Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia

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Resta, Thomas C., Louis G. Chicoine, John L. Omdahl, and Benjimem R. Walker. Maintained upregulation of pulmonary eNOS gene and protein expression during recovery from chronic hypoxia. Am. J. Physiol. 276 (Heart Circ. Physiol.: 45): H699–H708, 1999.—We previously demonstrated augmented endothelium-derived nitric oxide (EDNO)-dependent pulmonary arterial dilation and increased arterial endothelial nitric oxide synthase (eNOS) levels in chronic hypoxic (CH) and monocrotaline (nonhypoxic) models of pulmonary arterial hypertension. Therefore, we hypothesized that the long-term elevation of arterial eNOS levels associated with CH is related to pulmonary hypertension or some factor(s) associated with hypertension and not directly to hypoxia. To test this hypothesis, we examined responses to the EDNO-dependent dilator ionomycin in U-46619-constricted, isolated, saline-perfused lungs from control rats, CH (4 wk at 380 mmHg) rats, and rats previously exposed to CH but returned to normoxia for 4 days or 2 wk. Microvascular pressure was assessed by double-occlusion technique, allowing calculation of segmental resistances. In addition, vascular eNOS immunoreactivity was assessed by quantitative immunohistochemistry, and eNOS mRNA abundance was determined by RT-PCR assays. Our findings indicate that 4-day and 2-wk posthypoxic rats exhibit persistent pulmonary hypertension, likely due to maintained arterial remodeling and polycythemia associated with prior exposure to CH. Furthermore, arterial dilation to ionomycin was augmented in lungs from each experimental group compared with controls. Finally, arterial eNOS immunoreactivity and whole lung eNOS mRNA levels remained elevated in posthypoxic animals. These findings suggest that altered vascular mechanical forces or vascular remodeling contributes to enhanced EDNO-dependent arterial dilation and upregulation of arterial eNOS in various models of established pulmonary hypertension.

quantitative immunohistochemistry; reverse transcription-polymerase chain reaction; endothelial nitric oxide synthase; pulmonary hypertension; vascular remodeling

PULMONARY HYPERTENSION associated with long-term exposure to hypoxia is characteristic of chronic obstructive and severe restrictive pulmonary diseases, as well as residence at high altitude. This chronic hypoxia (CH)-induced pulmonary hypertension places a greater afterload on the right ventricle, resulting initially in right ventricular hypertrophy and, ultimately, in right heart failure in severe cases of lung disease. Three major physiological responses mediate the pulmonary arterial hypertension that occurs with CH: polycythemia, pulmonary arterial remodeling, and active arterial vasoconstriction. On restoration of normoxia, hypertension persists because of the slow regression of hematocrit and structural changes within the lung (17, 23).

Endothelium-derived nitric oxide (EDNO) is a potent pulmonary vasodilator that could potentially modulate the development of CH pulmonary arterial hypertension. For example, EDNO has been demonstrated to inhibit vasoconstrictor responses to acute hypoxia (4). Furthermore, reports suggest that EDNO exhibits anti-inflammatory properties in vascular smooth muscle (8) and may therefore inhibit the development of vascular remodeling in response to CH. Consequently, an increase in EDNO production with long-term hypoxic exposure could potentially alleviate some of the deleterious consequences associated with chronic pulmonary hypertension. Consistent with this possibility, several recent studies have demonstrated increases in pulmonary eNOS activity, protein, and mRNA levels after CH (9–11, 20, 32, 38). Studies from our laboratory have further demonstrated that CH selectively augments EDNO-dependent pulmonary arterial dilation, a response associated with upregulation of immunoreactive eNOS within the pulmonary arterial vasculature (25, 27). However, controversy exists concerning the relative roles of hypoxia per se vs. pulmonary hypertension or factors associated with hypertension, such as altered vascular shear forces or vascular remodeling, in mediating these responses to CH. We previously reported a similar increase in pulmonary arterial endothelial nitric oxide synthase (eNOS) levels and enhanced EDNO-dependent responsiveness within the arterial vasculature of rats with monocrotaline-induced pulmonary hypertension. Taken together, these findings suggest a primary role for altered vascular mechanical forces or vascular remodeling in mediating the augmented EDNO-dependent arterial dilation and upregulation of eNOS in hypoxic and nonhypoxic models of pulmonary hypertension. In contrast, a recent study by Le Cras et al. (10) demonstrated that reduction of blood flow to the left lung by left pulmonary arterial stenosis does not prevent the upregulation of eNOS after CH, suggesting that CH may increase eNOS expression independently of hemodynamic changes associated with pulmonary hypertension. Therefore, the purpose of the present study was to determine whether EDNO-dependent responses and eNOS gene/protein expression are augmented in an alternative nonhypoxic model of established pulmonary hypertension. Specifically, we examined responses to the non-receptor-mediated EDNO-dependent dilator ionomycin, as well as eNOS immunoreactivity, mRNA abundance, and vascular re-
modeling, in lungs isolated from rats previously exposed to CH but returned to normoxia for 4 days or 2 wk. These posthypoxic animals exhibit persistent pulmonary arterial remodeling and polycythemia associated with prior exposure to CH and, therefore, remain pulmonary hypertensive in the absence of hypoxia. We hypothesized that factors associated with pulmonary hypertension and not hypoxia per se contribute to the augmented vasoreactivity to EDNO-dependent dilators and upregulation of eNOS in established CH pulmonary hypertension.

MATERIALS AND 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

Rats were divided into four groups: control rats, CH rats, and rats previously exposed to CH but returned to normoxia for 4 days or 2 wk (posthypoxic groups). Control rats were housed at ambient barometric pressure (~630 mmHg). Animals designated for CH exposure were housed in a hypobaric chamber with barometric pressure maintained at 380 mmHg for 4 wk, as previously described (27).

Measurement of Pulmonary Arterial Pressure in Conscious Rats

Because anesthesia and acute surgical stress greatly alter cardiovascular control mechanisms, experiments were performed using chronically instrumented, unrestrained rats to demonstrate the persistence of pulmonary hypertension in posthypoxic groups.

Surgical preparation. Animals from each experimental group were chronically instrumented with pulmonary arterial and aortic catheters for measurement of pulmonary arterial pressure, systemic arterial pressure, and heart rate, as previously described (35). Catheters were implanted 2 days before measurement of basal hemodynamic variables. CH animals were returned to the hypobaric chamber on the morning after surgery.

Experimental protocols. Animals were prepared for experimentation and data were collected as previously described (26). Collection of baseline data was begun once rats were calm and mean arterial blood pressure (MABP), heart rate (HR), and pulmonary arterial pressure (PAP) had stabilized. Control and 2-wk posthypoxic rats were studied under normoxic conditions, and arterial blood samples were taken for measurement of blood gases (Radiometer ABL30 acid-base analyzer) and hematocrit after collection of baseline hemodynamic data. For CH rats, MABP, HR, PAP, and arterial blood gases were initially measured under hypoxic conditions (12% O2). This 12% O2 gas mixture was chosen to reproduce the inspired PO2 to which the CH rats had been acclimated. The gas mixture was then switched to room air, and animals were allowed ~5 min to become accustomed to normoxia. The atmosphere of the chamber was monitored continuously with Ametek CO2 and O2 analyzers during each experiment. Baseline MABP, HR, PAP, blood gases, and hematocrit were collected under normoxic conditions once animals were calm and demonstrated stable hemodynamic parameters. To demonstrate the persistence of pulmonary hypertension in 4-day posthypoxic rats, these same CH animals were maintained at ambient barometric pressure for 4 days, then studied again under normoxic conditions.

Assessment of Right Ventricular Hypertrophy

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 ventricle to total ventricle weight (RV/T).

Isolated Lung Experiments

We examined total, arterial, and venous vasodilatory responses to the non-receptor-mediated EDNO-dependent vasodilator ionomycin (calcium ionophore; 350 nmol/l) in U-46619-constricted, saline-perfused lungs from each group of animals, as described previously (27).

eNOS Immunohistochemistry

Procedures for eNOS immunostaining have been described in a previous report from our laboratory (25). Control sections were prepared by incubation with mouse IgG (1:2,500; Sigma Chemical) instead of primary antibody. Immunoprecipitation was achieved by incubating all sections for 10 min with 0.07% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB; Sigma Chemical) and 0.002% H2O2. Adjacent sections (10 μm from each eNOS-stained section) were stained for elastin and counterstained with Van Gieson solution (Sigma Accustain Elastic Stain) for accurate identification of arteries and veins and for measurement of vessel wall area and external diameter (see below).

Selection of Vessels for Densitometric Analysis

All vessels subjected to densitometric analysis were first identified as arteries or veins in the elastin-stained sections by the presence or absence of an internal elastic lamina, respectively. Because <60-μm-diameter arteries do not always possess internal elastic laminae (15) and are thus often indistinguishable from veins, only ≥60-μm-diameter veins were selected for analysis. The first 10–20 vessels observed in each elastin-stained section were subsequently identified in the adjacent eNOS-stained sections and then analyzed for eNOS staining intensity to provide an unbiased method for selection of arteries and veins for assessment of eNOS immuno-reactivity. All vessels on which densitometry measurements were made were additionally subjected to morphometric analysis for determination of vessel wall area and external diameter, as described below. Vessels that were sectioned at oblique angles were excluded from analysis. A total of 307 vessels from 23 rats were analyzed. Vessel images were generated with a cooled, digital CCD camera (Photometrics SenSys 1400) from a Nikon Diaphot 300 microscope and processed on a Dell Optiplex GXMT 5166 computer equipped with MetaMorph Imaging System hardware and software (Universal Imaging).

Densitometric Analysis of eNOS Staining

Densitometric analysis of immunocytochemical labeling was performed as described previously (3, 13, 19, 25, 33, 36). eNOS staining intensity is expressed in optical density (OD) units according to the following calculation

$$\text{OD} = -\log_{10}(\text{GL}_{\text{specimen}} - \text{GL}_{\text{background}})/\text{GL}_{\text{white}} - \text{GL}_{\text{background}}$$

where GLspecimen is the gray level of the image containing the vessel of interest, GLbackground is the gray level of the back-
ground reference image (the image obtained with no light to the camera), and GL_white is the gray level of the white reference image (the image obtained from an area of the microscope slide without tissue). Dividing the numerator by GL_white − GL_background compensates for uneven field illumination and for the OD contributed by the glass slide, Permount, and coverslip. GL_white was 3,497 ± 19 on a gray-level scale that ranges from zero (0% light transmittance) to 4,096 (100% light transmittance). Specific staining is defined as the difference in staining intensity between sections incubated with primary antibody and those treated with mouse IgG (negative control). eNOS staining intensity was calculated as the average OD of those areas containing specific staining. The maximum OD of any pixel of images from sections incubated in the presence of mouse IgG was 0.140. Therefore, areas of specific staining that were selected by the computer for calculation of average OD were those with an OD > 0.140. OD was calibrated using a stepped OD filter (Edmund Scientific) with OD steps ranging from 0 to 1 in increments of 0.1. All measurements were made using green-wavelength illumination.

**RT-PCR Assay**

Lungs from each group of rats were snap frozen in liquid nitrogen. Total RNA was isolated and purified from crushed frozen lungs by the acid-phenol-guanidinium method of Chomczynski and Sacchi (5). RT reactions (20 µl) contained 1.0 µg frozen lungs by the acid-phenol-guanidinium method of Chomczynski and Sacchi (5). RT reactions (20 µl) contained 1.0 µg of total cellular RNA, 200 U of murine Moloney leukemia virus reverse transcriptase (Perkin-Elmer), 5.0 µmol/l oligo(dT)16, 1.0 mmol/l dNTPs, and 3.0 mmol/l MgCl2. Reactions were incubated at room temperature for 10 min, at 42°C for 1 h, and then at 94°C for 5 min. PCR reactions contained 1.0 µmol/l specific oligonucleotide primers for eNOS [5’-TAGGGGACCAAATCACC-3’ (forward) and 5’-CAGGCTGCGACATCTTTTGTGAT (reverse)] and malate dehydrogenase [MDH; 5’-GAAGCCTGCGTATACAACT-3’ (forward) and 5’-TTTCA-GCTCAGGGATGACC-3’ (reverse)]. Oligomers were slightly modified from the published sequences of Ujiie et al. (34) and Schwartz et al. (31) for eNOS and MDH, respectively. The housekeeping gene MDH was used as a constitutively expressed internal control for cDNA quantity and quality. Preliminary experiments demonstrated that MDH expression did not change with exposure to hypoxia (data not shown). PCR reactions included 5.0 µl of RT product, 3 mmol/l MgCl2, 1× PCR Buffer (Invitrogen), 1.0 mmol/l dNTPs, and 1 U AmpliTag polymerase (Perkin-Elmer). The mixed samples were heated to 94°C for 4 min and then cycled as follows: 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min for 30 cycles. Final extension was 5 min at 72°C. PCR products were visualized and sized by 1.5% agarose gel (0.5 mg/ml ethidium bromide) electrophoresis. Gels were photographed using Polaroid 667 film and digitized using an Epson 636 scanner. Band density analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the National Institutes of Health). The PCR product sizes were the expected 819 and 507 bp for eNOS and MDH, respectively. Preliminary PCR reactions demonstrated that 30 cycles was well within the linear range for each reaction. This was assessed by running multiple PCR reactions, as described above, except with increasing cycle number (25, 30, 35, and 40 cycles) for each reaction. Reactions were linear up to 35 cycles for eNOS and MDH. The sequences of PCR products were determined with an ABI Prism Dye Terminator Cycle Sequencer with the Ready Reaction Kit (Perkin-Elmer). Briefly, portions of agarose gels containing the separated PCR products were cut out, and the cDNA band was extracted according to the QIAEX II protocol (QIAGEN) and processed for sequencing. Results confirmed PCR product identity.

**Assessment of Vascular Remodeling**

Digital images of vessels used for determination of remodeling were acquired from elastin-stained sections and processed using Metamorph Imaging System hardware and software (Universal Imaging). Measurements included medial circumference, assessed from the outer margin of the external elastic lamina, and luminal circumference. Vessel diameters were calculated from the medial circumference. Vessel wall area was determined by subtracting the calculated area from that of the luminal circumference. Vessels that were sectioned at oblique angles were excluded from analysis.

**Statistical Comparisons**

Values are means ± SE; n refers to the number of vessels in each group for those statistical comparisons of vessel wall area; for all other comparisons, n refers to the number of animals in each group. Where appropriate, one- or two-way ANOVA was used to make comparisons. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls test. P < 0.05 was accepted as statistically significant for all comparisons. All data expressed as percentages were normalized using the arcsine transformation before statistical analysis.

**RESULTS**

**Conscious Animal Experiments**

Blood gases and hematocrit. Table 1 shows arterial blood gas values for control and 2-wk posthypoxic rats under normoxic conditions and for CH rats in normoxia and hypoxia (12% O2). Hypoxia was associated with a significant decrease in arterial PO2 in CH rats. In addition, arterial PCO2 and pH showed the anticipated changes that occur with hypoxic stimulation of ventilation. Values for PO2 were significantly increased and PCO2 decreased in CH rats under normoxic conditions compared with control and 2-wk posthypoxic animals, thus reflecting the hyperventilation and acid-base adjustments known to result from CH exposure. Blood gas values did not differ between control and 2-wk posthypoxic rats. Blood gases were not measured in 4-day posthypoxic rats.

CH rats exhibited polycythemia as evidenced by significantly greater hematocrit (64 ± 1%, n = 7) than
posthypoxic rats. In hematocrit were observed between 4-day and 2-wk significantly declined by 4 days after hypoxia. No differences in hematocrit in 4-day and 2-wk groups compared with cessation of hypoxic exposure. Consistent with the fall in hematocrit 4-day and 2-wk groups compared with normoxic conditions is likely accounted for by the maintained pulmonary hypertension un-

certainly greater PAP than controls, indicating the persistence of pulmonary hypertension even 2 wk after cessation of hypoxic exposure. Consistent with the fall in hematocrit in 4-day and 2-wk groups compared with normoxic conditions. There were no differences in PAP between 4-day and 2-wk posthypoxic rats. CH rats, mean PAP also was significantly decreased by 4 days after hypoxia compared with CH rats under normoxic conditions. There were no differences in PAP between 4-day and 2-wk posthypoxic rats.

Right Ventricular Hypertrophy

CH was associated with significant right ventricular hypertrophy, as demonstrated by increased RV/T in CH rats (0.369 ± 0.006, n = 6) compared with controls (0.219 ± 0.005, n = 12). Values of RV/T were also significantly greater in 4-day (0.328 ± 0.007, n = 8) and 2-wk (0.293 ± 0.007, n = 6) posthypoxic rats than in controls, consistent with maintained pulmonary hypertension in these animals. However, some regression of right ventricular hypertrophy was observed at 4 days after hypoxia, as demonstrated by a significantly decreased RV/T compared with CH rats, which parallels the slight fall in PAP in these animals shown in Fig. 1. The mechanism by which this rapid regression of hypertrophy occurred is not clear but may involve decreased cellular mass, degradation of matrix components, or perhaps extracellular fluid loss. A further decrease in RV/T was observed in 2-wk compared with 4-day posthypoxic rats.

Isolated Lung Experiments

Baseline segmental resistances. Figure 2 shows total and segmental baseline vascular resistances in lungs isolated from each experimental group of rats. CH resulted in significant increases in total and arterial

| Table 3. Segmental vascular resistance changes in response to U-46619 |
|----------------|----------------|----------------|
|                | Total          | Arterial       | Venous         |
| Control        | 0.289 ± 0.011  | 0.153 ± 0.007  | 0.136 ± 0.006  |
| CH             | 0.317 ± 0.023  | 0.201 ± 0.018  | 0.116 ± 0.010  |
| 4-Day posthypoxic | 0.300 ± 0.012  | 0.192 ± 0.020  | 0.108 ± 0.013  |
| 2-Wk posthypoxic | 0.358 ± 0.035  | 0.251 ± 0.032  | 0.107 ± 0.018  |

Values are means ± SE in mmHg·ml⁻¹·min⁻¹·kg body wt. *P < 0.05 vs. corresponding control value.
baseline resistances, as previously demonstrated (27). A similar increase in total and arterial resistance was observed in 4-day posthypoxic rats, providing evidence for the persistence of arterial remodeling in these animals. Although total pulmonary vascular resistance also tended to be greater in 2-wk posthypoxic rats, this did not reach statistical significance. However, arterial resistance was significantly increased in 2-wk posthypoxic rats compared with controls. These findings suggest that arterial remodeling persists even 2 wk after cessation of hypoxic exposure, although some regression of remodeling is apparent, since total and arterial baseline resistances are lower in 2-wk posthypoxic animals than in CH rats. There were no differences in venous resistance among the four groups.

Segmental responses to U-46619. U-46619 elicited similar increases in total, arterial, and venous resistances among lungs from control, CH, and 4-day posthypoxic rats (Table 3). Although total and venous constrictor responses to U-46619 in 2-wk posthypoxic rats were also not different from the other groups, the response of the arterial segment was slightly, but significantly, greater in lungs from 2-wk posthypoxic rats than controls. As previously observed (6, 27), the concentration of U-46619 required to constrict lungs from CH rats (130 ± 10 nmol/l) was significantly less than that required to constrict control lungs (208 ± 10 nmol/l), suggesting that CH enhances the responsiveness of the pulmonary vasculature to U-46619. In contrast, there were no significant differences between the doses of U-46619 needed to constrict lungs from 4-day (165 ± 23 nmol/l) and 2-wk (179 ± 18 nmol/l) posthypoxic rats and those needed to constrict lungs from controls.

Segmental responses to ionomycin. Lungs isolated from CH rats exhibited greater total vasodilatory responses to the non-receptor-mediated EDNO-dependent vasodilator ionomycin than lungs from controls (Fig. 3), as previously described (27). This enhanced vasoreactivity to ionomycin is a consequence of augmented arterial dilation, since venous responses were unaltered by CH. A similar segmental vasodilatory profile was observed in lungs isolated from 4-day and 2-wk posthypoxic groups. There were no significant differences in vasodilatory responses among CH and 4-day and 2-wk posthypoxic groups in total, arterial, or venous segments of the pulmonary vasculature.

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![Fig. 3. Segmental vasodilatory responses (% reversal of U-46619-induced vasoconstriction) to endothelium-derived nitric oxide-dependent dilator ionomycin (350 nmol/l) in lungs from control (n = 14), CH (n = 7), 4-day posthypoxic (n = 8), and 2-wk posthypoxic (n = 6) rats. Values are means ± SE. *P < 0.05 vs. control.](attachment:image)

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![Fig. 4. Digitally acquired images of gray levels (A) and optical density (OD; B) of an artery from a CH rat. Areas of specific endothelial nitric oxide synthase (eNOS) immunostaining (OD > 0.140) that have been selected by computer for calculation of mean OD are depicted in red. Scale bar, 10 µm.](attachment:image)
eNOS Immunohistochemistry

Figure 4 shows an example of an artery (70 µm diameter) from a CH rat with areas of eNOS staining that have been selected by the computer for calculation of mean OD. A digitally acquired gray-level image (GLspecimen) of the artery shows dense staining selectively within the endothelium. In the corresponding OD image (see MATERIALS AND METHODS for calculation), the areas exhibiting specific staining for eNOS (i.e., OD > 0.140) are shown in red. No specific staining was observed in either parenchymal tissue or within the medial or adventitial layers of the vascular wall. eNOS staining intensity for a given vessel was calculated as the mean OD for all pixels contained within areas exhibiting specific staining.

Figure 5 shows examples of eNOS-stained arteries from a control rat (69 µm diameter), a CH rat (70 µm diameter), a 4-day posthypoxic rat (72 µm diameter), and a 2-wk posthypoxic rat (81 µm diameter). As we previously demonstrated (25), pulmonary arteries (20–300 µm diameter) from CH rats exhibit greater eNOS immunoreactivity than those from control animals, whereas no differences in eNOS staining intensity were detected in pulmonary veins (60–300 µm diameter) between the two groups (Fig. 6). A similar upregulation of arterial eNOS was evident in lungs from 4-day and 2-wk posthypoxic rats, whereas venous eNOS staining was unaltered. Furthermore, a significant decline in eNOS staining intensity was observed in arteries from the 2-wk posthypoxic animals compared with the CH rats. Interestingly, eNOS immunoreactivity was more pronounced in arteries than in veins in lungs from each experimental group, including controls, suggesting that arteries characteristically exhibit greater quantities of eNOS than veins, even under normal conditions. Data...
for all diameters of arteries and veins are grouped together, since eNOS staining intensity did not vary significantly as a function of arterial or venous diameter in any of the experimental groups. All arteries examined appeared to be fully dilated, as demonstrated by uncrenated internal elastic laminae.

eNOS mRNA Expression by RT-PCR

Figure 7 depicts representative RT-PCR products for eNOS and malate dehydrogenase (MDH) from each group of rats. B: quantitative densitometry data of RT-PCR products for eNOS mRNA normalized for MDH mRNA from control (n = 4), CH (n = 5), 4-day posthypoxic (n = 5), and 2-wk posthypoxic (n = 4) rats. Values are means ± SE. *P < 0.05 vs. control.

DISCUSSION

The major findings of this study are as follows: 1) Rats previously exposed to CH but returned to nor-

Fig. 7. A: representative RT-PCR products for eNOS and malate dehydrogenase (MDH) from each group of rats. B: quantitative densitometry data of RT-PCR products for eNOS mRNA normalized for MDH mRNA from control (n = 4), CH (n = 5), 4-day posthypoxic (n = 5), and 2-wk posthypoxic (n = 4) rats. Values are means ± SE. *P < 0.05 vs. control.

Vascular Remodeling

Figure 8 illustrates wall area (medial + intimal area) of arteries and veins from each group of animals. CH was associated with increased wall thickness in arteries with external diameters of 20–60, 61–100, and 101–150 µm compared with control rats. Although 151- to 300-µm diameter arteries also tended to exhibit greater wall areas in CH than in control rats, this did not reach statistical significance. Similar increases in wall area were observed in pulmonary arteries from 4-day and 2-wk posthypoxic rats compared with control animals, thus demonstrating the persistence of pulmonary arterial vascular remodeling in posthypoxic groups. Some regression of arterial remodeling did, however, occur at 2 wk after hypoxia in 61- to 100-µm-diameter arteries, as indicated by a significant decrease in wall area compared with the CH group. In contrast to arteries, no differences were observed in wall areas of veins among the four groups.
moxia for 4 days or 2 wk remain pulmonary hypertensive, apparently because of persistent polycythemia and pulmonary vascular remodeling associated with prior hypoxic exposure. 2) Lungs isolated from 4-day and 2-wk posthypoxic rats exhibit augmented EDNO-dependent arterial dilation, similar to that observed in lungs from CH rats. 3) The upregulation of pulmonary arterial eNOS and whole lung eNOS mRNA associated with CH exposure persists after restoration of normoxia for up to 2 wk.

Previous studies have demonstrated enhanced pulmonary vasoreactivity to EDNO-dependent dilators and upregulation of eNOS protein and mRNA in lungs from CH rats (9, 11, 20, 22, 28-30, 32, 38). Recent work from our laboratory has further localized the CH-induced upregulation of eNOS and associated augmentation of EDNO-dependent dilation to the pulmonary arterial circulation (25, 27). A similar increase in pulmonary arterial eNOS levels and enhanced EDNO-dependent responsiveness of the arterial vasculature were observed in a monocrotaline model of pulmonary arterial hypertension (25). In contrast to CH, however, monocrotaline-induced pulmonary hypertension can develop independently of arterial hypoxemia (26). These findings therefore suggest a role for pulmonary hypertension or factors associated with hypertension in maintaining the elevated levels of eNOS and altered vasoreactivity to EDNO-mediated agonists after CH. The correlation of the vascular site of hypertension (arterial) with those of enhanced EDNO-dependent dilation and increased eNOS expression provides additional evidence in support of this possibility.

The present study extended these previous observations by examining EDNO-mediated responses and eNOS mRNA/protein in rats with established pulmonary arterial hypertension resulting from prior hypoxic exposure. Similar to monocrotaline-treated rats, posthypoxic animals exhibited pulmonary hypertension and associated right ventricular hypertrophy in the absence of arterial hypoxemia. The persistence of pulmonary hypertension during recovery from hypoxia is likely a consequence of pulmonary arterial remodeling, as demonstrated functionally by increased baseline arterial resistance to flow in isolated perfused lungs and histologically by increased arterial wall area. Previous studies have described similar persistence of pulmonary hypertension, right ventricular hypertrophy, and vascular remodeling after 2 wk–3 mo of recovery from a degree of hypoxic exposure identical to that used in the present study (17, 23). We therefore hypothesized that if increased arterial eNOS protein/gene expression and augmented arterial reactivity to EDNO-dependent dilators after CH are related to pulmonary arterial hypertension, then lungs from posthypoxic rats should exhibit similar responses.

In agreement with our hypothesis, we have demonstrated enhanced arterial, but not venous, dilation to the non-receptor-mediated EDNO-dependent dilator ionomycin in lungs from CH and posthypoxic rats compared with controls, suggesting that eNOS activity is increased selectively within the arterial vasculature for ≥2 wk after cessation of hypoxic exposure. Previous work from our laboratory has demonstrated that responses to ionomycin in lungs isolated from control and CH rats are largely inhibited by Nω-nitro-L-arginine, a competitive inhibitor of NO synthase, indicating a primary role for NO in mediating these responses (27). Furthermore, the augmented arterial dilation to EDNO-mediated agonists in lungs from CH rats appears to be independent of prostaglandin or endothelium-derived hyperpolarizing factor release and does not likely represent enhanced arterial smooth muscle reactivity to NO after exposure to CH, since segmental pulmonary vascular responses to several NO donors are unaltered by CH (6, 27). Consistent with increased arterial eNOS activity in posthypoxic groups, arteries from 4-day and 2-wk posthypoxic rats exhibited more intense eNOS immunoreactivity than those from control animals, and the increased eNOS gene expression observed in lungs from CH rats is maintained even 2 wk after restoration of normoxia. The similar upregulation of arterial eNOS, whole lung eNOS mRNA levels, and enhanced EDNO-dependent arterial dilation in hypoxic and nonhypoxic models of pulmonary hypertension suggest a role for pulmonary hypertension as a mediator of these events.

The use of quantitative immunohistochemistry to identify changes in eNOS expression provides an advantage over Western blotting, in that it allows determination of differences in eNOS immunoreactivity between arterial and venous segments of the pulmonary vasculature. Western analysis has the additional disadvantage of being unable to distinguish differences between eNOS within the bronchial epithelium and eNOS within the vasculature, which complicates the interpretation of results. Quantitative immunohistochemistry has been used previously by our laboratory and others to demonstrate changes in tissue levels of intracellular peptides (3, 13, 19, 25, 33, 36). This technique is based on the principle that OD varies linearly with the concentration of DAB reaction product (33) and that the quantity of an antibody that binds to its antigen in a tissue section is proportional to the amount of antigen present (3, 19). However, it is not clear whether the development of DAB reaction product reached the saturation point for those arteries from experimental groups with the highest eNOS concentration (i.e., CH and 4-day posthypoxic groups). Therefore, DAB immunostaining intensity may not be linearly related to the amount of eNOS present across the entire range of tissue eNOS concentrations examined. Consequently, this method provides a means of identifying differences in eNOS staining intensity between vessels but does not allow quantification of absolute levels of eNOS.

Although the possibility exists that the maintained upregulation of eNOS in lungs from 4-day and 2-wk posthypoxic rats is secondary to hypoxic stimulation of eNOS gene expression, this seems unlikely considering the half-life of eNOS mRNA has been estimated at ~46 h in cultured bovine pulmonary artery endothelial cells (12), and eNOS protein levels within this same cell type have been demonstrated to decrease by 2.5-fold within 24 h of hypoxic exposure (12). Furthermore, eNOS...
protein and mRNA levels markedly decrease within 24 h of birth in rats (21), suggesting that stability of eNOS protein and transcript may be similar between in vivo and in vitro conditions. Therefore, if hypoxia were the only factor responsible for the maintenance of elevated eNOS levels during CH, then eNOS expression and vasoreactivity would be expected to return to control shortly after restoration of normoxia. In contrast, we have demonstrated maintained upregulation of eNOS protein/mRNA even after 2 wk of recovery from hypoxic exposure. Our present findings do not, however, preclude the possibility that hypoxia is responsible for the initial induction of eNOS associated with long-term hypoxia. Indeed, hypoxia increases the expression of several genes, including those that encode for various hormones, cytokines, and glycolytic enzymes (7), although controversy exists as to whether hypoxia exerts a stimulatory or inhibitory effect on eNOS synthesis in cultured endothelial cells (1, 12, 14). Consistent with a contributing role for hypoxia in the initial induction of pulmonary eNOS during CH are recent findings by Le Cras et al. (10) demonstrating that decreased blood flow to the left lung after surgical stenosis of the left pulmonary artery failed to prevent the upregulation of eNOS associated with CH.

Several mechanisms could potentially account for the increased arterial eNOS expression after CH. One possibility is that increased shear stress on the arterial wall associated with CH-induced pulmonary hypertension mediates this response. Indeed, shear stress increases eNOS mRNA and protein levels in cultured endothelial cells (24), and vessels exposed to chronic elevations in shear stress exhibit augmented endothelium-dependent relaxation (18). Alternatively, increased vascular eNOS levels after CH could potentially be a consequence of the hypertrophy and hyperplasia of endothelial cells associated with pulmonary vascular remodeling (16) or perhaps a result of polycythemia independent of pulmonary hypertension. This latter possibility is supported by evidence that renal vasodilation associated with erythropoietin-induced polycythemia is mediated by increased NO synthesis (37). The mechanism by which polycythemia increases NO synthesis is not understood, but it may be due to upregulation of eNOS secondary to increased wall shear stress or, rather, a consequence of decreased negative-feedback influences of NO on eNOS synthesis due to potentially greater NO-scavenging properties of polycythemic blood. Finally, it is not clear whether the maintained upregulation of pulmonary eNOS protein and mRNA in posthypoxic groups is dependent on increased rates of transcription or increased mRNA/protein stability.

In summary, we have demonstrated maintained pulmonary hypertension in rats after 4 days or 2 wk of recovery from CH. Pulmonary hypertension in posthypoxic animals likely results from persistent polycythemia and pulmonary arterial remodeling associated with prior exposure to hypoxia. We have additionally demonstrated augmented EDNO-dependent arterial dilation in lungs from posthypoxic rats, which is associated with upregulation of arterial eNOS, similar to that previously observed in CH- and monocrotaline-induced pulmonary arterial hypertension (25). Finally, pulmonary eNOS transcript levels remain elevated during recovery from CH. These findings suggest that factors other than hypoxia, such as altered vascular mechanical forces or vascular remodeling, contribute to the maintenance of increased arterial eNOS expression and enhanced arterial reactivity to EDNO-mediated agonists in various models of established pulmonary hypertension.

We thank Ryan Lindsey for expert technical assistance with RT-PCR procedures.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-42778 (R. W. Walker) and T32 HL-06960 (T. C. Resta) and by a fellowship from the American Heart Association with funds contributed in part by the New Mexico Affiliate (T. C. Resta).

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Received 24 March 1998; accepted in final form 28 September 1998.

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