Industries) were used for all experiments.

Experimental Groups

Animals designated for CH exposure were housed in a hypobaric chamber with barometric pressure maintained at 380 mmHg for a period of 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, animals 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 Po₂ (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.

Separate groups of animals were administered LPS (20 mg/kg ip; serotype 0127:B8; Sigma) or saline vehicle (2 ml/kg ip) 5 h before lung removal. LPS was solubilized in sterile saline at 10 mg/ml and was stored in aliquots at −80°C. This method of LPS administration has been previously reported to induce pulmonary iNOS in rats (9).

**Isolated Lung Experiments**

Effects of N-nitro-L-arginine and L-N^6-(1-iminoethyl)lysine dihydrochloride on segmental concentration-response curves to U-46619 in lungs from control and CH rats. The procedure for lung isolation has been described previously (25). Experiments were designed to examine the effect of iNOS inhibition on total, arterial, and venous constrictor responses to the thromboxane mimetic U-46619 (9,11-di-deoxy-9α,11α-methanoxyprostaglandin F₂α) in lungs from control and CH rats. U-46619 provides consistent and stable pressor responses in this preparation and, unlike hypoxia, constricts both arterial and venous segments of the pulmonary vasculature. Lungs were perfused with normal physiological saline solution (PSS) or PSS containing either the selective iNOS inhibitor L-N^6-(1-iminoethyl)lysine dihydrochloride (L-NIL) (10 µM; Calbiochem; see Refs. 5 and 17) or the nonselective NOS inhibitor N^-nitro-L-arginine (L-NNA; 300 µM; Sigma). This concentration of L-NIL is slightly above the reported IC₅₀ for mouse eNOS (1−3.3 µM) and below the reported IC₅₀ for human eNOS (10.4 µM) and rat nNOS (92 µM) as determined in NOS enzyme kinetic assays (5, 17). Furthermore, we have previously demonstrated that the concentration of L-NNA employed in the current study provides effective inhibition of EDNO-dependent pulmonary vasodilation in the isolated perfused rat lung, and this inhibition is reversed by administration of excess L-arginine but not of α-arginine (25, 28). At the end of the 30-min equilibration period, baseline capillary pressure (Pc) was assessed by a double-occlusion procedure as previously described (25). After assessment of baseline Pc, U-46619 was added to the perfusate reservoir to preconstrict both arterial and venous segments of the pulmonary vasculature until a stable arterial pressor response of ~10 mmHg was achieved. Pc was assessed at the plateau of the pressor response by double occlusion. The vasculature was then dilated with 2.5 nM AVP, and Pc was determined again at the point of maximal vasodilation by double occlusion. We have previously demonstrated that the nonselective NOS inhibitor L-NNA attenuates vasodilatory responses to AVP in this preparation (25, 28).

**Conscious Rat Experiments**

Surgical preparation of CH rats. CH rats were instrumented for measurement of pulmonary arterial pressure, systemic arterial pressure, heart rate (HR), and cardiac output (CO) for subsequent assessment of pulmonary and systemic hemodynamic responses to L-NIL. Implantation of pulsed Doppler blood flow probes for determination of CO as well as pulmonary arterial, femoral arterial, and femoral venous catheters was performed as previously described (24, 32). CH rats were returned to the hypobaric chamber the morning after combined catheter and Doppler flow probe implantation surgeries. Animals were allowed ≥5 days to recover before experimentation. All rats demonstrated normal behavior, including food and water intake, before the start of experiments.

Surgical preparation of vehicle- and LPS-treated rats. A separate group of animals was instrumented with femoral arterial and venous catheters (PE-10) as previously described (24) to demonstrate the ability of L-NIL to inhibit iNOS in vehicle- and LPS-treated conscious rats. Rats were allowed ≥2 days to recover from catheter implantation. On the day of experimentation, rats were administered either saline vehicle or LPS (20 mg/kg ip) 5 h before study.

Experimental protocols. Animals were placed in a Plexiglas chamber (25 × 15 × 10 cm) of sufficient size to allow free movement but small enough to discourage excessive exploration. The bottom of the chamber was covered with fresh bedding. The chamber was continuously flushed with either room air or a mixture of 12% O₂-88% N₂ depending on the protocol. All catheters were opened and flushed with sterile heparinized saline. Pulmonary arterial and aortic catheters were connected to Spectramed model P23 XL pressure transducers. Both pulsatile and mean pressures were recorded on separate channels of a Gould model RS-3600 chart recorder. The pulsatile arterial pressure signal was processed by a Gould Biotech amplifier to record HR continuously. For CH rats, CO was monitored by connecting the pulsed Doppler flow probe to a directional, pulsed Doppler flowmeter (VF-1; Crystal Biotech), and the mean signal was continuously recorded as kilohertz of Doppler shift on one channel of the chart recorder. Sensitivity and range gate controls of the Doppler unit were adjusted to obtain a maximal Doppler shift and to set the diastolic phase of the signal to electronic zero. Doppler shift varies with linear velocity of blood and therefore with volume flow as long as the radius of the vessel under the probe remains constant. Previous studies have shown a strong linear correlation between volume flow determined by either thermodilution or electromagnetic techniques and Doppler flowmetry (10). The atmosphere of the chamber was monitored continuously with Ametek CO₂ and O₂ analyzers during each experiment. Animals were allowed ≥30 min to acclimate to their surroundings before experimentation. Collection of baseline data was begun once rats were calm and once mean systemic arterial pressure (MSAP), mean pulmonary arterial pressure (MPAP), HR, and CO had stabilized. At

Refs. 25 and 28) in lungs from control animals. After assessment of baseline Pc, U-46619 was added to the perfusate reservoir to preconstrict both arterial and venous segments of the pulmonary vasculature until a stable arterial pressor response of ~10 mmHg was achieved. Pc was assessed at the plateau of the pressor response by double occlusion. The vasculature was then dilated with 2.5 nM AVP, and Pc was determined again at the point of maximal vasodilation by double occlusion. We have previously demonstrated that the nonselective NOS inhibitor L-NNA attenuates vasodilatory responses to AVP in this preparation (25, 28).
L124  iNOS BLOCKADE AFTER CHRONIC HYPOXIA

this time, one of the following experimental protocols was performed on each group of rats. Multiple protocols were performed on all animals, with a minimum of 24 h between experiments. LPS was administered to rats only after vehicle control studies had been completed. LPS was administered only one time to each animal.

Effect of L-NIL on pulmonary hemodynamics in CH rats. CH rats were studied under hypoxic conditions (12% O2) to reproduce the inspired PO2 to which the animals had been acclimated. Baseline data were collected for $\geq 5$ min, after which an infusion of either saline (20 µl·kg$^{-1}$·min$^{-1}$ iv) or L-NIL (100 µg·kg$^{-1}$·min$^{-1}$ iv) was begun and continued for 30 min. This dose of L-NIL is the reported ID$\text{50}$ for inhibition of LPS-induced plasma nitrate production in conscious rats (5), which is well below the ED$\text{50}$ for elevation of MSAP (540 µg·kg$^{-1}$·min$^{-1}$; see Ref. 5). Arterial blood samples were collected for measurement of blood gases (Radiometer ABL 30 acid-base analyzer) and hematocrit at the conclusion of the experiment.

Validation of L-NIL specificity in vehicle- and LPS-treated rats. Parallel protocols to those for CH rats were conducted to examine the effect of L-NIL on systemic arterial pressure in endotoxemic and vehicle control animals under normoxic conditions.

Western Blotting for iNOS

Separate sets of rats from each group were anesthetized with pentobarbital sodium (25 mg ip), and their lungs were quickly isolated and snap frozen in liquid nitrogen for use in both Western analyses and RPA (see below). Whole left lungs were fragmented with a mortar and pestle cooled in liquid nitrogen and then were homogenized on ice in 10 mM Tris-HCl buffer (pH 7.4) containing 255 mM sucrose, 2 mM EDTA, 12 mM leupeptin, 1 mM pepstatin A, 0.3 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma). Tissue homogenates were centrifuged at 1,500 g at 4°C for 10 min to remove insoluble debris. Protein concentrations of samples were determined by the Bradford method (Bio-Rad Protein Assay). Tissue sample proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 7.5% acrylamide. In addition to samples, each gel included molecular-weight standards (Bio-Rad) and both iNOS and eNOS standards (mouse macrophage and human endothelial lysates, respectively; Transduction Laboratories). The separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) and were blocked overnight at 4°C with 5% nonfat milk, 3% bovine serum albumin (Sigma), and 0.05% Tween 20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.5). Blots were incubated for 4 h at room temperature with a mouse monoclonal antibody raised against mouse iNOS (1:10,000; Transduction Laboratories) in TBS. Immunchemical labeling was achieved by incubation for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Bio-Rad) in TBS followed by chemiluminescence labeling (Amersham enhanced chemiluminescence). iNOS protein bands were detected by exposure to chemiluminescence-sensitive film. Membranes were stained with Coomassie brilliant blue to confirm equal protein loading per lane.

Construction of ProbeTemplates for RPA

Probe templates for RPA were constructed for rat iNOS and rat malate dehydrogenase (MDH). To amplify segments of rat cDNA for these genes, polymerase chain reaction (PCR) primers were developed based on the archived mouse DNA sequences (GenBank accession nos. M29462 and MMU 43428 for MDH and iNOS, respectively). PCR primer sequences were 5'-TCCGACACCCATCAGAC-3' (forward) and 5'-GATACGACGGTACATCAGGGAC-3' (reverse) for MDH and 5'-GACATGCACAGAAGCTGC-3' (forward) and 5'-GGGCTCTTGTGGATGCTAAAG-3' (reverse) for iNOS. These primers generate products of 110 and 253 bp, respectively. Primers were used to amplify their respective target sequences in a PCR using Pyrococcus furiosus (Pfu) DNA polymerase (Stratagene). cDNA was reverse transcribed from rat lung RNA in reactions containing 0.1 µl of total RNA, 10 µM oligo (dT)$_8$ (Perkin-Elmer), 200 µM each dNTP, and 20 U of avian myeloblastosis virus reverse transcriptase (Promega). Pfu PCR consisted of 5 µl of rat cDNA, 5 units of Pfu DNA polymerase, 0.1–0.5 µM each primer, and 100–250 µM each dNTP. PCR runs consisted of 4-min incubation at 94°C, followed by 20–30 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min. Final extension was performed at 72°C for 10 min. To prepare the MDH and iNOS PCR products for use as probe templates, alternate reverse (antisense) PCR primers were obtained for these genes that had the sequence for the T7 RNA polymerase promoter (5'-TAATACGACTCACTATAGGGAGGA-3') added to the 5'-end of the original reverse primer. The new primer was used to reamplify the original PCR products and the T7 sequence incorporated in the new PCR product in an orientation that allows for the expression of the antisense strand. The identities of the PCR products were confirmed by sequencing.

RPA for iNOS mRNA

iNOS and MDH probes were amplified by Pfu PCR as described above, purified using the StratPrep PCR Purification Kit (Stratagene), and ethanol precipitated. Radiolabeled antisense cDNA was prepared by incubating 1 µg of template DNA in the presence of 10 units of T7 RNA polymerase and 50 µCi of [α-32P]UTP (800 Ci/mmol; 20 mCi/ml; Amersham), using the MAXiScribe in vitro transcription kit (Ambion) according to the manufacturer’s recommendations. Full-length probes were purified by polyacrylamide gel electrophoresis on a 5% Tris-borate-EDTA (TBE)-urea gel. RPA was performed using reagents supplied by the RPA II kit (Ambion) according to the manufacturer’s recommendations. Aliquots of the labeled probes containing 4 × 10$^5$ counts/min (cpm) of the labeled and 6 × 10$^5$ cpm of MDH were mixed with 10 µg of total RNA and incubated overnight at 42°C. Hybridization reactions were digested with a mixture of denatured RNase T1 (100 U/ml) and RNase A (2.5 U/ml) for 30 min at 37°C. Digestion products were precipitated, resuspended in 8 µl of formamide loading buffer, loaded on a 5% TBE-urea gel, and electrophoresed on 200 volts (constant) for 25 min. Gels were used to expose a Phosphor Storage Screen (Molecular Dynamics), the screens were scanned with a STORM 860 PhosphorImager (Molecular Dynamics), and the respective bands were quantitated using ImageQuant software. MDH was used as a constitutively expressed internal control for RNA quantity and quality. Preliminary experiments demonstrated that MDH expression in lung tissue was unaltered by CH. iNOS mRNA abundance was determined by dividing the band volumes for iNOS bands by those of the corresponding MDH bands. Additional hybridizations containing 2.5, 5, or 10 µg of lung RNA isolated from a CH rat were performed and subjected to RPA as above to demonstrate the linearity of the relationship between iNOS and MDH mRNA band volumes and input RNA quantity.
Assessment of Polycythemia and Right Ventricular Hypertrophy

Blood samples were obtained from control and CH rats by direct cardiac puncture for measurement of hematocrit at the time of lung isolation for Western and RPA assays. Right ventricular hypertrophy was also assessed as an index of CH-induced pulmonary hypertension using previously described methods (24). Briefly, after isolation of the heart, the atria and major vessels were removed from the ventricles, and the right ventricle was dissected from the left ventricle and septum. The degree of right ventricular hypertrophy was assessed by calculating the ratio of right ventricular to total ventricular weight.

Calculations and Statistics

Total pulmonary vascular resistance in isolated, perfused lungs was calculated as the difference between arterial pressure and venous pressure divided by flow. Pulmonary arterial resistance was calculated as the difference between arterial pressure and \( PC \) divided by flow. Similarly, pulmonary venous resistance was calculated as the difference between \( PC \) and venous pressure divided by flow. Vasodilatory responses were calculated as a percent reversal of U-46619-induced vasoconstriction for the total pulmonary vasculature as well as for arterial and venous segments.

Total peripheral resistance (TPR) was calculated by dividing MSAP by CO for conscious animal experiments. Similarly, total pulmonary resistance (TPUR) was calculated by dividing MPAP by CO. Because Doppler flow probes were not calibrated for actual volume flow, all CO, TPR, and TPUR data are expressed as a percent of baseline values.

All data are expressed as means \( \pm SE \). Values of \( n \) refer to the number of animals in each group. Where appropriate, a one-way analysis of variance (ANOVA), two-way ANOVA or Student's t-test was used to make comparisons. The Mann-Whitney rank sum test or Kruskal-Wallis one-way ANOVA on ranks test was used instead of a Student's t-test or one-way ANOVA, respectively, for data that failed the tests for normality or equal variance. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls test for all pairwise comparisons or Dunnett's test for comparisons versus a control. All data expressed as percentages were normalized using the arcsine transformation before statistical analysis with appropriate parametric tests. A level of \( P < 0.05 \) was accepted as statistically significant for all comparisons.

RESULTS

CH-Induced Polycythemia and Right Ventricular Hypertrophy

CH rats exhibited polycythemia as evidenced by a significantly greater hematocrit (69 \( \pm \) 1\%, \( n = 9 \)) compared with that in control animals (47 \( \pm \) 1\%, \( n = 8 \)). Furthermore, greater right ventricular-to-total ventricular weight ratios were observed for CH rats (0.347 \( \pm \) 0.009, \( n = 9 \)) compared with controls (0.212 \( \pm \) 0.006, \( n = 8 \)), thus demonstrating the development of right ventricular hypertrophy indicative of pulmonary hypertension associated with long-term hypoxic exposure.

Isolated Lung Experiments

Baseline segmental vascular resistances. Table 1 shows total, arterial, and venous baseline resistances in lungs from each group of animals. CH resulted in significant increases in total and arterial baseline resistances as previously demonstrated (25), providing functional evidence for the development of pulmonary arterial remodeling. As expected, venous resistances were not different between control and CH groups. Neither nonselective NOS inhibition with L-NNA nor selective iNOS blockade with L-NIL altered total or segmental baseline resistances in lungs from either control or CH rats, suggesting that endogenous NO synthesis is not involved in determining basal tone in this preparation.

Treatment with LPS was without effect on either total, arterial, or venous baseline resistances. Interestingly, L-NIL significantly elevated arterial resistance in lungs from LPS-treated rats while having no effect on venous baseline resistance. However, arterial resistance was also slightly but significantly greater in L-NIL-treated lungs from the vehicle group compared with untreated lungs.

Effects of L-NNA and L-NIL on segmental concentration-response curves to U-46619 in lungs from control and CH rats. Figure 1 depicts vasoconstrictor responses to U-46619 in lungs from control rats in the presence of 300 \( \mu M \) L-NNA or 10 \( \mu M \) L-NIL. Whereas NO synthesis inhibition with L-NNA significantly augmented total and segmental responses to U-46619, the selective iNOS inhibitor L-NIL had no effect on U-46619-induced vasoconstriction. Similarly, total vascular reactivity to U-46619 was largely augmented by L-NNA in lungs from CH rats (Fig. 2A). This enhanced vasoconstrictor responsiveness after NO synthesis inhibition in lungs from CH rats was primarily a consequence of profound augmentation of arterial constriction to U-46619 (Fig. 2B), since venous reactivity was only modestly influenced by L-NNA (Fig. 2C). These data are consistent with our previous finding that eNOS is upregulated selectively within the arterial vasculature after exposure to CH (23). In contrast, L-NIL was without effect on either total or segmental vasoconstrictor responsiveness in lungs from CH rats.

Table 1. Baseline segmental vascular resistances in isolated, saline-perfused lungs

<table>
<thead>
<tr>
<th></th>
<th>( n )</th>
<th>Total</th>
<th>Arterial</th>
<th>Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>0.091 ( \pm ) 0.004</td>
<td>0.047 ( \pm ) 0.003</td>
<td>0.044 ( \pm ) 0.004</td>
</tr>
<tr>
<td>Control + L-NNA</td>
<td>5</td>
<td>0.095 ( \pm ) 0.004</td>
<td>0.050 ( \pm ) 0.004</td>
<td>0.045 ( \pm ) 0.003</td>
</tr>
<tr>
<td>Control + L-NIL</td>
<td>11</td>
<td>0.090 ( \pm ) 0.004</td>
<td>0.048 ( \pm ) 0.004</td>
<td>0.043 ( \pm ) 0.002</td>
</tr>
<tr>
<td>CH</td>
<td>6</td>
<td>0.123 ( \pm ) 0.013*</td>
<td>0.083 ( \pm ) 0.009*</td>
<td>0.039 ( \pm ) 0.005</td>
</tr>
<tr>
<td>CH + L-NNA</td>
<td>6</td>
<td>0.109 ( \pm ) 0.010</td>
<td>0.076 ( \pm ) 0.007*</td>
<td>0.032 ( \pm ) 0.003</td>
</tr>
<tr>
<td>CH + L-NIL</td>
<td>6</td>
<td>0.122 ( \pm ) 0.010*</td>
<td>0.083 ( \pm ) 0.008*</td>
<td>0.038 ( \pm ) 0.005</td>
</tr>
<tr>
<td>Vehide</td>
<td>5</td>
<td>0.083 ( \pm ) 0.007</td>
<td>0.045 ( \pm ) 0.003</td>
<td>0.039 ( \pm ) 0.005</td>
</tr>
<tr>
<td>Vehide + L-NIL</td>
<td>5</td>
<td>0.089 ( \pm ) 0.003</td>
<td>0.057 ( \pm ) 0.003†</td>
<td>0.032 ( \pm ) 0.002</td>
</tr>
<tr>
<td>LPS</td>
<td>5</td>
<td>0.087 ( \pm ) 0.003</td>
<td>0.039 ( \pm ) 0.002</td>
<td>0.047 ( \pm ) 0.003</td>
</tr>
<tr>
<td>LPS + L-NIL</td>
<td>5</td>
<td>0.104 ( \pm ) 0.004</td>
<td>0.065 ( \pm ) 0.005‡</td>
<td>0.039 ( \pm ) 0.005</td>
</tr>
</tbody>
</table>

Values are means \( \pm SE \); \( n \), number of rats. Resistances are in mmHg \( \cdot \) ml \( \cdot \) min \( \cdot \) kg body wt. CH, chronic hypoxia; LPS, lipopolysaccharide; L-NNA, N\(^{-}\)-nitro-L-arginine; L-NIL, L-N\(^{-}\)-(1-iminoethyl)lysine dihydrochloride. *\( P < 0.05 \) vs. corresponding control value. †\( P < 0.05 \) vs. vehicle. ‡\( P < 0.05 \) vs. LPS.
Effects of L-NIL on segmental concentration-response curves to U-46619 in lungs from vehicle-treated and LPS-treated rats. Total, arterial, and venous responses to U-46619 were unaffected by L-NIL pretreatment in lungs from vehicle-treated rats (Fig. 3). Consistent with induction of iNOS, lungs isolated from LPS-treated rats demonstrated markedly attenuated vasoconstrictor responsiveness compared with vehicle controls. iNOS inhibition with L-NIL significantly increased responses to U-46619 for both arterial (Fig. 3B) and venous (Fig. 3C) segments of the pulmonary vasculature in LPS lungs. Interestingly, total vasoconstrictor responses in the LPS + L-NIL group (Fig. 3A) were significantly greater than those observed in lungs from vehicle-treated rats. This effect of L-NIL was due entirely to greater arterial responsiveness to U-46619 (Fig. 3B), since venous responses were not different between the LPS + L-NIL and vehicle groups (Fig. 3C).

Effects of L-NIL on segmental responses to an EDNO-dependent dilator in lungs from control rats. Preconstriction with U-46619 resulted in similar increases in total (0.270 ± 0.021 vs. 0.275 ± 0.018 mmHg·ml⁻¹·min⁻¹·kg body wt), arterial (0.142 ± 0.021 vs. 0.142 ± 0.013 mmHg·ml⁻¹·min⁻¹·kg body wt), and venous (0.127 ± 0.006 vs. 0.133 ± 0.007 mmHg·ml⁻¹·min⁻¹·kg body wt) resistances between L-NIL-treated (n = 6) and untreated lungs (n = 6) from control rats, respectively. Furthermore, there was no significant difference in the concentration of U-46619 required to preconstrict lungs in each group (202 ± 8 nM for L-NIL treated vs. 250 ± 29 nM for untreated). Total and arterial vasodilatory responses to the EDNO-dependent pulmonary vasodilator AVP (2.5 nM; see Refs. 25 and 28) were unaltered by L-NIL pretreatment (Fig. 4). However, a slight but significantly greater venodilatory response to AVP was observed in lungs pretreated with L-NIL compared with untreated lungs.

Conscious Rat Experiments

Baseline hemodynamics. Table 2 illustrates baseline data for MSAP, MPAP, and HR in vehicle-treated, LPS-treated, and CH rats. No differences in hemodynamic variables were observed before infusion of either saline or L-NIL within each group of rats. However, LPS treatment was associated with significant systemic hypertension. This finding is in contrast to previous work demonstrating prolonged hypotensive responses...
to LPS in anesthetized rats (27). However, anesthesia and acute surgical stress greatly alter cardiovascular control mechanisms (4) and may therefore attenuate compensatory responses to LPS that would otherwise maintain systemic pressure. Consistent with this possibility are earlier studies in conscious rats indicating that, although hypotension occurs initially after administration of LPS, the animals return to normotensive values after 4–6 h (1, 7). CH rats exhibited pulmonary arterial pressures consistent with pulmonary hypertension and further showed elevated HR compared with controls as previously described (24).

Hemodynamic responses to L-NIL in CH rats. Consistent with our isolated lung data, MPAP and TPuR were unaltered by L-NIL in conscious CH rats breathing 12% O2 (Table 3), suggesting that iNOS does not alter hypoxic vasoconstrictor responsiveness in vivo. MSAP, CO, and TPR were similarly unaffected by L-NIL infusion in CH rats. Blood gas values were not different after 30 min of saline vs. L-NIL infusion (pH 7.524 ± 0.009 vs. 7.506 ± 0.005; PCO2 19.9 ± 2.0 vs. 23.0 ± 0.9; PO2 39.5 ± 1.9 vs. 39.7 ± 2.1, respectively).

Hemodynamic responses to L-NIL in vehicle- and LPS-treated rats. L-NIL significantly elevated MSAP in LPS-treated rats as shown in Table 3, demonstrating the effectiveness of L-NIL in inhibiting iNOS in conscious rats and supporting a role for iNOS in modulating basal arterial pressure in endotoxemia as previously suggested (7, 27). This ~11-mmHg increase in MSAP with L-NIL infusion is substantial considering LPS-treated animals exhibited significant hypertension under baseline conditions (Table 2). In contrast, L-NIL was without effect on MSAP in vehicle-treated rats. HR also was unchanged with L-NIL infusion in all groups (Table 3).

### Table 2. Baseline hemodynamic variables for vehicle-treated, LPS-treated, and CH conscious rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>MSAP, mmHg</th>
<th>MPAP, mmHg</th>
<th>HR, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>7</td>
<td>105.2 ± 1.8</td>
<td>103.5 ± 2.1</td>
<td>371 ± 9</td>
</tr>
<tr>
<td>L-NIL</td>
<td>9</td>
<td>118.6 ± 1.5*</td>
<td>117.7 ± 3.3*</td>
<td>376 ± 24</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>100.3 ± 2.7</td>
<td>35.5 ± 1.8</td>
<td>426 ± 8.5*</td>
</tr>
<tr>
<td>L-NIL</td>
<td>5</td>
<td>96.2 ± 2.1</td>
<td>36.8 ± 0.7</td>
<td>439 ± 3.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. MSAP, mean systemic arterial pressure; MPAP, mean pulmonary arterial pressure; HR, heart rate. Data from vehicle-treated and LPS-treated animals were obtained during normoxia; data from CH animals were obtained during hypoxia (12% O2). All data are before infusion of either saline or L-NIL. *P < 0.05 vs. corresponding vehicle value.

Figure 3. Total (A), arterial (B), and venous (C) resistance changes to U-46619 in lungs from vehicle-treated rats perfused in the presence (vehicle + L-NIL; n = 5) or absence (vehicle; n = 5) of L-NIL (10 µM) and lipopolysaccharide (LPS)-treated rats perfused in the presence (LPS + L-NIL; n = 5) or absence (LPS; n = 5) of L-NIL (10 µM). Data are means ± SE. *P < 0.05, LPS + L-NIL vs. LPS. †P < 0.05 vs. vehicle.

Figure 4. Segmental vasodilatory responses to arginine vasopressin (2.5 nM) in untreated (n = 6) and L-NIL-treated (10 µM; n = 6) lungs from control rats. Data are means ± SE. *P < 0.05 vs. untreated.

Figure 5 depicts Western blots for iNOS in lungs from each group of rats. Similar to previous reports (13, 34), faint bands for iNOS were detected at ~120 kDa, a molecular mass identical to that observed for the mouse macrophage iNOS protein standard (not shown). The protein band detected immediately above the iNOS band migrated to the known molecular mass for eNOS (~135 kDa) and comigrated with the human endothelial lysate eNOS protein standard (not shown), suggesting some cross-reactivity of the antibody with eNOS. Additionally, the protein in the upper band was de-
tected in greater quantities in lungs from CH rats compared with controls, consistent with previous reports that eNOS is upregulated after long-term exposure to hypoxia (12, 13, 23, 29, 34). Although iNOS was detected in abundance in lung homogenates of LPS-treated rats, relatively low levels of the enzyme were detected in lungs from control, CH, and vehicle-treated rats. iNOS bands derived from control, CH, and vehicle-treated lungs did not provide sufficient signal above background to allow quantitation of band density.

### iNOS mRNA levels

Figure 6A shows RPA bands for iNOS and MDH mRNA in lungs from control and CH rats, as well as examples of bands from an LPS lung. Similar to results from Western analyses, only faint bands for iNOS message were detected in control, CH, and vehicle groups, whereas relatively large quantities of iNOS mRNA were apparent in lungs from LPS-treated rats. Figure 6B depicts mean data for iNOS mRNA levels normalized to MDH mRNA. Data from control and CH rats represent the means of 2 separate RPA assays. As expected, iNOS message levels were largely increased by LPS administration (25-fold). Furthermore, exposure to CH resulted in an approximately twofold increase in iNOS transcript levels. Levels of MDH mRNA did not differ between groups.

### DISCUSSION

The present study examined the putative role of iNOS in modulating pulmonary vasoconstrictor responsiveness after exposure to CH. The major findings from this study are 1) selective iNOS inhibition with L-NIL had no effect on pulmonary vasoconstriction to U-46619 in isolated lungs from either control or CH rats, although nonselective NOS blockade with L-NNA significantly augmented vasoconstrictor reactivity in both groups; 2) L-NIL markedly augmented responses to U-46619 in lungs from rats treated with LPS to induce pulmonary iNOS; 3) responses to the EDNO-dependent pulmonary vasodilator AVP were unaltered by L-NIL in lungs from control animals; 4) L-NIL elevated MSAP in conscious LPS-treated rats but had no effect on either pulmonary or systemic hemodynamics in conscious CH rats; and 5) CH was associated with an approximately 2.5-fold increase in iNOS transcript levels.

### Table 3. Hemodynamic responses to L-NIL in vehicle-treated, LPS-treated, and CH conscious rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>ΔMSAP, mmHg</th>
<th>ΔMPAP, mmHg</th>
<th>ΔHR, beats/min</th>
<th>CO, %</th>
<th>TPR, %</th>
<th>TPuR, %</th>
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<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>7</td>
<td>2.6 ± 1.8</td>
<td>1.4 ± 1.5</td>
<td>14 ± 14</td>
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<tr>
<td>L-NIL</td>
<td>9</td>
<td>1.4 ± 1.5</td>
<td></td>
<td>−2 ± 9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>0.8 ± 1.5</td>
<td></td>
<td>7 ± 5</td>
<td></td>
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</tr>
<tr>
<td>L-NIL</td>
<td>5</td>
<td>10.8 ± 2.3*</td>
<td>−16 ± 9</td>
<td>93.9 ± 3.5</td>
<td>89.9 ± 6.2</td>
<td></td>
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<tr>
<td><strong>LPS (12% O₂)</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>4</td>
<td>0.0 ± 3.5</td>
<td>−2.2 ± 1.4</td>
<td>1 ± 5</td>
<td>106.4 ± 3.0</td>
<td></td>
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</tr>
<tr>
<td>L-NIL</td>
<td>3</td>
<td>3.5 ± 2.0</td>
<td>−0.6 ± 0.2</td>
<td>−2 ± 7</td>
<td>99.5 ± 3.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. Δ, Change in; CO, cardiac output; TPR, total peripheral resistance; TPuR, total pulmonary resistance. Data from vehicle-treated and LPS-treated animals were obtained during normoxia; data from CH animals were obtained during hypoxia (12% O₂). All data are after 30-min infusion of either saline (20 µl·kg⁻¹·min⁻¹) or L-NIL (100 µg·kg⁻¹·min⁻¹). CO, TPR, and TPuR data represent percentage of preinfusion values. *P < 0.05 vs. saline.

![Fig. 5. Western blots for inducible nitric oxide synthase (iNOS) in lungs from control (n = 6), CH (n = 6), vehicle-treated (n = 6), and LPS-treated (n = 6) rats. iNOS was identified as a single band at ~120 kDa.](image)

![Fig. 6. A: iNOS and malate dehydrogenase (MDH) mRNA bands from lung homogenates of control, CH, and LPS-treated rats. Lane on left depicts undigested probes (P) for iNOS and MDH. Single species were identified for iNOS and MDH at 253 and 110 bp, respectively. B: mean data showing iNOS mRNA normalized to MDH mRNA in lungs from control (CON; n = 5), CH (n = 5), vehicle-treated (VEH; n = 5), and LPS-treated (n = 5) rats. Data from control and CH rats represent the means of 2 separate ribonuclease protection assays. Data are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. vehicle treated.](image)
twofold induction of pulmonary iNOS mRNA, whereas a 25-fold increase was observed in response to LPS. These results suggest that iNOS does not influence vasoconstrictor reactivity in chronic hypoxic pulmonary hypertension.

Previous studies suggested that CH is associated with increased iNOS protein and mRNA expression in whole lung tissue (13, 20, 34). This increased expression has been localized further to the smooth muscle and endothelial layers of the pulmonary vasculature as demonstrated by immunohistochemistry and in situ hybridization (20, 34). Considering iNOS is tonically active and exhibits high efficacy for NO synthesis (18), such an upregulation of pulmonary vascular iNOS would be expected to have profound effects on pulmonary vascular reactivity. Consequently, we hypothesized that lungs isolated from CH rats would exhibit attenuated vasoconstrictor responsiveness due to increased pulmonary iNOS expression. Contrary to our hypothesis, however, the present findings indicate that vasoconstriction to U-46619 was unaltered by L-NIL in lungs from either control or CH rats. These results suggest that iNOS does not attenuate vasoconstrictor responses after CH and are consistent with previous studies demonstrating maintained or even increased reactivity to vasoconstrictors in lungs from CH rats compared with controls (14, 15). In contrast to L-NIL, L-NNA augmented responses to U-46619 in lungs from both groups of animals, demonstrating that eNOS activity likely buffers resistance changes to U-46619 in this preparation as previously reported (33). Consistent with these findings from isolated lungs, iNOS inhibition with L-NIL was without effect on pulmonary hemodynamics in conscious CH rats breathing a hypoxic gas mixture.

The selectivity of L-NIL for iNOS inhibition in isolated lungs was evident by the lack of effect of L-NIL on pulmonary vasoconstrictor responses to U-46619. If L-NIL had affected eNOS activity, an increased responsiveness to U-46619 similar to that observed with L-NNA would have been expected. Our finding that vasodilatory responses to the EDNO-dependent pulmonary vasodilator AVP (25, 28) are unaltered by L-NIL pretreatment provides additional support that L-NIL did not exhibit nonspecific inhibitory effects on eNOS in this preparation. Furthermore, L-NIL greatly potentiated U-46619-induced vasoconstriction in lungs from rats treated with LPS, a compound that we have presently demonstrated to induce pulmonary iNOS, suggesting that L-NIL effectively inhibited iNOS activity. It is possible that the lack of responsiveness to L-NIL in lungs from CH rats is a consequence of decreased accessibility of L-NIL to iNOS compared with lungs from LPS-treated rats. However, preliminary immunohistochemistry data for iNOS in a lung from an LPS-treated rat demonstrated that iNOS was localized to macrophages widely distributed throughout the lung parenchyma (data not shown). In contrast, iNOS was undetectable in a lung from a CH rat. However, it is clear from our isolated lung data that iNOS was effectively inhibited by L-NIL despite its extravascular location in lungs from LPS-treated rats. Therefore, one would expect greater accessibility of L-NIL to iNOS in CH lungs, considering previous reports demonstrating iNOS immunoreactivity within the medial and endothelial layers of pulmonary arteries from CH rats (20). L-NIL further appeared to exhibit specificity for iNOS inhibition in conscious rats, since infusion of the drug elevated MSAP in LPS-treated animals but not in vehicle-treated rats.

An interesting observation from these studies is that L-NIL did not restore vasoreactivity to U-46619 to levels comparable to lungs from vehicle-treated animals but rather augmented vasoconstrictor responsiveness beyond that observed in vehicle controls. Furthermore, baseline pulmonary arterial resistance was increased by L-NIL in lungs from LPS-treated animals. This enhanced responsiveness to U-46619 after L-NIL administration and increased baseline resistance in lungs from LPS-treated rats occurred selectively within the arterial vasculature, suggesting that iNOS inhibition unmasked an LPS-induced vasoconstrictor influence within the arterial circulation. The reason for this altered arterial reactivity associated with acute endotoxemia is not clear but may represent the stimulated release of a vasoconstrictor or increased arterial smooth muscle sensitivity to U-46619.

Considering the lack of effect of L-NIL on pulmonary vasoreactivity to U-46619 in lungs from CH rats and on pulmonary hemodynamics in conscious rats, we questioned whether iNOS gene and protein expressions are increased in lungs from CH rats. Consistent with previous reports (13, 20, 34), pulmonary iNOS transcript levels were elevated ~2-fold after chronic hypoxic exposure, whereas a 25-fold increase in iNOS mRNA expression was observed with LPS-induced endotoxemia. Although Western analysis revealed distinct iNOS bands in lungs from control and CH rats, they were below the sensitivity required for quantitation. In contrast, LPS treatment considerably increased pulmonary iNOS protein expression, findings that are entirely consistent with our physiological data from isolated lungs. It is possible that our Western assays did not provide the required sensitivity to detect any differences in iNOS protein that may have existed between lungs from control and CH rats. Nevertheless, the relatively low levels of iNOS protein in lungs from CH rats do not appear to play a significant role in modulating pulmonary vascular tone.

In summary, we have demonstrated that iNOS inhibition with L-NIL does not alter pulmonary vasoconstrictor responsiveness in isolated, saline-perfused lungs from CH rats. Similarly, L-NIL infusion had no effect on pulmonary hemodynamics in conscious CH rats. Consistent with these physiological responses, iNOS mRNA and protein were expressed at relatively low levels in our rat model of chronic hypoxic pulmonary hypertension. Together, these findings suggest that iNOS activity is of minor physiological significance with respect to vasoreactivity in the setting of chronic hypoxic pulmonary hypertension.
REFERENCES


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