Heme Oxygenase-mediated Resistance to Oxygen Toxicity in Hamster Fibroblasts

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The role of heme oxygenase (HO)-1 was evaluated in the oxygen-resistant hamster fibroblast cell line, O2R95, which moderately overexpress HO when compared with the parental cell line, HA-1. To suppress HO-1 expression, O2R95 were transfected with HO-1 antisense oligonucleotide or treated with tin-mesoporphyrin (SnMP). To increase HO-1 expression, cells were transfected with HO-1 cDNA in a pRC/cytomegalovirus (CMV) vector. All cells were challenged with a 48-h exposure to 95% O2 (hyperoxia). When HO activity was suppressed, O2R95 cells had significantly decreased cell viability, increased susceptibility to lipid peroxidation, and increased protein oxidation in hyperoxia. In contrast, further overexpression of HO-1 did not improve resistance to oxygen toxicity. Antisense-transfected cells and SnMP-treated cells with lowered HO activity showed increased levels of cellular heme compared with controls. In the HO-1 cDNA-transfected O2R95 cells, cellular heme was lowered compared with controls; however, cellular redox active iron levels were increased. We conclude that HO mediates cytoprotection to oxygen toxicity within a narrow range of expression. We speculate that this protective effect may be mediated in part through increased metabolism of the pro-oxidant heme but that higher levels of HO activity obviate protection by increased redox active iron release.

Heme oxygenase (HO-1), the rate-limiting enzyme in the conversion of heme to bilirubin, is known to be induced by various oxidant stresses. However, it is not clear whether HO serves in protection against hyperoxia and, if so, by which mechanisms. HO-1 antisense transfection experiments have shown that higher HO-1 protein levels were associated with protection against UVA radiation (1). Furthermore, transfection of coronary vessel endothelial cells with an overexpression vector containing HO-1 cDNA resulted in resistance against hemoglobin-induced injury (2). We have previously shown that HA-1 hamster fibroblasts made stably resistant to oxygen toxicity (O2R95) had 1.8-fold higher HO activity (3), suggesting that moderate increases in HO activity may be beneficial in resistance to oxygen toxicity. However, O2R95 cells have increases in other antioxidants that may also contribute to their resistance to oxygen toxicity (4), and no direct evidence currently exists linking HO to resistance to oxygen toxicity.

Investigators have hypothesized that HO may serve a role in protection against oxidative injury by forming the antioxidant molecules biliverdin and bilirubin (5, 6). Additionally, induction of ferritin with enhanced HO activity has been observed (1). This could lead to sequestration of redox active iron, thereby conferring protection against oxidative stress (7). Another possible antioxidant mechanism of HO could involve the destruction of heme itself. Heme and hemoproteins have been shown by several investigators to be instrumental in exacerbating oxidative injury (8, 9). This has lead to the hypothesis that reduction of the cellular heme pool by HO may diminish the interaction of heme with oxygen radicals or other reactive oxygen intermediates.

To investigate mechanisms by which HO-1 plays a causal role in resistance to oxygen toxicity, we examined the effect of reducing or increasing HO activity in O2R95 cells in the absence of nonspecific effects on other antioxidants believed to provide protection from oxygen toxicity. This was achieved by transfection with antisense oligonucleotides to HO-1 and HO-1 cDNA in a pRC/cytomegalovirus (CMV) overexpression vector, respectively. All transfected cells were also evaluated for levels of major cellular antioxidants other than HO and were not shown to demonstrate nonspecific effects.

Cells were then exposed to hyperoxia for 48 h, and cellular injury was measured by LDH release and Trypan Blue dye exclusion. Oxidative damage was assessed by measuring protein oxidation, glutathione depletion and susceptibility to lipid peroxidation as determined by formation of thiobarbituric acid reactive substances (TBA-RS). The injury response of the antisense-transfected cells was then compared with that of O2R95 cells transfected with sense or random oligonucleotides. To further corroborate the causal role of HO-1 in hyperoxic resistance, oxygen-resistant cells were treated with tin-mesoporphyrin (SnMP), a competitive inhibitor of HO, and exposed to hyperoxia for 48 h. Cellular injury and oxidative injury parameters were also assessed and compared with controls not treated with SnMP.

In all models of HO manipulation, the possible mechanism by which HO confers protection against oxygen toxicity were probed by comparing heme content and iron content of control and treated cells. This was done to determine whether accumulation of heme could explain increased oxidative injury when HO activity was suppressed and whether iron accumulation occurred with higher levels of HO activity.

EXPERIMENTAL PROCEDURES

Cell Line—A hamster fibroblast cell line with stable resistance to oxygen toxicity (O2R95) was used in all experiments. These cells have been extensively studied as to their antioxidant levels, growth charac-
teristics, and morphology (4). O$_2$R$_5$ cells were isolated following chronic exposure (>200 days) of the HA-1 parental cell line to progressively increasing concentrations of oxygen (80–95%). O$_2$R$_5$ cells were then passaged in normoxia (up to 75 days) and were shown to maintain a stable oxygen-resistant phenotype, relative to HA-1 cells (4). Additionally, these cells are known to have increased levels of HO-1 and HO-2 protein as well as total HO activity compared with the parent cell line HA-1 (3).

**Cell Culture Conditions**—Hamster fibroblasts were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum (HyClone), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were grown in a 5% CO$_2$ humidified atmosphere at 37 °C and maintained at subconfluency by passaging every 3–4 days with trypsin-EDTA. The cells were counted and 1 × 10$^5$ cells for each cell type was seeded in 75-mm$^2$ flasks and allowed to proliferate for 3 days for experiments.

**Transfection with Oligonucleotides**—Upon attaining 50% confluence, cells were incubated in serum-free medium for 5 h. The cells were then transfected with HO-1 antisense oligonucleotides using a liposomal transfection reagent, DOTAP (Boehringer Mannheim). The oligonucleotides were comprised of the HO-1 transcription initiation codon and 6 base pairs on either side. Negative controls were the sense oligonucleotide, a complementary sequence to the antisense, and a random oligonucleotide, which comprised all of the base pairs of the antisense codon in random order. DOTAP and oligonucleotides were each prepared in HEPES buffer and then mixed at a ratio of 6:1. This ratio was determined in preliminary experiments to allow for optimal transfection efficiency in our system (data not shown). These two solutions were then combined and added to medium containing 0.5% serum, and this mixture was then added to the culture dishes. The cells were allowed to grow in the media containing oligonucleotides for a 24-h period. Thereafter, the complete growth medium was added, and the culture flasks were exposed to hyperoxia (95% oxygen, 5% CO$_2$) for a 48-h period in a 37 °C humidified incubator. At 24-h intervals, the cells and media were collected for analysis. The 48-h time point was chosen in these experiments since it is known that no significant cellular injury occurs in O$_2$R$_5$ cells exposed to hyperoxia within this time frame (3).

**Incubation with Tin-mesoporphyrin**—In other experiments, cells cultured to growth of 60–70% confluence were rinsed in Hank’s balanced salt solution and incubated in Eagle’s MEM supplemented with 1% fetal bovine serum (HyClone), glutamine (2 µmol), penicillin (100 units/ml), and streptomycin (100 µg/ml) and 10 µM tin-mesoporphyrin (Porphyrin Products, Logan UT) immediately prior to exposure to hyperoxia for 48 h, as described above. The metalloporphyrin solution was prepared in a darkened room, and the cell cultures were shielded from the light to avoid photoreactivity.

**Determination of HO Activity and Immunoactive HO-1 Protein Levels**—Cell homogenates were analyzed for HO activity by gas chromatography, as described previously (10), in subdued lighting. Homogenates were analyzed for protein content by the method of Bradford (11) and read at 585 nm.

Polyclonal rabbit anti-HO-1 antibodies were raised against a 30-kDa soluble cold-soluble protein expressed in *Escherichia coli* from rat liver cDNA (12) (gift of Angela Wilks, University of California San Francisco, CA) by Berkeley Antibodies Inc., Berkeley, CA, as described previously (13). Rabbit anti-rat HO-2 were obtained from Stressgen (Vancouver, BC), and human ferritin antibodies were obtained from Sigma. For detection of HO-1 and HO-2 immunoactive protein, 20-µg aliquots of cell sonicates were electrophoresed on a 12% polyacrylamide gel and incubated overnight with a 1:600 dilution of rabbit anti-HO-1 IgG. Antigen antibody complexes were visualized with the alkaline phosphatase chemiluminescence system according to the manufacturer instructions (Bio-Rad). Blots were subsequently washed in Tris-buffered saline with 0.1% Tween 20 overnight and reincubated for 2 h with a 1:800 dilution of rabbit anti-HO-2 IgG, and antigen antibody complexes were visualized as described above. For ferritin analysis, the Western method was modified in that a 15% gradient polyacrylamide gel (Bio-Rad) was used for electrophoresis, and a 1:1000 dilution of ferritin antibodies was incubated with the membranes for 2 h. In all gels, equal loading was verified by Coomassie Blue staining. Quantification of protein signal was performed by densitometry (PDI, Sunnyvale, CA). Additionally, to allow for normalization of ferritin protein signal between samples, quantification of bands on the membranes was performed, and values for the antigen-antibody signal were expressed as a ratio of the membrane signal.

Immunohistochemical staining of HO-1 protein was accomplished with cells grown on glass slides to >80% confluence. The slides were washed in phosphate-buffered saline (PBS) and then fixed in ice-cold 100% acetone. The cells were permeabilized in 0.3% saponin in PBS and blocked in a PBS solution containing 5% milk, 1% bovine serum albumin, and 0.03% saponin. The slides were then incubated with a 1:25 dilution of rabbit anti-rat HO-1 antibodies overnight in a humidified chamber. After incubation, the slides were washed twice in PBS containing 0.03% saponin and 1% milk and further incubated with a 1:50 dilution of goat anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA) conjugated to fluorescein isothiocyanate (FITC) for 2 h at 37 °C. The slides were then mounted in phenylene diamine and viewed with an Axioskop fluorescent microscope (Zeiss, Germany) fitted with a 100-watt Mercury HBO100W/2 (Zeiss) lamp at excitation of 493 nm and photographed with a Nikon camera.

**Determination of Cellular Injury**—Cell culture medium was assayed for LDH release after hyperoxic exposure. In brief, samples of media (0.1 ml) were mixed with 0.2 mg NADH in 0.1 M KPO$_4$ buffer and allowed to incubate for 10 min in a multwell plate. Sodium pyruvate (2.3 µmol) was then added, and samples were read at 340 nm at 2 s intervals for 2 min. LDH concentration was calculated automatically from the slope of the absorbance curve with comparison to standard LDH enzyme solutions (Enzyme control 2-E, Sigma) (14). As another measure of cytotoxicity, cells were also stained with Trypan Blue and counted on a hemocytometer (>500 cells scored per sample). The percentage of cells that excluded Trypan Blue was used as an assessment of cell viability.

**Determination of Oxidative Damage**—Protein oxidation was estimated by Western analysis of protein carbonyl content by taking 4 volumes of the cell sonicates (a total of 20 µg of protein) in 2 volumes of 10% SDS buffer and reacting with 1 volume of 10 mM dinitrophenyldrazine in trifluoroacetic acid for 30 min at room temperature. The samples were then neutralized by addition of 2 ml Tris base, 30% glycerol (v/v), subjected to SDS-PAGE (15% gradient gels), and transferred to PVDF membrane (Immobilon, Millipore). The blots were incubated with mouse monoclonal IgG anti-DNP antibody (Sigma) and then complexed to rat IgE anti-mouse HRP-labeled antibody (Southern Bio-technologies) and visualized by chemiluminescence (ECL kit, Amersham Corp.) (15). To compare the extent of protein oxidation, densitometric quantification of the band consistently showing the strongest signal in all samples was performed (PDI). Additionally, to allow for normalization of anti-DNP signal between groups, quantification of the Coomassie Blue-stained band on the membranes was performed, and values for the anti-antigen antibody signal was expressed as a ratio of the membrane signal.

Susceptibility to lipid peroxidation was assessed in cells scraped from flasks and incubated with buffer containing 50 µM ADP and 1 mM FeCl$_3$ for 1 h at 37 °C. Thereafter, 0.3 ml of 10% trichloroacetic acid and 0.6 ml of 0.5% thiobarbituric acid (TBA) solution were added, and samples were incubated at 60 °C for 15 min. The samples were centrifuged at 5,000 × g, absorbance was read at 535 nm, and values were determined using an extinction coefficient of 1.55 × 10$^{-5}$ cm$^{-1}$ (16).

To ensure that the effect of antisense transfection was specific to HO-1 and did not result in altered levels of antioxidants, cellular antioxidants were measured 24 h after transfection. For total glutathione and antioxidant enzyme analysis, cells were washed twice with cold (4 °C) Puck’s saline, scraped into cold saline, centrifuged, and the cell pellets were frozen at −80 °C. Frozen cell pellets were thawed, 50 mM phosphate buffer containing 1.34 µM diethylenetriaminepentaacetic acid was added, and the samples were sonicated for five bursts of 5 s each on ice. An aliquot of each sample was assayed for protein content by the method of Lowry, et al. (17). An aliquot of each sample was then mixed with 5% sulfosalicylic acid to obtain a diluted, protein-precipitated sample for determination of total glutathione using the method of Anderson (18) and expressed as micrograms of total GSH per milligrams of protein. The γ-glutamyl transferase activity of samples was detected using a commercially available kit (Sigma 419). Glutathione S-transferase activity was determined by the methods of Simmons and Van der Jagt (19) using chlorodinitrobenzene as substrate. Glutathione peroxidase activity was assayed by the method of Lawrence and Burk (20) using cumene hydroperoxide as substrate. Catalase activity was determined by the method of Beers and Sizer (21) and expressed as k units/mg of protein as described by Aebi (22). Superoxide dismutase activity was determined by the methods of Spitz and Oberley (23) and expressed as units per mg of protein. Cu/Zn superoxide dismutase activity was distinguished from Mn superoxide dismutase activity by the method of Sies (24). The determination of the Cu/Zn superoxide dismutase activity with 5 mM sodium cyanide.

**Determination of Heme and Iron Content**—Heme content was determined in cell homogenates (20 µg of protein) solubilized in 2.5 ml of 1% cetyltrimethylammonium bromide in 0.2 N NaOH and scanned at absorbance 350–450 nm. The absorbance peak corresponding to the heme Sorret band (387.5 nm) was quantitated by comparing to an external solution.
Fig. 1. Total HO activity in transfected cells exposed to hyperoxia. HO activity in cell homogenates was determined by gas chromatography allowing for the detection of CO. The clear bar (A) represents antisense-transfected cells, the hatched bar (S) represents sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of six experiments.

The effect of antisense transfection on other antioxidants was also assessed to better understand the effect of this technique on susceptibility to hyperoxic injury. A small but significant elevation in total glutathione content was observed with HO-1 antisense transfection prior to hyperoxic exposure. None of the other measured antioxidant enzymes were modified by antisense transfection (Table I).

Evaluation of Cellular Injury—Antisense transfection led to significantly decreased resistance to oxygen toxicity. After hyperoxic exposure, cells treated with antisense oligonucleotides had a 1.8- and 1.7-fold increase in LDH release compared with sense or random transfected controls, respectively (Fig. 3A) and significantly decreased cell viability compared with sense and random transfected controls, respectively (Fig. 3B). More specific markers of oxidant injury were also assessed. After 48 h of hyperoxic exposure, antisense-transfected cells showed lower levels of total glutathione than sense or random transfected controls (83% of sense and 77% of random) (Fig. 4). Protein oxidation, as determined by protein carbonyl content, increased 2.02- and 1.81-fold in antisense-transfected cells exposed to hyperoxia compared with sense and random controls, respectively (Fig. 5). Furthermore, susceptibility to TBA-RS formation was increased 1.9- and 2.5-fold in antisense-transfected hamster fibroblasts compared with sense and random transfected controls, respectively (Fig. 6). These results strongly support the hypothesis that the oxygen-resistant phenotype noted in the O2R95 cells is in part causally related to the modified HO-1 protein and activity in these cells compared with the parent cells. These results are also consistent with the observations of Vile et al. (2), which show, using antisense transfection, that lowering HO-1 protein altered resistance to UVA radiation. However, no reports to date have documented a causal link between decreased HO-1 protein or decreased HO activity on resistance to oxygen toxicity, further suggesting that HO is important in physiological antioxidant defenses.

Effect of Overexpression of HO-1 in O2R95 Cells—Others
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TABLE I

Antioxidants in transfected O2R95 cells

| Antioxidant | Antisense | Sense | Random | HO-1 cDNA |
|-------------|-----------|-------|--------|-----------|
| GSH*        | 5.23 ± 0.20 | 4.52 ± 0.07 | 4.21 ± 0.07 | 5.76 ± 0.36 |
| γ-GT†       | 1.39 ± 0.06 | 1.35 ± 0.09 | 1.22 ± 0.11 | 1.07 ± 0.21 |
| GPX         | 45.33 ± 0.23 | 41.03 ± 2.62 | 39.07 ± 1.15 | 44.2 ± 2.1 |
| GST         | 547.67 ± 15.07 | 538 ± 17.32 | 477 ± 30.04 | 581.33 ± 21.3 |
| CAT         | 976 ± 88 | 916.33 ± 42.53 | 914 ± 40.39 | 1260 ± 87.9 |
| Total SOD   | 201.33 ± 11.56 | 188 ± 6 | 224 ± 14.47 | 205.67 ± 24.13 |
| Cu,Zn-SOD   | 168.33 ± 12.92 | 160.33 ± 5.81 | 195.33 ± 15.32 | 183 ± 25.58 |
| Mn-SOD      | 33.33 ± 4.33 | 27.67 ± 2.4 | 28.67 ± 2.03 | 22.67 ± 1.45 |

* µg/mg of protein.
† p < 0.05 versus sense or random control.
‡ p < 0.05 versus sham-transfected control.
§ Units/mg of protein.
α Milliunits/mg of protein.
β sites units/mg of protein.

FIG. 4. Total glutathione content in transfected cells exposed to hyperoxia. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of four experiments.

Fig. 3, A, relative LDH release in transfected cells exposed to hyperoxia. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of six experiments. B, relative cell viability in transfected cells exposed to hyperoxia as determined by Trypan Blue exclusion. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus sense control; †, p < 0.05 versus random control. Values are expressed as mean ± S.E. of nine experiments.

have investigated the effects of transfection of HO-1 cDNA in other models of injury. A 3-fold increase in HO activity resulted from cytoprotection against heme-mediated injury (2). If lowered HO activity is associated with lowered resistance to oxygen toxicity and overexpression of HO is associated with improved resistance to oxygen toxicity, as previously suggested (3), we hoped to further improve oxygen resistance in the O2R95 cells by further increasing HO activity with transfection with HO-1 cDNA in a prC/CMV overexpression vector. With this strategy, HO activity and HO-1 protein were 1.8- and 2.3-fold higher, respectively, in the transfected O2R95 than in the O2R95 controls (Fig. 1), whereas HO-1 protein was increased 2.3-fold in the HO-1 cDNA-transfected cells compared with sham-transfected controls (Fig. 2A), and no changes in HO-2 protein were noted (Fig. 2B). The latter observation was further corroborated with immunohistochemistry where HO-1 protein was visibly increased in the HO-1-cDNA transfected cells compared with sham-transfected controls (data not shown). Therefore, we could assume that the HO-1 cDNA transfected cells had approximately a 4-fold increase in HO activity compared with HA-1 parent cells. However, the transfected cells did not show any further protection against oxygen toxicity despite relatively higher total GSH and catalase levels than controls (Table I). There was no decrease in LDH release in HO-1 cDNA transfected cells exposed to hyperoxia compared with controls (Fig. 3A). Additionally, no changes in total glutathione (Fig. 4) and in susceptibility to TBA-RS formation (Fig. 6) were associated with overexpression of HO-1. In fact, in some instances, deleterious effects were observed with overexpression of HO-1. There was a significant loss of cell viability (Fig. 3B), and protein oxidation was increased 1.67-fold (Fig. 5, A and B) in HO-1 cDNA-transfected cells compared with sham-transfected controls after hyperoxic exposure. This seemingly paradoxical effect may be similar to what is observed with other antioxidants, albeit within a tighter range. For example, maximal overexpression of Cu,Zn superoxide dismutase results in increased rather than decreased toxicity compared with moderate overexpression (27). Perhaps HO serves as an antioxidant in oxygen toxicity but only within a narrow range of HO expression.

Evaluation of Heme, Iron, and Ferritin—To begin to better understand how HO mediates antioxidant effects, we examined heme and iron contents in all experimental conditions. We hypothesized that heme regulation by HO may allow for lowering of this known pro-oxidant. Several investigators have shown that heme is an oxidant in several model systems (8, 9). Furthermore, it was recently demonstrated that HO-1 overexpression can protect against exogenously provided heme (2). However, it is not known whether HO sufficiently modifies endogenous heme levels to alter outcome of oxidative injury in hyperoxia, especially in a cell culture model where no exoge-
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**Fig. 5.** A. Shown is a representative of six Western analyses of protein carbonyls detected with anti-DNP antibodies in antisense-transfected cells. Lane U, negative control, cells not treated with an oxidizing agent; lane O, positive control, cells treated with ascorbic acid and ferric chloride for 4 h to cause oxidative damage; lane A, antisense-transfected cells; lane S, sense-transfected cells; lane R, random transfected cells, and lane V, HO-1 cDNA-transfected cells. Equal loading of the experimental samples was verified with Coomassie Blue. B, densitometric quantitation of the protein carbonyl content of transfected cells exposed to hyperoxia. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus control; †, p < 0.05 versus random control. Values are derived from the Western analyses illustrated in panel A) and expressed as mean ± S.E. of six experiments.

**Fig. 6.** TBA-RS formation in transfected cells exposed to hyperoxia and incubated with FeCl3 and ADP for 1 h (see “Experimental Procedures”). The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of six experiments.

**Fig. 7.** Heme content in transfected cells exposed to hyperoxia. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of three experiments.

**Fig. 8.** Iron content in transfected cells exposed to hyperoxia. The clear bars (A) represent antisense-transfected cells, the hatched bars (S) represent sense controls, the gray bars (R) represent random transfected cells, and the solid bars (V) represent the HO-1 cDNA-transfected cells. *, p < 0.05 versus controls. Values are expressed as mean ± S.E. of three experiments.

SnMP, a competitive inhibitor of HO. This compound was chosen since it has been used in clinical trials in human neonates without adverse effects (31), and it lowers HO activity effectively without induction of HO-1 mRNA or changes in HO-1 protein within 48 h, as with other metalloporphyrins (32). Cells incubated with 10 μM SnMP showed a significant decrease in total HO activity compared with untreated controls (65 ± 3% of control; p < 0.05 in a total of 3 experiments in each group). Although HO activity changed with SnMP incubation, HO-1 or HO-2 protein content did not change (Fig. 9).

As with antisense transfection, incubation of O2R95 with SnMP and subsequent exposure to hyperoxia resulted in loss of resistance to oxygen toxicity. In the SnMP-treated cells, we noted decreased cell viability as assessed by Trypan Blue dye exclusion (80.1 ± 3 versus 95.1 ± 2%; mean of five experiments; p < 0.05), glutathione depletion (3.3 ± 0.01 versus 5.35 ± 0.04 μg/mg of protein; mean of four experiments; p < 0.05), and increased susceptibility to lipid peroxidation (34.02 ± 3.5 versus 13.58 ± 4.4 nmol/mg of protein; mean ± S.E. of 5 groups, p < 0.05) but, surprisingly, no increased LDH release compared with the antisense-transfected cells. This could not be attributed to a direct effect of SnMP on the LDH assay since we did not observe a reduction in LDH with addition of SnMP to samples. In addition, although hyperoxic exposure alone resulted in increased protein oxidation compared with air controls (relative densitometric units of 0.48 ± 0.05 versus 0.25 ± 0.00 mean ± S.E. of three experiments; p < 0.05), incubation with SnMP did not further increase protein oxidation (0.48 ± 0.05 versus 0.53 ± 0.03; mean ± S.E. of three experiments).

These differences may be due to cellular effects of SnMP since this agent may be metabolized by the cells and may inhibit or alter other enzymes or cellular functions (33). Nonetheless, these experiments are consistent with the antisense transfection experiments, where only HO-1 was altered, and demonstrate that lowering HO activity increases susceptibility to oxygen toxicity.

As with the transfection experiments, we wanted to evaluate...
FIG. 9. Representative of six Western analyses of HO-1 (upper band) and HO-2 (lower band) immunoreactive protein in SnMP-treated cells. Lane C, positive control, liver from CoCl-treated adult rat for HO-1 or adult rat brain for HO-2; lane OC, control, oxygen exposed cells; lane OS, SnMP-treated oxygen-exposed cells. Equal loading of the experimental samples was verified with Coomassie Blue.

FIG. 10. Representative of 4 polyacrylamide gels treated with luminol for the detection of hemoproteins in O2R95 cells incubated with 10 μM SnMP and exposed to hyperoxia (see "Experimental Procedures"). The images represent an inverted pixelated image of the photonic emission collected from the electrophoresed protein samples by a CCD camera. Lane 1, negative control, 500 μg albumin; lanes 2–6, positive controls, cytochrome c (10 μg), hemoglobin (20 μg), myoglobin (40 μg); lane OC, control, oxygen exposed cells; and lane OS, SnMP-treated oxygen-exposed cells. Equal loading of the experimental samples was verified with Coomassie Blue. The lower band in each lane (arrow) represents free heme migrating with the dye front.

the role of heme and iron in the protective role of HO using this model. However, SnMP absorbs at 387 nm. Therefore, detection of the heme peak would be altered in the presence of SnMP. We alternatively evaluated heme and hemoprotein content of cells by chemiluminescent detection. Using this technique, SnMP-treated cells exposed to hyperoxia had visibly higher heme and hemoprotein content than untreated hyperoxia exposed controls (Fig. 10). SnMP alone did not result in luminescence. In hyperoxia, SnMP treated cells had a 1.9-fold increase in total cellular iron compared with untreated controls exposed to hyperoxia (2.79 ± 0.54 versus 1.45 ± 0.53 μg/mg protein, mean ± S.E. of four experiments, p < 0.05). This could not be explained by interference of SnMP with the ferrozine reaction. These results further corroborate that degradation of heme and accumulation of iron may play a significant role in HO-mediated cytoprotection.

Effect of HO Expression on Ferritin Protein Levels—In many model systems, iron (34, 35) and increased HO activity (35, 36) have shown to regulate ferritin. It is thought that in model systems, iron (34, 35) and increased HO activity (35, 36) accumulate, thereby negating the beneficial antioxidant properties of HO.

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