Lung-specific induction of heme oxygenase-1 and hyperoxic lung injury

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Taylor, Jennifer L., Martha Sue Carraway, and Claude A. Piantadosi. Lung-specific induction of heme oxygenase-1 and hyperoxic lung injury. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L582–L590, 1998.—Heme oxygenase (HO)-1, which catalyzes heme breakdown, is induced by oxidative stress and may protect against oxidative injury. We hypothesized that induction of HO-1 by hemoglobin (Hb) in the lung would protect the rat from pulmonary O2 toxicity. Rats given intratracheal (IT) Hb showed lung-specific induction of HO-1 by 8 h by Western analysis. Rats were then pretreated for 8 h before 60 h of exposure to 100% O2 with either IT normal saline, Hb, or Hb plus the HO-1 inhibitor tin-protoporphyrin (SnPP). Both the Hb+O2 and Hb+O2+SnPP animals had less lung injury than normal saline controls as indicated by lower pleural fluid volumes and wet-to-dry weight ratios (P < 0.01). The improvement in injury in the two Hb-treated groups was the same despite a 61% decrease in HO enzyme activity in the Hb+SnPP group after 60 h of O2. In addition, inhibition of HO activity with SnPP alone before O2 exposure did not augment the extent of hyperoxic lung injury. These results demonstrate that IT Hb induces lung HO-1 in the rat and protects against hyperoxia; however, the protection is not mediated by increased HO enzyme activity.

Oxidative stress; reactive oxygen species; antioxidant enzymes

REACTIVE OXYGEN SPECIES (ROS) have been implicated in the pathogenesis of many pulmonary diseases including acute respiratory distress syndrome (ARDS) (12). An in vivo model of acute lung injury with features of ARDS can be produced by administering O2 to animals. In rats, exposure to continuous hyperoxia leads to excessive ROS production and destruction of the pulmonary capillary endothelium, resulting in pulmonary hemorrhage, interstitial and alveolar edema, and inflammatory cell infiltration (8). After 48 h of hyperoxia (>95% O2), pulmonary edema and pleural effusions are observed. These pathophysiologic changes increase significantly between 48 and 60 h. Exposure to 100% O2 for 60–72 h is fatal in rats (7).

Tolerance to O2 has been shown to occur through increased gene expression of antioxidant enzymes such as manganese superoxide dismutase (MnSOD) and Cu,ZnSOD. Crapo et al. (8) suggested that the structural changes in the alveolar and capillary epithelial cells in the lungs of rats displaying tolerance to O2 might be related to the enhanced activity of the SODs induced by O2 exposure. Hyperoxia also has been shown to increase other stress-responsive genes in vivo, including heme oxygenase (HO)-1 (14). Two isoforms of HO have been identified: HO-1, which is inducible, and HO-2, which is expressed constitutively (14). HO catalyzes the initial and rate-limiting step in the catabolism of heme to biliverdin, resulting in the release of iron and the production of carbon monoxide. Biliverdin is then converted to bilirubin by biliverdin reductase. These heme degradation products have been shown to have antioxidant activity (23).

While heme, the main substrate of HO-1, induces the expression of the enzyme, nonheme compounds such as endotoxin, hormones and heavy metals also induce HO-1 (6). More recently, it has been found that tumor-promoting phorbol esters, sodium arsenite, H2O2, ultraviolet (UV) irradiation, hyperthermia, hyperoxia, and sulfhydryl reagents induce HO-1. Importantly, these different agents share the ability to induce oxidative stress either by production of ROS or by interfering with endogenous antioxidant compounds such as glutathione (6). Because of the correlation between oxidative stress and HO-1 induction and the antioxidant properties of bilirubin, it has been hypothesized that HO-1 induction is part of a general response to oxidative stress and may confer protection from oxidative tissue damage.

In vitro and in vivo studies have supported a role for HO in protecting against oxidant injury. For example, HO protects from radiation-induced injury in human skin fibroblasts (27) and from heme-induced and hemoglobin (Hb)-induced toxicity in rabbit coronary endothelial cells (1). In addition, HO-1 induction was shown to be protective in oxidant-mediated rhabdomyolysis in the rat (17). Finally, pretreatment of rats with Hb was found to induce HO-1 and protect against subsequent lethal endotoxemia (21).

To date there have been no in vivo experiments performed to specifically investigate the protective effects of HO-1 induction in the lung against hyperoxia. It has been demonstrated that intratracheal (IT) injection of erythrocytes decreases hyperoxic pulmonary injury in rats and lowers mortality (26). Those rats pretreated with erythrocytes demonstrated 75% survival at 12 days compared with death of saline control animals within 4 days of hyperoxic exposure. The protective effect of this therapy was attributed to the antioxidant activity of glutathione present in erythrocytes. Measurements of HO activity were not made.

Hb, which accumulates in the lung under certain pathological conditions, is also known to be a powerful inducer of HO-1. On the basis of the possible protective role of HO-1 against oxidative stress, we developed the hypothesis that Hb, by inducing lung-specific HO-1 expression, would protect the rat from hyperoxic lung injury. In this study, we first demonstrate that administration of IT Hb produces lung-specific induction of HO-1. We then show that pretreatment with IT Hb...
protects rats from hyperoxic lung injury. To determine whether HO was responsible for the protection, we administered tin-protoporphyrin (SnPP), a competitive inhibitor of HO activity, alone and before IT Hb and O2 administration. Inhibition of HO activity, however, did not augment hyperoxic lung injury or reverse the protective effects of IT Hb against hyperoxia in the rat.

METHODS

Materials. Pathogen-free adult male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA); Powergen homogenizer was purchased from Omni International (Marietta, GA); Hoefer minigel system and Hoefer TE series Transphor electrophoresis unit were purchased from Hoefer Scientific Instruments (San Francisco, CA); Branson sonifier was purchased from Branson Ultrasonics (Danbury, CT); model U2000 UV/Vis spectrophotometer was purchased from Hitachi Instruments (Tokyo, Japan); Nikon Optiphot-2 microscope, HFX-IIA photomicrographic attachment, and Nikon FX-35DX camera were purchased from Nikon (Garden City, NY); background Stats, Stat-Q secondary linking antibody, and peroxidase- streptavidin labels were purchased from Innovex Biosciences (Richmond, CA); Tris-HCl, SDS, and polyacrylamide were purchased from Bio-Rad (Hercules, CA); polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA); anti-HO-1 antibody was purchased from Stress Gen (Victoria, BC, Canada); enhanced chemiluminescence kits and Hyperfilm were purchased from Amersham Life Science (Arlington Heights, IL); acetic acid, paraformaldehyde, pyridine, and n-butanol were purchased from Mallinkrodt Baker (Paris, KY); SnPP was purchased from Porphyrin Products (Logan, UT); ethanol was purchased from AAPER Alcohol and Chemical (Shelbyville, KY); Nembulator was purchased from Abbott Laboratories (N. Chicago, IL); halothane was purchased from Halocarbon Laboratories (North August, CA); horseradish peroxidase-labeled goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch (West Grove, PA); the MnSOD antibody was a gift from Dr. Ling Yi Chang (Denver, CO); and the ferritin antibody was from Dako (Carpinteria, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Experimental design. To establish a time course and to determine specificity of organ induction of HO-1 by Hb, rats received either IT rat Hb or normal saline. Hb was tested for endotoxin by the Limulus assay and found to contain <3 ng endotoxin/dose of Hb. For IT administration, the rats were anesthetized with halothane, the trachea was cannulated transorally, and 0.5 ml of sterile normal saline or 0.5 ml of rat Hb (62.5 mg) dissolved in saline (9.69 × 10−7 M) was injected. There was one fatality from anesthesia. There were no fatalities from IT Hb or SnPP injections. After pretreatment of rats with IT Hb or IT normal saline, the animals were killed at several time points by intraperitoneal injection of Nembutal. One rat receiving IT normal saline was killed for each time point at 8, 16, and 24 h. For rats receiving IT Hb, one rat was killed for each time point at 8, 16, 24, and 48 h. After anesthesia, the peritoneal and chest cavities were opened, and the blood was removed by flushing with normal saline via the right ventricle. Lungs, kidneys, heart, and spleen were harvested for Western blot analysis.

To determine whether induction of HO-1 by Hb was protective against hyperoxia, six groups of rats were studied. The animals were anesthetized briefly with halothane before pretreatments. Two groups of five rats each received IT normal saline or IT Hb and were exposed only to room air. Four groups of 10 rats each were exposed to hyperoxia. One group of these rats received IT normal saline, and one group of rats received IT Hb. To investigate the role of HO-1 induction, the third group of 10 rats received IT Hb subcutaneously. A final group of rats received only SnPP without IT Hb before hyperoxia. All pretreatments for the experimental animals were given 8 h before exposure to hyperoxia, during which time animals were allowed to acclimate to their exposure cages. In the SnPP-treated animals, the subcutaneous injections were repeated at 24 h by briefly removing the animal from the exposure chamber, injecting it, and immediately returning it to the chamber.

O2 exposures. O2 exposures were conducted in Plexiglas chambers using 100% O2 for 60 h. Rats were allowed free access to rat food and water during the exposures. The O2 concentration in the chambers was monitored continuously and kept at 98% or above throughout the experiments. At 60 h, the surviving rats were removed from the O2 and killed with intraperitoneal injections of Nembutal. The chest cavity was opened, and pleural fluid volumes were measured using a syringe. For two rats (chosen at random) in each group, the right branch of the pulmonary artery was occluded with a temporary ligature and the right lung was harvested for wet-to-dry weight ratios and biochemical assays. The left lung was flushed with normal saline via the right ventricle. The left lung, heart, kidneys, and spleen were harvested for Western blotting. For the remaining animals in each group and for control animals, both lungs were immediately harvested for wet-to-dry weight ratios and biochemical assays.

SnPP preparation. SnPP was prepared in the dark by dissolving ~100 mg in 2 ml of 200 mM NaOH. Normal saline was added (8 ml), and the pH of the solution was adjusted to 7.4–8.0. Each rat was subcutaneously injected with 50 µmol/kg in 1 ml of solution.

Western blotting. The tissues were harvested and immediately homogenized with a Powergen homogenizer on ice in cold lysis buffer [50 mM Tris, pH 7.6, 3% Nonidet P-40, 150 mM NaCl, 1 mM MgCl2, 5 mM EDTA, 2 mM 1,10-phenanthroline, 2 mM 3,4-dichloroisocoumarin, and 0.4 mM trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane]. The homogenates were centrifuged at 10,000 g at 4°C for 10 min. The supernatants were decanted, and an aliquot was stored at −20°C for determination of protein concentration. The remaining supernatant was mixed 1:1 with double-strength Lamelli sample buffer (250 mM Tris, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol, pH 6.8), aliquoted, and stored at −80°C.

Proteins were separated by PAGE using a Hoefer mini gel system. For HO blot, samples containing 15 µg of protein were loaded in each lane of 12% polyacrylamide gels. The proteins were separated over 2 h. A 0.1% gelatin solution was loaded in each lane of 15% polyacrylamide gels. The proteins were separated over 2 h. A Hoefer TE series Transphor electrophoresis unit was used to transfer proteins to PVDF membrane over 1 h. The membranes were blocked at 4°C overnight in TBST (100 mM Tris, 1,000 mM NaCl, and 1% Tween 20, pH 7.5) with 5% milk. The next day, membranes were washed for 30 min with TBST. For these and all subsequent washes, the TBST was changed every 6 min during the 30-min wash. The blots were incubated with HO-1 antibody at a 1:1,000 dilution with 4% milk or MnSOD antibody diluted 1:10,000 in TBST over 1 h. The membranes were then washed for 30 min with TBST. A 4% milk solution with goat anti-rabbit antibody in a 1:20,000 dilution (HO-1) or in a 1:35,000 dilution (MnSOD) was used to incubate the secondary antibody for ~1 h. The membranes were then washed with TBST for 30 min. Detection of signal by 10.220.32.246 on June 21, 2017 http://ajplung.physiology.org/ Downloaded from
was performed by enhanced chemiluminescence. The membranes were stained with 0.1% Coomassie blue solution for 1 min followed by destaining (50% methanol-15% acetic acid) to check for equivalent loading of protein for each sample.

Protein concentration. All protein concentrations were determined using the bicinchoninic acid assay (16).

Wet-to-dry weight ratios. A portion of the freshly harvested right lung tissue was placed immediately on preweighed tin foil and weighed. The lungs were allowed to dry for 96 h at 60°F in a vacuum oven. The lungs were then reweighed, and the wet-to-dry weight ratios were calculated.

Myeloperoxidase assay. Myeloperoxidase (MPO), an enzyme found in neutrophils, is a marker of tissue neutrophil content. After lung tissue was harvested, it was frozen immediately in liquid nitrogen and stored at −80°C. The frozen tissues were pulverized with a mortar and pestle under liquid nitrogen and stored at −80°C. Before the assay, the samples were weighed in precooled test tubes, and 50 mM phosphate buffer (pH 6) was added to a ratio of 1 ml/200 mg. This original homogenate was homogenized again and stored at −80°C. For the assay, the original homogenate was brought to 4°C, and 250 µl aliquots were placed in micropipette tips and diluted with 50 mM phosphate buffer (pH 6) and equal volumes of 1% hexadecyltrimethylammonium bromide in phosphate buffer. The remaining diluted homogenate was stored at −80°C for protein determination. Samples were disrupted ultrasonically using a Branson sonifier at 20% power output and 20% duty cycle for 10 pulses. The homogenates were centrifuged at 18,800 g for 27 min at 4°C, and the supernatant was decanted. One aliquot of supernatant was stored at −20°C, and the remainder was stored at −80°C.

Because constitutive expression of HO is high in the spleen under normal conditions, spleen tissue homogenate was used as the positive control for HO activity. Frozen lung and spleen tissues were brought to 4°C and dispersed in a Polytron in one or three volumes of MgCl₂-phosphate buffer (pH 7.4), respectively. The samples were disrupted ultrasonically using a Branson sonifier at 20% power output and 20% duty cycle for 10 pulses. The homogenates were centrifuged at 18,800 g for 15 min at 4°C. The supernatants were decanted and aliquotted. One aliquot was used for protein determination, and the remaining aliquots were stored at −80°C until the assay was performed. Lung, spleen, and liver supernatants and 20 mM glucose 6-phosphate stock solution were brought to 4°C. The remaining stock solutions (8 mM NADPH, 0.016 µM of glucose-6-phosphate dehydrogenase, and 400 µM NADP) were prepared at the time of assay. In a 500-µl reaction mixture, 2 mg of liver supernatant, 20 µM NADP, 0.8 mM NADPH, 2 mM glucose 6-phosphate, and 0.0016 µM of glucose-6-phosphate dehydrogenase were added to microfuge tubes. Aliquots of 200 µl of lung supernatant (2–6 mg of protein) were added to the reaction mixture for each sample. Spleen supernatant was used for the positive control. For the negative control, NADPH was replaced with MgCl₂-phosphate buffer (pH 7.4). The mixture was then incubated at 37°C for 1 h in the dark. After the incubation, 500 µl of chloroform were added to each sample tube. All tubes were vortexed and then centrifuged in a microfuge. The lower chloroform layer was removed, and the OD was measured between 464 and 530 nm. The assay was run in duplicate. Enzyme activity was calculated in picomoles of bilirubin formed per milligram of protein per hour (4, 24, 25).

Immunocytochemistry. Rat lungs were inflation fixed in situ at 20 cm of fixative pressure in 2% glutaraldehyde. They were embedded in paraffin and cut into 4-µm sections. The tissue sections were deparaffinized in xylene, rehydrated in graded alcohol solutions, blocked with H₂O₂ in absolute methanol (2 ml of 30% H₂O₂ in 30 ml of methanol) for 8 min, rinsed in 95% alcohol for 2 min, and placed in deionized H₂O. The slides were washed three times with PBS, and the sections were incubated with background buster for 10 min at room temperature. Primary antibody diluted to 1:100 for ferritin or 1:1,100 for HO-1 in 1% PBS was applied to the sections for 45 min at 37°C. Slides were then washed as above and incubated with Stat-Q secondary linking antibody for 10 min at room temperature. The slides were then washed three times with PBS and incubated with Stat-Q peroxidase-streptavidin label for 10 min at room temperature. The slides were incubated with diaminobenzidine for 3 min, rinsed with tap water, and counterstained with hematoxylin. Sections were photographed using a Nikon Optiphoto-2 microscope with an HFX-IIA photomicrographic attachment and a Nikon FX-35DX camera.

Ferritin assay. Lung tissue from the rats was homogenized (1.0 g/5.0 ml) in lysis buffer, and aliquots were vacuumed into a 10 ml of nitrocellulose in a saline buffer containing 100 mM Tris, pH 8.0. The blot was air-dried, blocked with 5% powdered milk, and then incubated with a 1:2,000 dilution of rabbit anti-human ferritin antibody in 5% dry milk for 2 h. The blot was washed in PBS-0.05% Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG in 5% dry milk for 1 h. The signal was developed using enhanced chemiluminescence reagents. Band ODs were measured on a Millipore digital bioimaging system.

Statistical methods. All results are expressed as means ± SE. Statistical analyses were performed on surviving animals
using an ANOVA for comparison between groups of three or more or an unpaired t-test for comparison between two groups. Results were considered significant for \( P \) values < 0.05. All \( P \) values were rounded to either \( P < 0.01 \) or \( P < 0.05 \).

RESULTS

HO induction in rat tissues. Western blot analyses were performed on lung, heart, kidney, and liver homogenates to determine whether IT administration of Hb would produce lung-specific HO-1 induction. Spleen homogenate served as a positive control. As seen in Fig. 1A, the blots show strong induction of HO-1 in the lungs of rats after IT Hb administration compared with minimal induction in lungs of rats treated with IT normal saline. In contrast to the lung, IT Hb produced minimal or no induction of enzyme in the kidney, heart, and liver. To determine the time course of HO-1 induction, IT Hb was administered to rats 8, 16, 24, and 48 h before death. Maximal induction was seen at 16 h but was detectable at 8 h. The HO-1 signal began to decline 24–48 h after Hb treatment (Fig. 1B).

Immunocytochemistry was performed on samples of lung tissue from rats receiving either IT normal saline or IT Hb at the 16-h time point to localize HO-1 induction in the lung. Figure 2A shows a photomicrograph of lung tissue from a rat that received IT normal saline incubated with nonimmune globulin to control for nonspecific staining. Figure 2B shows the minimal induction of HO-1 caused by IT normal saline administration. Figure 2C is a photomicrograph of staining of lung tissue from a rat that received IT Hb incubated with nonimmune globulin to control for nonspecific staining. In the lung of a rat treated with IT Hb, diffusely increased staining was found throughout the alveolar epithelium and in alveolar macrophages in the lung (Fig. 2D).

To correlate the increased protein induction demonstrated by Western blotting with HO activity, lung samples of rats killed 8 h after administration of IT Hb or IT normal saline were assayed for enzyme activity. Figure 3A shows that IT Hb administration induced a 74% increase in HO activity compared with IT normal saline administration (101.48 ± 12.71 vs. 26.22 ± 2.80 pm·mg protein\(^{-1}\)·h\(^{-1}\), respectively; \( P < 0.01 \); Fig. 3A).

IT Hb protects from hyperoxia. Because HO-1 protects against some forms of oxidative stress, we sought to determine whether HO-1 induction before hyperoxic exposure would protect rats from lung injury. In the IT Hb group, all 10 animals survived 60 h of hyperoxic exposure. There were two fatalities in the IT normal saline group. Figure 4 shows that pleural fluid volumes in animals that received IT normal saline or IT Hb in room air were minimal. After 60 h of hyperoxia, however, rats receiving IT Hb had significantly lower pleural fluid volumes compared with those receiving IT normal saline (7.8 ± 0.87 vs. 11.5 ± 1.21 ml, respectively; \( P < 0.01 \)). Figure 5 shows normal wet-to-dry weight ratios in IT normal saline- and IT Hb-treated animals in room air. After 60 h of hyperoxia, wet-to-dry weight ratios were increased in both the IT normal saline and IT Hb groups but were lower in animals that received IT Hb compared with those that received IT normal saline (5.62 ± 0.08 vs. 5.96 ± 0.10, respectively; \( P < 0.01 \)). These results demonstrate protection of the lung with less pulmonary edema formation. This finding, however, did not correlate with biochemical determinants of injury. Lung samples from control rats receiving IT Hb or IT normal saline showed no difference in TBARS. After exposure to hyperoxia, rats in the IT Hb group had higher TBARS levels than those receiving IT normal saline as seen in Fig. 6 (98.9 ± 3.8 vs. 79.8 ± 3.2 OD/g protein, respectively; \( P < 0.01 \)).

The results of the MPO assay performed on lung samples from all groups (Fig. 7) demonstrated a higher neutrophil enzyme content in the lungs of control rats receiving IT Hb than in those receiving IT normal saline (1,502.1 ± 323.0 vs. 468.8 ± 96.0 OD/g protein, respectively; \( P < 0.05 \)). This finding was confirmed by hematoxylin and eosin staining of lung tissue from rats receiving either IT normal saline or IT Hb. These sections demonstrated minimal neutrophil influx in rats receiving IT normal saline compared with those receiving IT Hb (data not shown). There was an in-
crease in MPO activity in the IT normal saline-treated group after exposure to 100% O₂ that was not significant (NS) (468.8 ± 96.0 vs. 916.1 ± 233.6 ΔOD/g protein; P = NS) and no further increase in MPO activity in Hb-treated animals after O₂ exposure (1,502.1 ± 323.0 vs. 1,571.4 ± 186.9 ΔOD/g protein; P = NS).

Inhibition of HO-1 by SnPP. If HO-1 activity is integral to the protection against hyperoxia conferred by IT Hb, inhibition of HO-1 activity by SnPP would be expected to reverse this protection. To test this possibility, 10 rats were given 1 ml (50 µmol/kg) of SnPP solution subcutaneously at the time of IT Hb administration (8 h before starting the 60-h O₂ exposure). The injections were repeated 24 h later. One fatality occurred in this group after 60 h of hyperoxic exposure. HO activity assays were performed on the lung tissues of these rats after O₂ exposure to determine whether enzyme inhibition had been achieved. As seen in Fig. 3B, SnPP reduced HO enzyme activity after 60 h of O₂ exposure by 61% in the rats receiving the inhibitor at the time of IT Hb administration compared with those receiving IT Hb alone (22.76 ± 6.96 vs. 58.55 ± 8.59 pm·mg protein⁻¹·h⁻¹, respectively; P < 0.01). The protection against lung edema, however, was not reversed. Neither pleural fluid volumes (7.2 ± 1.17 vs. 7.8 ± 0.99 ml; P = NS) nor wet-to-dry weight ratios (5.63 ± 0.08 vs. 5.62 ± 0.08; P = NS) were different between the two groups (Figs. 4 and 5). Animals in the IT Hb+SnPP group exposed to hyperoxia had significantly higher MPO activity than those in the IT Hb group exposed to hyperoxia (2,678.4 ± 241.2 vs. 1,571.4 ± 186.9 ΔOD/g protein, respectively; P < 0.01; Fig. 7). TBARS were significantly lower in the lungs of rats exposed to hyperoxia receiving IT Hb+SnPP compared with those that only received IT Hb as seen in Fig. 6 (70.4 ± 2.1 vs. 98.9 ± 3.8 ΔOD/g protein; P < 0.01).

To determine whether endogenous HO activity protects against hyperoxic lung injury, a group of 10 rats was given SnPP without IT Hb. Seven of these 10 animals survived 60 h of O₂ compared with 8 of 10 animals exposed to O₂ without SnPP treatment. This regimen of SnPP administration almost completely inhibited endogenous HO activity in the treated animals, but there were no significant differences in pleural fluid volumes or wet-to dry weight ratios between these rats and rats in the normal saline+O₂ control group (Fig. 8).

MnSOD after IT administration of Hb. Because inhibition of HO activity did not augment O₂ toxicity or reverse the protection against hyperoxia conferred by IT Hb administration, we hypothesized that Hb may have induced an alternative antioxidant enzyme. Because MnSOD is highly protective against pulmonary

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Fig. 2. Immunocytochemistry of rat lung stained with anti-HO-1 antibody. A: immunocytochemistry with nonimmune globulin of lung from a rat that received IT normal saline shows minimal staining. B: immunocytochemistry with anti-HO-1 antibody in IT normal saline rat lung shows minimal staining. C: immunocytochemistry with nonimmune globulin of a lung section from a rat that received IT Hb shows nonspecific background staining. D: immunocytochemistry with anti-HO-1 antibody in IT Hb lung shows diffuse, heavy staining throughout alveolar epithelium, endothelium, and in alveolar macrophages. A–D: original magnification approximately ×200.
O₂ toxicity, we performed Western blot analysis using anti-MnSOD antibody on lung tissue homogenate from rats receiving no treatment, IT normal saline, or IT Hb. As seen in Fig. 9, MnSOD protein was not induced by IT Hb instillation compared with IT normal saline.

Ferritin induction by IT Hb. Ferritin has been shown to be induced by systemic administration of methemoglobin (metHb) (4) and, as an iron-storage protein, has been postulated to provide cytoprotection against iron-mediated free radical injury. Therefore, we utilized anti-rat ferritin antibody to study lung tissue from rats receiving either IT normal saline or IT Hb to determine whether ferritin synthesis was also being induced by IT Hb administration. Immunochemical staining of lung tissue for ferritin showed diffuse, heavy increases in the lung after IT Hb that were most apparent in alveolar epithelium and alveolar macrophages (data not shown). These microscopic findings were confirmed by a slot blot for ferritin (Fig. 10) on tissue harvested 16 h after IT normal saline, Hb, or Hb+SnPP. Figure 10 demonstrates the more than threefold increase in ferritin content in lungs of animals treated with IT Hb compared with normal saline. This increase in ferritin was sustained in the Hb+SnPP-treated animals.
Discussion

Because protection from oxidative stress has been associated with HO-1 induction, we sought to determine whether organ-specific induction of HO-1 would confer protection against pulmonary O2 toxicity in the rat. During many acute lung injuries such as pulmonary hemorrhage and ARDS, the lung parenchyma is exposed to increased amounts of Hb. Whether this exposure injures the lung or leads to induction of protective responses is unknown. In this paper we have shown that Hb is a potent inducer of HO-1 in the lungs and that IT Hb given before hyperoxia results in reduced injury to the rat lung as indicated by less pulmonary edema and lower pleural fluid volumes. Inhibition of HO enzyme activity, however, did not augment hyperoxic lung injury or reverse the protection conferred by IT Hb administration. At this time, the mechanism by which Hb protects the lung from O2 toxicity remains uncertain.

HO-1 induction in the lung has been accomplished previously by administration of intravenous metHb (4, 21); however, systemic administration of metHb induces HO-1 in the liver, heart, spleen, and kidneys (21). To investigate HO-1 induction in the lungs and to determine whether it might exert a protective effect against pulmonary O2 toxicity, we gave Hb intratracheally to the rat to induce lung HO-1 specifically. The time course of induction of HO-1 after IT instillation agrees with the findings after systemic Hb administration in which enzyme induction is present at 8 h and peaks at 16 h (4, 21, 22).

Several studies have demonstrated that induction of HO-1 before oxidative stress protects from subsequent tissue injury (1, 17, 27, 28). HO-1 induction by Hb is protective against lethal endotoxemia (21). The role of HO-1 in protection from O2 has also been studied in O2-resistant fibroblasts. These fibroblasts have greater constitutive HO-1 expression compared with controls, which led the authors (9) to postulate that HO-1 plays a role in protection against hyperoxic damage. HO inhibitor studies however, were not reported in these experiments. In human pulmonary epithelial cells that over-express HO-1, survival in hyperoxia is increased; this is reversible by administration of SnPP (15). By comparison, we show that IT Hb administration protects against hyperoxic lung injury in vivo; however, inhibition of HO enzyme activity does not reverse this protection. Although the SnPP did not completely inhibit the enzyme, allowing for the possibility that the residual 39% of the original active enzyme was sufficient to protect the lung, it is important to note that the remaining HO activity in the lungs of rats receiving SnPP was the same as the HO activity in the lungs of the saline control rats that received O2. These results argue against HO activity providing protection against O2 toxicity. That HO activity did not account for the protection against hyperoxic lung injury was supported by administering SnPP to normal rats and exposing them to hyperoxia for 60 h. Despite almost total inhibition of HO activity, no significant exacerbation of lung injury developed compared with normal saline controls.

When we were unable to demonstrate that HO-1 induction conferred the protection against O2 toxicity produced by Hb, we further investigated the mechanism by which IT Hb might protect against hyperoxic injury. It has been demonstrated that heme from...
methemoglobin is taken up by endothelial cells (4). The release of free iron causes injury to endothelium and activates neutrophils. Therefore, it was not unexpected that control animals pretreated with IT Hb had higher MPO activity in the lungs than those treated with normal saline or that microscopy of lung tissue of the control animals treated with Hb demonstrated a greater inflammatory response than animals that received normal saline. Interestingly, hemoglobin did not cause a significant increase in MPO activity in animals treated with IT normal saline or IT Hb. Animals in the hyperoxic IT Hb+SnPP group showed significantly more lung MPO activity than the animals in the hyperoxic IT SnPP group. We did not determine whether the animals in the IT Hb+SnPP group had more MPO activity at baseline or whether the inhibition of HO-1 played a role in this response. The fact that Hb-treated animals had higher MPO activity after O2 exposure than normal salinetreated rats suggests that the protective effect of IT Hb is not mediated by an antineutrophil effect of Hb administration.

An alternative explanation for the MPO data is that the influx of neutrophils caused by IT Hb may have played a role in protection against hyperoxia. The lungs of rats exposed to hyperoxia in the Hb and Hb+SnPP groups had significantly higher MPO activity than normal saline controls. Release of iron increases NADPH oxidoreductase and lactoferrin in the neutrophil. Iron (Fe3+) is complexed by lactoferrin released from the neutrophil. In this bound form, iron is less likely to undergo oxidation-reduction reactions and can be transported to macrophages for storage in ferritin (10). Thus it is possible that the inflammatory reaction caused by the administration of Hb enhanced iron storage mechanisms to make it unavailable for participation in the generation of hydroxyl or ferryl radicals.

Because tolerance of the lungs to hyperoxia is usually accompanied by an increase in the level of antioxidants in the lung (7), we expected to find that the protected animals would demonstrate less nonspecific tissue oxidation products. TBARS values, however, were highest in the Hb-treated animals exposed to 100% O2. One possibility for these results involves free iron liberated by heme breakdown. After administration of intravenous Hb, HO induction peaks at 16 h, whereas ferritin induction does not peak until 48 h (4). Greater HO activity would liberate more free iron than initially could be stored by ferritin. Of the three groups exposed to O2, the Hb+O2 group had the highest HO values followed by the normal saline+O2 and Hb+O2+SnPP groups. Therefore, the Hb+O2 group would be the most susceptible to hydroxylation reactions because of the increased availability of iron for the Fenton reaction.

Thus lower TBARS could be found in animals that had minimal induction of HO (normal saline+O2) or animals in which HO activity had been inhibited (Hb+SnPP+O2). The finding that nonspecific tissue oxidation products were highest in the protected group does not support the concept that the protection afforded by IT Hb administration occurs through a direct antioxidant mechanism. In addition, we determined that the major intracellular antioxidant enzyme associated with protection against hyperoxic injury, MnSOD, was not induced by prior IT Hb administration to the lung.

Balla et al. (3) demonstrated that ferritin, possibly through iron sequestration or reoxidase activity, acts as a cytoprotective agent of endothelial cells against oxidative stress. Although it has been postulated that HO-1 catabolism of heme and the subsequent release of iron are responsible for the ferritin induction associated with heme exposure, it has been shown that ferritin can be induced and is associated with cellular protection even when HO-1 induction is inhibited (3). We therefore hypothesized that the protection afforded by IT Hb may be through direct induction of ferritin by Hb. Lung ferritin content was clearly increased by both immunocytochemistry and slot-blot analysis after administration of IT Hb. These data provide circumstantial evidence for a role for ferritin in the protection conferred against hyperoxia by IT Hb administration and open an avenue for further investigation of the
mechanisms of protection against hyperoxic lung injury. It is also possible that Hb protects the lung through other mechanisms, perhaps involving scavenging of NO- and prevention of peroxynitrite formation. This possibility awaits further investigation.

In conclusion, IT Hb administration specifically induces HO-1 in the lung and protects against hyperoxia; however, in the rat lung, the protection is not mediated by HO enzyme activity. Although HO-1 induction is protective in some types of oxidative stress, induction of HO activity in other settings has not been protective against oxidative injury (2, 19). This contradictory evidence raises questions about the nature of the role of HO-1 as an antioxidant enzyme and emphasizes the need for further biochemical study of the cellular mechanisms affected by HO-1 under conditions of oxidative stress. Our results also suggest that the mechanism by which IT Hb protects against pulmonary O2 toxicity may not be through antineutrophil or direct antioxidant action. Further studies should explore the role ferritin might play as a means of protecting the lung from hyperoxia.

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