Carbon monoxide promotes hypoxic pulmonary vascular remodeling

MARTHA SUE CARRAWAY, ANDREW J. GHIo, HAGIR B. SULIMAN, JACQUELINE D. CARTER, A. RICHARD WHORTON, and CLAUDE A. PIANTADOSI. Carbon monoxide promotes hypoxic pulmonary vascular remodeling. Am J Physiol Lung Cell Mol Physiol 282: L693–L702, 2002. First published November 16, 2001; 10.1152/ajplung.00211.2001.—CO is a biologically active gas that produces cellular effects by multiple mechanisms. Because cellular binding of CO by heme proteins is increased in hypoxia, we tested the hypothesis that CO interferes with hypoxic pulmonary vascular remodeling in vivo. Rats were exposed to inspired CO (50 parts/million) at sea level or 18,000 ft of altitude [hypobaric hypoxia (HH)], and changes in vessel morphometry and pulmonary pressure-flow relationships were compared with controls. Vascular cell single strand DNA (ssDNA) and proliferating cell nuclear antigen (PCNA) were assessed, and changes in gene and protein expression of smooth muscle α-actin (sm-α-actin), β-actin, and heme oxygenase-1 (HO-1) were evaluated by Western analysis, RT-PCR, and immunohistochemistry. After 21 days of HH, vascular pressure at constant flow and vessel wall thickness increased and lumen diameter of small arteries decreased significantly. The presence of CO, however, further increased both pulmonary vascular resistance (PVR) and the number of small muscular vessels compared with HH alone. CO + HH also increased vascular PCNA and nuclear ssDNA expression compared with hypoxia, suggesting accelerated cell turnover. CO in hypoxia downregulated sm-α-actin and strongly upregulated β-actin. CO also increased lung HO activity and HO-1 mRNA and protein expression in small pulmonary arteries during hypoxia. These data indicate an overall propensity of CO in HH to promote vascular remodeling and increase PVR in vivo.

pulmonary hypertension; hypoxic pulmonary vasoconstriction; actin; heme oxygenase

CHRONIC HYPOXIA ALTERS the structure of pulmonary arterial vessels by inducing proliferation and hypertrophy of smooth muscle in larger and growth of smooth muscle in small arteries that are normally nonmuscular (14, 20, 24). Pulmonary hypertension gradually becomes irreversible, accompanied by right heart hypertrophy and failure. These responses occur in chronic lung and cyanotic heart diseases and in natives and sojourners to high altitude (12). Among the complex cellular and molecular mechanisms regulating the vascular responses to hypoxia is increased expression of heme oxygenase-1 (HO-1) (11), which produces carbon monoxide (CO) endogenously during heme degradation (22). CO, like nitric oxide (NO), can activate guanylate cyclase (15), resulting in smooth muscle relaxation, and the molecule may have other regulatory effects (26). HO-1 gene expression in vascular smooth muscle cells (VSMC) can be activated by hypoxia inducible factor-1 (HIF-1) (11), which regulates transcription of several hypoxia-responsive proteins, including erythropoietin and vascular endothelial growth factor (9, 10). Thus regulation of HO-1 gene expression by HIF-1 suggests a unique function for the enzyme in hypoxia, which could be mediated by CO production and its effects on heme proteins (Hp).

The effects of CO on the pulmonary vessels in hypoxia are unclear. A previous study has shown that inhaled CO does not attenuate hypoxic pulmonary vasoconstriction in isolated rat lungs after chronic hypoxia (3). Exogenous CO inhibits in vitro VSMC growth (16) and HO-1 knockout mice develop more right ventricular hypertrophy in chronic hypoxia than wild-type mice (28), implying HO-1 activity inhibits pulmonary vascular responses to hypoxia. The latter changes are difficult to attribute solely to endogenous CO due to the multifaceted consequences of failing to express HO-1 including increased tissue heme and iron content.

Pulmonary vascular phenotype changes in response to chronic hypoxia differently among species, but in the rat, new smooth muscle in small arteries correlates with increased medial thickness after 3 wk of hypoxia (14), and previously nonmuscular arterioles become muscular (20). Subpopulations of pulmonary VSMC also have been described that have multiple differentiation profiles in hypoxia (1, 19). The VSMC express different contractile and structural elements in hypoxia. For example, expression of smooth muscle-α-actin
(sm-α-actin), a structural protein specific to smooth muscle (19), decreases in proliferating neonatal lung vascular cells after hypoxia but is maintained in mature muscle cells (7).

In the rat lung, HO-1 protein and mRNA increase in the first week of hypoxia, return to control values by 2 wk, followed by a second increase in HO-1 mRNA at 3 wk (4). Because this bimodal change in HO-1 in hypoxic lung makes it difficult to assess the effects of endogenous CO production in specific phases of hypoxia, we studied the effects of exogenous CO on pulmonary vascular structure and function. Based on observations that CO interferes with VSMC proliferation in hypoxia in vitro (16) and the known effects of CO binding on Hp function, we hypothesized that sustained CO exposure would influence pulmonary vascular remodeling in hypoxia by altering vascular metabolism. These effects of CO could either inhibit or promote vessel hypertrophy by its affects on cell turnover rate. We tested the hypothesis in rats exposed to hypoxia or hypoxia plus inspired CO [50 parts/million (ppm)] for 3 wk and found CO accentuated the hypoxic increases in vascular resistance and promoted the expected structural changes in small pulmonary arteries.

The structural changes correlated with changes in cell proliferation, apoptosis, sm-α-actin, β-actin, and HO-1 gene and protein expression in the lungs and pulmonary vasculature.

METHODS

Materials. All materials were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Animal exposures. Rats were exposed to hypobaric hypoxia (HH) using an altitude chamber simulating altitude of 18,000 ft (380 Torr) for 1, 3, 7, 14, or 21 days (n = 8 animals per group) on a protocol approved by the Duke University Animal Care and Use Committee. The chamber was returned briefly to sea level once daily to clean the cages and replenish the food and water supply for the animals. CO exposures were performed at 50 ppm in rats either in air (21% O₂) or at altitude as above (Air + CO and HH + CO). CO levels were monitored continuously using a Toxilog gas detector (Biosystems, Rockfall, CT). After the exposures, the animals were anesthetized with pentobarbital (50 mg/kg ip), an arterial catheter was placed, and blood samples drawn at the altitude to which the rats were exposed. Arterial blood gases, pH, hemoglobin, and carboxyhemoglobin (COHb) were measured on a calibrated blood gas analyzer (Instrumentation Laboratories model 1640) and CO-oximeter (Instrumentation Laboratories model 480).

Tissue preparation. The thorax was opened, and the lungs were flushed gently with 0.9% NaCl through the right ventricle, excised from the hilar structures, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen lungs were later used for Western blot and RT-PCR analysis and to measure HO enzyme activity. The heart was excised and immediately dissected, and the wet hearts were weighed to determine right ventricle to left ventricle plus septum (R/L heart) ratio as a measure of right heart hypertrophy. Lungs from three rats exposed to air, CO + air, hypoxia, or CO + hypoxia were inflation and perfusion fixed with 4% paraformaldehyde for morphometry studies according to the method of Meyrick and Reid (14). Lungs from additional rats in the 14- and 21-day exposure groups were inflation fixed for immunohistochemistry to localize sm-α-actin and HO-1 and assess apoptosis and cell proliferation by staining for single strand DNA (ssDNA) and proliferating cell nuclear antigen (PCNA). Control lung tissue came from animals exposed to air at sea level or air containing 50 ppm CO for 1–21 days.

Pulmonary vessel morphology. Oriented lung sections were cut from both upper and lower lobes in a plane perpendicular to the hilum. Sections were paraffin embedded, cut into 6-μm-thick sections, and stained with hematoxylin and eosin. We avoided large central vessels (>200 μM) by analyzing only vessels in the peripheral 2 mm of lung parenchyma. In addition, the measurements were limited to vessels with a complete circumferential smooth muscle layer. Digital image analysis was used to measure the area of the lung sections that were sampled. Using an ocular grid on a Nikon Optiphot-2 light microscope, an observer unaware of the exposure conditions measured external diameter and wall thickness for each vessel. Eyepiece grid sampling dimensions were determined using a micrometer calibration slide. The grid was used as a systematic guide to ensure 100% sampling of selected tissue areas. The slides were scanned using an EPSON Expression 800, and scanned images were printed and printed with Adobe Photoshop. Printed images were digitized and analyzed with a commercial software package (Quickmeasure 1.6; Tally Systems, San Diego, CA), and the surface area was calculated for each tissue sample.

Muscular vessels were counted and grouped according to diameter into three categories: small, <50 μm; medium, 50–100 μm; and large, >100 μm. Vessel wall thickness as percent diameter [(2 × measured wall thickness/diameter) × 100] was determined from the measurements for small and medium arteries. Lumen diameter was calculated from the measurements of vessel diameter and wall thickness.

Measurement of pulmonary pressure-flow curves. Pulmonary pressure-flow measurements were used to estimate fixed vascular resistance ex vivo in air control rats, after 21 days of Air + CO (50 ppm) or 21 days of HH or HH + CO (50 ppm). Animals were removed from the exposure chambers to room air and anesthetized with halothane, and tracheal catheters were placed. The thorax was opened, and the lungs were inflated with 5 ml of air. The main pulmonary artery (PA) was cannulated through an incision in the right ventricle with fluid-filled PE-70 tubing connected to a calibrated pressure transducer and a calibrated infusion pump. The left atrium was opened, and the lungs were perfused with Ringer lactate at constant flow until PA pressure stabilized. Then, pulmonary pressure-flow curves were recorded at different flows for both increasing and decreasing flow rates. The average of the two values was taken for the pressure at each flow rate.

Western blot analysis. Tissue was homogenized on ice in cold lysis buffer [150 mM NaCl, 50 mM Tris, pH 7.6, 1% SDS (Bio-Rad, Carpenteria, CA), 3% Nonidet P-40, 5 mM EDTA, 1 mM MgCl₂, 2 mM 1,3-dichloroisocoumarin, 2 mM 1,10-phenanthroline, and 0.5 mM E-64]. The homogenate was centrifuged at 10,000 g for 10 min. The supernatant was decanted, and an aliquot was stored at −20°C for measurement of protein concentration. The remaining supernatant was mixed with an equal volume of double-strength Laemmli sample buffer [250 mM Tris-HCl (Bio-Rad), pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% β-mercaptoethanol], divided into aliquots, and stored at −80°C.

Electrophoresis was performed on 12% polyacrylamide gels under reducing conditions using a minigel system (Hoefer Scientific Instruments, San Francisco, CA). All lanes were loaded with 15 μg of protein, and electrophoresis was performed over 1.5 h under constant current of 30 mA. The proteins were electrotransferred on a TE Series Transphor...
unit at 100 V (Hoefler Scientific) to a polyvinylidene fluoride membrane (Millipore, Amersham Life Sciences, Cleveland, OH) and blocked overnight at 4°C in Tris-buffered saline with 0.1% polyoxyethylene sorbitan monolaureate (TBST) containing 5% nonfat dry milk. The following day, the membranes were washed six times over 30 min in TBST at room temperature. Western blots were performed with a rabbit polyclonal antibody against rat HO-1 (Stress-Gen, Vancouver, BC, Canada), mouse sm-α-actin (Sigma), actin (Sigma), and PCNA (Santa Cruz Biotech). Incubation with the primary antibody was performed for 1 h at room temperature at dilutions of 1:1,000 (HO-1) and 1:2,000 (actin) in TBST with 5% milk. After multiple washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated IgG secondary antibody (Jackson Laboratories) diluted 1:10,000 in TBST with 5% milk. The membranes were then washed in TBST, and the signal was detected on Biomax film (Eastman Kodak, Rochester, NY) using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Equal protein loading was confirmed by Coomassie blue staining of the membranes after developing the blots.

**HO activity assay.** HO activity was measured as reported (5, 21) with modification from the method of Tenhunen et al. (22). Briefly, lungs were homogenized on ice in one volume of 100 mM phosphate buffer with 2 mM MgCl2. The homogenates were sonicated and centrifuged, and HO activity in the supernatant was measured using liver cytosol as a source of biliverdin reductase. The reaction mixture also contained 20 mM hemin, 0.8 mM NADPH, 2 mM glucose 6-phosphate, and 0.0016 U/μl of glucose-6-phosphate dehydrogenase. An NADPH-free reaction mixture provided a background sample. Bilirubin was extracted with chloroform and measured on a spectrophotometer based on optical density (OD) at 464 nm minus 530 nm using an extinction coefficient of 40 mM⁻¹ cm⁻¹.

**Immunohistochemistry for sm-α-actin and HO-1.** Inflamed fixed lungs were paraffin embedded and cut into 6-μm sections. Before labeling, the tissue sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. The sections were blocked in a solution of 5% nonfat dry milk, 1% BSA, 5% goat serum in 0.01 M PBS, and 0.1% Triton X-100 before overnight incubation at 4°C with a monoclonal antibody to sm-α actin (Sigma) and HO-1 (Stress-Gen) in 1% milk and 1% BSA in 0.01 M PBS and 0.1% Triton X-100 using a 1:200 dilution. The sections were washed three times with PBS with 0.1% Triton X-100 (5 min each) and incubated with the secondary antibody, biotinylated goat anti-mouse IgG (Jackson Laboratories), at a dilution of 1:1,000 in 1% milk in 0.01 M PBS and 0.1% Triton X-100 at room temperature for 1 h. The signal was detected with peroxidase-conjugated avidin and diaminobenzidine. The slides were counterstained with 1% hematoxylin. Negative control sections were processed as above except primary incubations were performed with nonimmune rabbit serum (Jackson Laboratories) instead of primary antibody.

**Detection of ssDNA and PCNA.** Paraffin-embedded fixed lung tissue was cut into 6-μm sections and placed on slides for labeling PCNA and ssDNA. Immunohistochemical labeling for PCNA was performed using a commercial protocol and antibody to PCNA (Boehringer Mannheim, Indianapolis, IN). The ssDNA method, a specific and sensitive cellular marker for detection of apoptosis before frank internucleosomal DNA fragmentation, required sections to be incubated in 60°C hot formamide (50%) for 30 min and washed in PBS before being stained with monoclonal antibody to ssDNA, diluted 1:100 (Chemicon International, Temecula, CA) using our standard immunohistochemical protocol (see above).

**RT-PCR.** Lung tissue was homogenized (1 g/5 ml) with 4 M guanidine thiocyanate (Boehringer Mannheim), 50 mM sodium citrate, 0.5% sarcosyl, and 0.01 M dithiothreitol. RNA was pelleted by ultracentrifugation through cesium chloride (Boehringer Mannheim) and 0.1 M EDTA. Total RNA (100 ng) was reverse transcribed (M-MLV Reverse transcriptase; Life Technologies), and the resultant cDNA was amplified for 27 and 37 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and HO-1, respectively, in separate reactions using gene-specific primers. Oligonucleotide sequences were synthesized using an Applied Biosystems 391 DNA synthesizer (Foster City, CA) based on sequences published in the GenBank DNA database. The following sense and anti-sense sequences were employed: GAPDH: 5′-CTT AGG AGA AGG CTG GGG-3′ and -5′-CAA AGT TGT CAT GGA TGA CC-3′; HO-1: 5′-ATT GGA GGC TGG AGC TAT GT TCT G-3′ and 5′-CCT TCG GTG CAT CTC CTC AG-5′; sm-α-actin: 5′-CGA TAG AAC AGC GCA TCA TC-3′ and -5′-CAT CAG GCA GGT CGT AGC TC-3′; β-actin: 5′-CCT TCC TGG CCA TGG AGT CCT G-3′ and 5′-GGA GCA ATG ATC TTG ATC TTC-3′.

Amplification products were separated on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The resulting negative (type 655 film; Polaroid, Cambridge, MA) was quantitated using a BioImage Densitometer (BioImage, Ann Arbor, MI). For each experimental condition, the integrated OD of the HO DNA bands were divided by that of the GAPDH DNA band (as a reference gene) to correct for variations in the amount of amplified cDNA in each sample.

**Statistical analysis.** Experimental data from each group were expressed as means ± SE. Statistical analyses were performed with two-way ANOVA (factorial design) with a post hoc comparison test (Fisher’s exact test) using commercially available software (Statview 4.0, Calabasas, CA). A P value of 0.05 or less was accepted as significant.

**RESULTS**

**Physiological responses.** The altitude exposures in this study produced a stable range of arterial Po2 values of 40–46 mmHg both without and with added CO. The responses in hematocrit and R/L heart ratios for rats at each point of study during HH and HH + CO are shown Fig. 1, A and B. In rats exposed to HH alone, the hematocrit increased steadily for 21 days. In rats exposed to HH + CO, hematocrit was significantly lower after 7 and 14 days than HH alone (50.6 ± 2 and 56.2 ± 1.2, respectively, in hypoxia, and 57.6 ± 1.7 and 61 ± 1.7, respectively, in hypoxia, P ≤ 0.05). By 21 days, however, hematocrit in HH + CO–exposed rats approached the values of the HH animals. R/L heart ratios increased similarly in both HH and HH + CO groups at all time points. COHb levels measured in arterial blood at the end of each study (Fig. 1C), used as a marker of exposure, indicate consistent CO exposures. In the HH animals, COHb averaged 1.5 ± 0.14% after 3 days and was stable until 21 days, when COHb reached 2.8 ± 0.1%. This was significantly higher than the 14-day values (P ≤ 0.05). In rats exposed to HH + CO, the mean COHb level remained stable between 3.5% and 3.9% during the entire experiment.

**Pulmonary vascular morphometry.** Changes in pulmonary vascular structure were measured to assess the effect of inspired CO on hypoxic vascular remodel-
ing in the lung. Quantitative measurements were made in four groups of rats including air control, 21-day air + CO (50 ppm), 21-day HH, and 21-day HH + CO (50 ppm). Figure 2 shows summarized data for small (<50 μm; A–C) and medium-sized (50–100 μm; D–F) muscular vessels including wall thickness, lumen diameter, and number of muscular arteries after 21 days of HH + CO or 21 days of HH + CO. Vessel wall thickness as a percentage of total diameter is shown in Fig. 2, A and D. After 21 days of HH + CO, the mean wall thickness of small, muscular arteries had increased significantly compared with air control ani-

Fig. 1. Physiological responses of rats exposed to hypobaric hypoxia (HH) and HH + CO. Mean values are shown for control rats [time (t) = 0] and after exposure to HH (18,000 ft) (●) and HH + CO (50 parts/million (ppm); ●) for up to 21 days. A: hematocrit (Hct) increased significantly in the HH group compared with HH + CO after 7 and 14 days (*P < 0.05 vs. HH + CO). B: right to left (R/L) heart calculated from right ventricle wet weight divided by left ventricle plus septum wet weight. R/L heart ratios increased similarly in the HH and HH + CO groups at all time points. C: carboxyhemoglobin (COHb). In hypoxic animals, COHb increased significantly (to 2.8%) after 21 days of exposure. In rats exposed to HH + CO, COHb levels were stable at 3.5–4% over 21 days (*P < 0.05 compared with control).

Fig. 2. Pulmonary vessel morphometry. A and D: pulmonary vessel wall thickness (WT) expressed as %total diameter (%D) in small (<50 μm) and medium (50–100 μm) muscular vessels. After 21 days of HH, small-artery WT %D increased significantly over control. After 21 days of HH + CO, WT %D in small arteries increased over air control but less than in HH animals. No differences were found in WT %D in medium vessels. B and E: lumen diameter of small and medium-sized muscular vessels. After HH and HH + CO, lumen diameter significantly decreased in small but not medium vessels. C and F: the number of small and medium muscular arteries/cm² in air or HH for 21 days. After 21 days of hypoxia, no difference was found in number of small, muscular arteries/cm² compared with control animals. After 21 days of HH + CO, the number of small muscular vessels/cm² increased relative to control and HH (*P < 0.05 vs. control, #P < 0.05 vs. hypoxia). No differences were found in number/cm² of muscular medium-sized vessels (50–100 μm) compared with control (*P < 0.05 vs. control, #P < 0.05 vs. HH).
mals (Fig. 3A, 22.3% vs. 14.9%, \( P < 0.01 \)). When CO was present during hypoxia, small vessel wall thickness averaged \( -20\% \) after 3 wk, which was greater than control, but significantly less than HH animals (\( P < 0.05 \)). CO alone had no significant effect on small-vessel wall thickness. In medium-sized vessels (Fig. 3D), no differences were found in wall thickness after exposure to HH or HH + CO, whereas air + CO resulted in a slight decrease in wall thickness.

The effects of HH and HH + CO on lumen diameters of small and medium-sized muscular vessels are shown in Fig. 2, B and E. After 3 wk of HH or HH + CO, statistically significant and approximately equivalent decreases were found in the average lumen diameter of small arteries. There was no measurable effect of CO on medium-sized vessels during hypoxia.

The number of small and medium-sized muscular pulmonary arteries per unit surface area (cm\(^2\)) was measured in peripheral lung regions from the lungs of the same animals (Fig. 2, C and F). After 21 days of HH, the number of muscular small arteries per unit area increased from \( \sim 11 \) to 13 vessels/cm\(^2\), but this difference was not statistically significant (\( P = 0.16 \)). After 21 days of HH + CO, however, the number of small, muscular vessels per unit lung had increased to more than 20/cm\(^2\), which was significantly increased over air control and HH rats (\( P < 0.05 \)). There were no differences among any of the groups in number of medium-sized vessels per unit area of lung.

Figure 3 shows the histograms of distribution of vessel size by internal diameter of muscular vessels derived from direct measurements of total vessel diameter and wall thickness. These data demonstrate that HH shifted the distribution of vessels to include more muscular arteries with small diameters. This effect was accentuated by addition of CO in hypoxia, whereas air + CO had no effect relative to air alone. These data indicate that CO increased the number of muscular vessels in the lungs during hypoxia.

Measurement of pulmonary pressure-flow curves. Pressure-flow relationships for the pulmonary vasculature were determined ex vivo in lungs of rats after 21 days of CO exposure in air, HH, and HH + CO, and was determined ex vivo in lungs of rats after 21 days of CO exposure in air, HH, and HH + CO, and

![Fig. 3. Histograms of effects of hypoxia or CO + hypoxia on muscular arteries in the rat lung. A and B: distribution of muscular vessels by internal lumen area in control rats and rats receiving air + CO (50 ppm) for 21 days. C and D: distribution of muscular vessels in rats after HH or HH + CO (50 ppm) for 21 days. Hypoxia shifts the frequency histogram to the left; this effect is accentuated by CO exposure during hypoxia.]

![Fig. 4. Pulmonary pressure-flow measurements in rat lungs. Pressure-flow relationships were determined from stable pulmonary artery (PA) pressure at constant flow rate in control lungs (Air) and lungs of rats after exposure to 21 days of HH, air + CO, or HH + CO (50 ppm). Pulmonary vascular resistance (PVR) was estimated from the ratio of the PA pressure measured ex vivo in perfused lungs at constant flow of 5, 10, 20, and 30 ml/min. Previous exposure to CO in air had no effect on PVR compared with air controls. PVR increased significantly after 21 days of HH (* \( P < 0.01 \)) compared with air and air + CO. After 21 days of HH + CO, PVR was significantly increased over animals exposed to HH (# \( P < 0.01 \)).]
compared with air control rat lungs. PA pressure was measured during perfusion at four controlled flow rates, and mean pressure values are plotted for each of the four groups in Fig. 4. The pulmonary vascular resistance (PVR) was estimated from the ratios of pressure to flow on the curves. Chronic HH significantly increased vascular resistance over air control animals (P < 0.01), whereas 21 days of air + CO exposure had no effect. The administration of CO during 21 days of HH increased PVR significantly more than HH alone (P < 0.01).

Immunohistochemistry for ssDNA and PCNA. To assess cell turnover in the vessels of the lung, immunohistochemistry was performed for ssDNA (as an early marker of apoptosis) and PCNA (as a marker of cell proliferation). Representative immunohistochemistry stains for ssDNA are shown in Fig. 5, from control lungs and after 21 days of HH and HH + CO. In control lung, minimal staining for ssDNA was present (Fig. 5A). After 21 days of hypoxia, nuclear staining for ssDNA was detected in alveolar macrophages and occasional vascular cells (Fig. 5B). After HH + CO (Fig. 5C), ssDNA staining was prominent in both macrophages and vascular cells. Figure 5C, inset, shows strong nuclear staining for ssDNA was present in some cells while neighboring cells were undergoing mitosis.

Figure 6 shows immunostained lung sections for cell proliferation in the same animals using anti-PCNA antibody. In Fig. 6A, staining for PCNA is rarely detected in control lung, most often in the cytoplasm. After 21 days of HH, occasional nuclei and cytoplasm stain positively for PCNA throughout the lung (Fig. 6B). After 21 days of HH + CO, however, many VSMC and endothelial cells show strong nuclear staining for PCNA (Fig. 6C). Western blots for PCNA in whole lung homogenate also showed that expression of this cyclin increased for the first 7 days during HH as expected (data not shown). In animals exposed to HH + CO, however, PCNA initially decreased in the first 7 days but then became strongly activated by 21 days as indicated by the immunohistochemical stains. Interpreted together, the features of the positive ssDNA and PCNA staining suggest continuous CO exposure promotes cell turnover in the lung during hypoxia in alveolar macrophages, VSMC, and endothelial cells.

Lung sm-α-actin expression. The sm-α-actin, a major structural and functional protein in vascular smooth muscle, was measured on Western blots of the lung after HH and HH + CO. The sm-α-actin mRNA content of the lungs was measured semiquantitatively by RTPCR and compared with control; sm-α-actin was localized by immunohistochemistry to determine whether the measured changes occurred in vascular smooth muscle. Figure 7A shows a representative Western blot for sm-α-actin in the lungs of rats exposed to air, air + CO, HH, and HH + CO. Control rat lung expresses sm-α-actin, which appears as a single strong band at 42 kDa, and the signal is not affected by exposure to 50 ppm CO in air. After hypoxia, sm-α-actin expression increased slightly in the lung by 14 and 21 days. In animals exposed to HH + CO, however, sm-α-actin was markedly decreased in the lung after 14 and 21 days. Thus HH + CO significantly decreased sm-α-actin expression in the lung after 14–21 days, whereas after HH alone, sm-α-actin content of the lung increased over control. After air + CO, sm-α-actin in the lung was similar to control and hypoxia-exposed animals.

Fig. 6. Vascular expression of proliferating cell nuclear antigen (PCNA) in hypoxia by immunohistochemistry. A: control lung shows minimal vascular staining for PCNA. B: after 21 days, HH lung shows increased staining of cytoplasm and nuclei in vascular smooth muscle and alveolar macrophages after 21 days. C: after 21 days, HH + CO lung shows intense staining for PCNA in smooth muscle and endothelial cell nuclei after 21 days. Magnification ×400. Insets in B and C: vascular detail at ×1,000.
Figure 7B shows densitometry data for sm-α-actin mRNA by RT-PCR (left) and β-actin (right) in the lungs after HH or HH + CO. The mRNA values are normalized to GAPDH and are expressed as a fraction of mRNA in control lungs. The sm-α-actin mRNA was 3-fold and 2.5-fold higher in HH than air control after 1 and 3 days, respectively (P < 0.01). After 7, 14, and 21 days of HH, sm-α-actin mRNA was no longer significantly increased compared with control rat lungs. In contrast, sm-α-actin mRNA was not significantly different than the control values at any time in rats exposed to HH + CO. In addition, after 1, 3, and 21 days of HH + CO, sm-α-actin mRNA in the lungs was significantly less than in the lungs of animals exposed to HH alone (P < 0.01). To measure changes in expression of β-actin isoform in the lung, RT-PCR for β-actin mRNA also was performed. In contrast to sm-α-actin mRNA, β-actin mRNA in the lung after 7 and 14 days of exposure to HH + CO was more than twofold higher than in control rats (P < 0.01). In HH animals, β-actin was not significantly different than control at any time point and, after 7 and 14 days, was significantly lower than in the HH + CO group (P < 0.01).

Lung HO-1 expression. HO-1 protein, activity, and mRNA were measured in the lungs after HH and HH + CO and compared with control lungs. In Fig. 8A, a Western blot for HO-1 shows representative results in the lungs of one control animal and one from each time point after HH and HH + CO. HO-1 protein expression increased in the lungs after hypoxia over the first 7 days of exposure to HH. After 7 and 14 days of HH, HO-1 protein expression was again similar to control and was higher than in HH-exposed animals after 3, 7, and 14 days (*P < 0.05 vs. HH).
days, and, after HH + CO, HO-1 expression remained elevated for 21 days. In the lungs of rats breathing air + CO, minimal or no increase in HO-1 was seen over 21 days of exposure (data not shown).

HO enzyme activity increased transiently during exposure to HH alone and then declined to values similar to control lungs at 7–21 days (Fig. 8B). In contrast, HO activity increased significantly in the lungs of the HH + CO animals after 1 day, peaked at 14 days, and remained significantly higher than the control and HH animals for the entire 21 days.

HO-1 mRNA was measured by RT-PCR in the lungs of control rats and after HH and HH + CO. Figure 8C shows mean HO-1 mRNA signal intensity from densitometry data of three animals from each group normalized to GAPDH. In rats exposed to HH and HH + CO for 1 day, HO-1 mRNA was fivefold higher than control values. In rats exposed to HH + CO, HO-1 mRNA was twofold higher than the hypoxia animals after 3 and 14 days. By 21 days, HO-1 mRNA was increased significantly over control in HH rats but had decreased to near control values in the lungs of rats exposed to HH + CO.

**Immunohistochemistry for sm-α-actin and HO-1.** Figure 9 shows representative photomicrographs of immunohistochemistry in lungs of an air-exposed rat and after 14 days of exposure to HH or HH + CO. Figure 9, A–C, shows immunohistochemistry for sm-α-actin, and immunohistochemistry for HO-1 is shown in Fig. 9, D–F. The sm-α-actin staining in control lung was limited to bronchial and vascular smooth muscle. In control lung, sm-α-actin is present in the thin smooth muscle layer of a small arteriole (Fig. 9A). After 14 days of HH, sm-α-actin staining increased significantly in walls of small blood vessels, reflecting the thickened smooth muscle layer (Fig. 9B). In addition, sm-α-actin staining was present in the alveolar region, likely representing new smooth muscle in small vessels. In contrast, staining for sm-α-actin after 14 days of HH + CO was attenuated in the vessel walls and alveolar region. In control lungs (Fig. 9D), HO-1 was present primarily in lung macrophages. After 14 days of HH, HO-1 was present diffusely in macrophages, in the alveolar region, and in the walls of thickened blood vessels (Fig. 9E). After 14 days of HH + CO, HO-1 was present throughout the alveolar region, in the macrophages, and in blood vessel walls (Fig. 9F).

**DISCUSSION**

The main finding of this study is that continuous exposure of rats to low concentrations of CO alters...
vascular remodeling during HH in vivo. This result was somewhat unanticipated, but it correlates with a greater increase in the lung’s pressure-flow curve in chronic HH with CO than without it. This effect was independent of right heart hypertrophy and differences in erythrocyte response between the groups. The CO-related changes in vascular structure and PVR were associated with decreased expression of sm-α-actin in small pulmonary arteries during hypoxia. Because CO in air at sea level did not produce similar effects, the combination of CO and hypoxia appears to be critical and implicates an Hp-based mechanism for the interaction.

The lack of difference in morphometric and pressure-flow data between CO control and air control animals confirms that the amount of CO used in these experiments was not sufficient to cause significant hypoxia in the lung. Therefore, the finding that the presence of CO accelerates hypoxic vascular remodeling cannot be attributed to an exaggerated vascular response to hypoxia. Higher concentrations of CO in air do result in significant tissue hypoxia, and previous studies report that neonatal rats exposed to 500 ppm CO in air develop right heart remodeling and polycythemia but not increased PVR (13, 17, 25). COHb levels of 3–4% in the current experiments are too low to cause significant changes in lung tissue P 02 that would promote more rapid remodeling of the lung to chronic HH. Although the accuracy of optical measurements of COHb in the 2% range is a problem, the low values do indicate that lung cell concentrations of CO during HH were in the range of those attained by increases in endogenous CO production in vivo (14).

In an earlier study, CO was found to decrease smooth muscle cell growth in vitro at a concentration 1,000 times greater than used in this study (16). The living rat lung, however, shows evidence of a higher degree of vascular cell turnover when CO is persistently present during long-term hypoxia. This interpretation is suggested by increases in both ssDNA consistent with apoptosis and nuclear PCNA consistent with cell proliferation. If true, the biochemical linkage of CO to the regulation of growth responses is quite complex. Such complex effects could occur by a variety of biochemical mechanisms, e.g., when CO interactions with reduced cellular hemoproteins increase during hypoxia.

Some of our findings suggest that CO increases muscular pulmonary vessels during hypoxia by stimulating smooth muscle cell proliferation. Although mature contractile VSMC contain sm-α-actin as a major structural and functional protein, proliferating VSMC show low expression of sm-α-actin and other mature muscle proteins after mechanical injury or during hypoxic vascular remodeling (2, 7). A less well-differentiated phenotype of proliferating smooth muscle cells possibly results from either a phenotypic shift or the expansion of a less-differentiated subpopulation of smooth muscle cells (7). The relative decrease in sm-α-actin mRNA with CO in hypoxia suggests that its lack of expression is due to decreased gene transcription or altered mes-sage stability. The twofold increase in β-actin mRNA transcript in the lungs after hypoxia + CO confirms the switch in actin isoform expression, as reported in proliferating smooth muscle cells (2). This raises several intriguing possibilities for future investigation of how CO modifies actin message expression, translation, and/or mRNA or protein degradation in hypoxia.

The mechanisms responsible for the effects of CO during hypoxia potentially involve HO enzyme activity in the lung. It is known that initial expression of many hypoxia-responsive genes is regulated by HIF-1, including HO-1. Although HIF-1 DNA binding activity was not measured, prolonged expression of HO-1 in the lung after CO in hypoxia may be associated with early HIF-1 activation, whereas HO-1 expression at later time points may be regulated by either HIF-1 or other factors. For example, hypoxia in cells leads to induction of c-Fos and appearance of c-Jun/c-Fos activator protein-1 heterodimer, promoting gene expression of proteins involved in cell proliferation, including HO-1 (8). Recent studies have found that hypoxia alters expression of cytokines such as interleukin-6 and stimulates cellular reactive oxygen species (ROS) associated with nuclear factor-κB activation (6, 27). Such mechanisms may allow CO to augment the effect of hypoxia through HIF-1-independent pathways.

In this study, CO increased HO-1 expression in the lung in vivo only during HH; however, in endothelial cells in vitro, CO (at 100 ppm) also increased HO-1 expression in normoxia (23), where, it was proposed, to increase cellular NO metabolites, which then serve as toxic or signaling mediators. However, we detected minimal to no overall increase in HO-1 expression in air control lung exposed to CO at 50 ppm for up to 3 wk. The effects of HO-1 induction by CO in hypoxic lung are not clear, and multiple biochemical mechanisms are possible for enhanced HO-1 expression by a metabolic product like CO. Enzyme inhibition by CO substrate binding leading to HO-1 induction by heme is the only such mechanism demonstrated to date (5).

A unifying mechanism for the vascular effects of CO in hypoxia would have to explain apparent increases in apoptosis, cell proliferation, and HO-1 induction. Perhaps such responses could be triggered by enhanced generation of reactive species of oxygen or nitrogen. For instance, high concentrations of CO actually increase oxidative stress in the brain (18), where ROS generation by mitochondria has been demonstrated (29). In the present study, however, such oxidative mechanisms have not been explored and remain for investigation.

The increase in smooth muscle content of pulmonary arteries in hypoxia has important functional consequences for the processes that influence vascular resistance. Although CO in HH increased resistance, our technique did not independently assess the contribution of small arteries. However, the morphometry provides additional perspective because the histograms show a greater number of muscular vessels in the lungs after CO + hypoxia than hypoxia alone. These vessels may represent previously nonmuscular arter-
ies not accounted for in hypoxia but which contribute to an apparent increase in PVR after CO. On the other hand, total lumen cross-sectional area of muscular vessels in rats exposed to CO in hypoxia increase relative to air control animals (~25%), whereas lumen area of muscular vessels decreased in hypoxia relative to control (~20%). The extent to which any of this increase in lumen area by CO represented vasculogenesis would tend to counteract an increase in PVR in HH. Alternatively, CO may have slowed smooth muscle growth in one size of vessel but stimulated vessels of another size, which contributed more to an overall increase in PVR.

In conclusion, we find that exogenous CO can promote vascular remodeling and the increase in vascular resistance in HH in the lung. These responses are associated with complex cellular changes comprising evidence for both apoptosis and increased proliferation of resident cell populations suggestive of increased cell turnover. Structural changes induced by CO in hypoxia correlate with changes in sm-α-actin and HO-1 expression in small pulmonary arteries. The effects of CO require hypoxia, as CO are absent after the same CO exposure in air, strongly implicating PO2-dependent Hp-based mechanisms in the responses. These new findings may have direct relevance to environmental exposures and human lung disease, for example, in smokers, where alveolar CO levels reach hundreds of parts per million, and hypoxic lung cells may take up and retain CO for long periods of time.

Funding for this work was provided by an American Lung Association Research Grant and National Heart, Lung, and Blood Institute Grant HL-4-2444.

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