# Protective Effects of Heme Oxygenase-1 against Oxidant-Induced Injury in the Cultured Human Tracheal Epithelium

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To examine whether increases in heme oxygenase (HO)-1 activity have protective effects on the oxidant-induced injury of airway epithelial cells, human tracheal epithelial cells were cultured on a porous filter membrane, and electrical conductance (*G*) and mannitol flux across epithelial membrane were measured with Ussing's chamber methods and D-[$^3$H]mannitol, respectively. Hydrogen peroxide ($H_2O_2$; 1 mM) increased *G* with time from the baseline value of 6.0 ± 0.6 to 17.8 ± 0.9 mS/cm$^2$ at 6 h after administration ($P < 0.001$). Likewise, $H_2O_2$ significantly increased mannitol flux through the cultured epithelium ($P < 0.01$). Pretreatment of cultured epithelial cells with hemin (10 μM; 8 h) or interleukin (IL)-1β (10 ng/ml; 16 h) completely inhibited increases in *G* and mannitol flux induced by $H_2O_2$. Tin protoporphyrin IX (50 μm) and zinc protoporphyrin IX (10 μM), inhibitors of HO-1, reduced hemin-induced and IL-1β–induced inhibitory effects. Hemin treatment increased HO-1 messenger RNA expression, HO-1 protein production, and HO activity and bilirubin content as well as ferritin content in the cultured epithelial cells. Pretreatment with hemin and desferoxamine, which, like ferritin, can bind iron, inhibited $H_2O_2$-induced increases in *G* and mannitol permeability. Although exogenous bilirubin mimicked hemin-induced inhibitory effects, exogenous apoferritin failed to inhibit $H_2O_2$-induced effects on *G* and mannitol permeability. These findings suggest that HO-1 induction provides protection against $H_2O_2$-induced injury of the cultured human airway epithelial cells in part via the HO–bilirubin pathway. **Yamada, N., M. Yamaya, S. Okinaga, R. Lie, T. Suzuki, K. Nakayama, A. Takeda, T. Yamaguchi, Y. Itoyama, K. Sekizawa, and H. Sasaki. 1999. Protective effects of heme oxygenase-1 against oxidant-induced injury in the cultured human tracheal epithelium. Am. J. Respir. Cell Mol. Biol. 21:428–435.**

Heme oxygenase (HO) oxidatively degrades heme to biliverdin, which is reduced enzymatically to bilirubin by biliverdin reductase (1). Two isoforms of HO, HO-1 and HO-2, exist and are the products of separate genes (2, 3). HO-2 is a constitutive enzyme found primarily in the central nervous system, whereas HO-1 is induced by heme, heavy metals, cytokines, endotoxin, and hormones as well as by agents causing oxidative stress (2–6).

Recent reports have shown that increased HO-1 production provides cellular protection against heme- and non–heme-mediated oxidant injury (7–10). HO-1 prevents kidney failure and reduces mortality caused by infusion of hemoglobin (7), and induction of HO-1 by hemoglobin results in protection against lethal endotoxemia (8). Further, overexpression of HO-1 in cultured endothelial cells also provides protection against heme and hemoglobin toxicity (9).

Hydrogen peroxide ($H_2O_2$) and xanthine–xanthine oxidase (X-XO) increase electrical conductance (*G*) across the cultured rat alveolar and ferret tracheal epithelium (11, 12). Likewise, $H_2O_2$, glucose oxidase, and X-XO increase both *G* and mannitol flux across the cultured Madin Darby canine kidney cells (13) and cultured human tracheal epithelial cells (14). These findings suggest that oxidants cause epithelial injury, resulting in increased permeability across the epithelium. An increase in resistance to hyperoxia is observed in human pulmonary epithelial cells with increased HO-1 activity induced by gene transfection (10). Pretreatment with nitrogen oxide impairs resistance to reactive oxygen toxicity in rat hepatocytes, and tin protoporphyrin IX (SnPP-9) prevents the protection (15). However, increased constitutive HO-1 expression is associated with resistance to hyperoxia in oxidant-resistant hamster fibroblasts (16). Pretreatment with hemoglobin

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*Abbreviations:* apoferritin, APO; desferoxamine, DFO; electrical conductance, *G*; hydrogen peroxide, $H_2O_2$; heme oxygenase, HO; interleukin, IL; messenger RNA, mRNA; phosphate-buffered saline, PBS; standard error of the mean, SEM; tin protoporphyrin IX, SnPP-9; ultraviolet, UV; zinc protoporphyrin IX; ZnPP-9.

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protects against hyperoxia in the rat, but the protection is not mediated by increased HO enzyme activity (17). Further, the mice lacking HO-2 were sensitized to hyperoxia despite increased HO-1 expression (18). Thus, the effects of HO-1 induction on oxidant-induced injury of airway epithelium remain uncertain.

We therefore investigated whether induction of HO-1 has protective effects against $H_2O_2$-induced increases in permeability of the cultured human tracheal epithelium. We also examined mechanisms responsible for protection against $H_2O_2$-induced epithelial injury by HO-1.

## Materials and Methods

### Cell Culture

Tracheas from 62 patients without overt pulmonary disease (mean age, 64 ± 4 yr; range, 24 to 89 yr) were obtained 3 to 6 h after death, under a protocol passed by the Tohoku University Ethics Committee. Human tracheal epithelial cell culture was performed as described previously (14, 19).

### Ussing Chamber Study

For studies in Ussing chambers, Millicell inserts with their attached cells without edge damage were mounted in a modified Ussing chamber (14). Experiments were performed on Days 7 to 14 of culture in a Krebs–Henseleit solution with the following composition (in mM): 118 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 25.5 $NaHCO_3$, and 5.6 glucose. The solution was maintained at 37°C and aerated continuously by bubbling with a mixture of 95% $O_2$–5% $CO_2$ (pH 7.4). Transepithelial resistance and *G* were determined from the current produced by the fixed transepithelial potential difference (pulse width, 200 ms; intensity, 0.5 mV; frequency, 0.05 Hz).

When cultured cells become differentiated, they have a value of over 40 $\Omega \cdot cm^2$ for resistance and show a multilayered structure with cilia and secretory granules (19). Therefore, cultured cells with high resistance ($> 40\ \Omega \cdot cm^2$) were judged as differentiated and used for subsequent experiments.

### Experimental Protocols

To examine the effects of $H_2O_2$ on *G* in the cultured human tracheal epithelium, we added $H_2O_2$ (1 mM) to both sides of the Krebs–Henseleit solution in the Ussing chambers. In preliminary studies, we found that $H_2O_2$ caused changes in *G* at a concentration higher than 1 mM. Therefore, we used this concentration of $H_2O_2$ in the following experiments.

To determine whether HO-1 inhibits $H_2O_2$-induced increases in *G*, the cultured human tracheal epithelial cells were preincubated with hemin (10 μM), an inducer of HO-1 in a variety of tissues (6), for 8 h (20). To study the mechanisms responsible for hemin-induced inhibitory effects on increases in *G* induced by $H_2O_2$ (1 mM), cells were preincubated with hemin (10 μM) and either SnPP-9 (50 μM) or zinc protoporphyrin IX (ZnPP-9; 10 μM), competitive inhibitors of HO-1 (3, 6, 10, 17, 21), for 8 h. Because metalloporphyrins are photosensitive and light-exposed metalloporphyrins do not inhibit HO (21), the cells treated with either SnPP-9 or ZnPP-9 were covered from light throughout the experiments.

To determine the mechanisms responsible for HO-1–mediated protection against epithelial injury induced by $H_2O_2$, cells were preincubated with the iron chelator desferoxamine (DFO; 5 mM) 15 min before the administration of hemin or the vehicle of hemin. We also studied the effects of apoferritin (APO) and bilirubin on $H_2O_2$-induced increases in *G*. Cells were preincubated with either APO (1 μM) or bilirubin (1 μM) for 1 h before the administration of $H_2O_2$.

To determine whether induction of HO-1 by stimuli other than hemin has a protective effect on epithelial injury induced by $H_2O_2$, the cultured human tracheal epithelial cells were preincubated with interleukin (IL)-1β (10 ng/ml) (4) for 16 h. In preliminary studies, we found that the maximal expression of HO-1 messenger RNA (mRNA) was observed at a 16-h incubation time.

### Mannitol Flux Studies

Measurement of permeability across epithelial cell sheets was performed by the methods described by Cooper and colleagues (22) with D-[$^3$H]mannitol, producing a behavior similar to albumin (23). Cell sheets cultured on Millicell inserts, put on 24-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ), were rinsed with phosphate-buffered saline (PBS) and culture medium was added to both sides of the cell sheets. Culture medium contained 2 mM nonradioactive mannitol to minimize the D-[$^3$H]mannitol (0.05 μM)–induced changes in osmolarity. To match the fluid level, we added 0.2 ml and 0.7 ml of medium to the apical and basolateral sides, respectively. D-[$^3$H]mannitol (1 μCi/ml, 0.05 μM; Daiichi Kagaku Yakuhin, Tokyo, Japan) was added to either the serosal or mucosal side of the tissue (hot side). Cell sheets were then reincubated at 37°C in a 5% $CO_2$ incubator. Whole volumes of medium (0.2 or 0.7 ml) were taken for liquid scintillation counting from either the mucosal or serosal side of the cell sheets (cold side) every 6 h, and the same volume of fresh cold medium was replaced to the cold side of the cell sheets.

For the first 6 h, the medium on both sides (hot and cold sides) did not contain $H_2O_2$. After the first sampling at 6 h, the hot-side medium containing D-[$^3$H]mannitol was taken and replaced by fresh radioactive medium containing D-[$^3$H]mannitol supplemented with $H_2O_2$ (1 mM) or the vehicle. On the cold side, fresh medium with $H_2O_2$ (1 mM) or the vehicle was replaced after the first sampling period at 6 h. Whole medium was then taken for counting and fresh medium supplemented with $H_2O_2$ (1 mM) or the vehicle was replaced to the cold side every 6 h. In preliminary studies we found that apparent increases in *G* were observed 6 h after the addition of $H_2O_2$ (1 mM). Therefore, mannitol permeability was studied in cell sheets preincubated with $H_2O_2$ (1 mM) for 6 h. To examine the protective effects of HO-1 on $H_2O_2$ (1 mM)-induced increases in mannitol permeability, cell sheets were preincubated with either hemin (10 μM) alone, hemin (10 μM) plus SnPP-9 (50 μM), or hemin (10 μM) plus ZnPP-9 (10 μM) for 8 h.

We also studied the effects of either DFO, APO, or bilirubin, and those of IL-1β pretreatment (10 ng/ml, 16 h) on

$H_2O_2$-induced increases in mannitol permeability in a way similar to the Ussing chamber study described previously.

### Northern Blot Analysis

Human tracheal epithelial cells were cultured in human placental collagen–coated glass tubes. To study the effects of hemin and IL-1β on the expression of HO-1 gene in the human tracheal epithelial cells, cells were incubated in medium containing either hemin (10 μM) or IL-1β (10 ng/ml) for 0, 8, 16, and 24 h. Total cellular RNA was prepared from each glass tube according to the method of RNAzol B (Cinna/Biotex Laboratories, Inc., Houston, TX) and subjected to Northern blot analysis as described previously (24). Total RNA (10 μg) extracted from human tracheal epithelial cells was electrophoresed in 1.1% agarose/formaldehyde gel and transferred onto a nylon membrane (Hybond-$N^+$; Amersham Life Science, Little Chalfont, UK) via capillary action. The RNAs blotted on the filter were fixed by brief exposure to ultraviolet (UV) irradiation and were hybridized with $^{32}$P-labeled *Xho*I (position −64)–*Xba*I (position 923) fragment derived from human HO-1 complementary DNA, pHHO1 (25). Hybridization with the probe labeled with [α-$^{32}$P]deoxycytidine triphosphate, using the Random Primer DNA Labeling Kit Ver.2 (Takara, Ohtsu, Japan), was performed overnight at 42°C. After washing the hybridized filter, autoradiographic detection of the labeled probe was performed by exposing the filter to Kodak Scientific Imaging film for 48 to 72 h at −80°C. Quantitation of autoradiographic bands was accomplished with an image analyzer (Bio Imaging Analyzer, BAS-2000; Fuji Photo Film, Minamiashigara, Japan), and expressed as the intensity of the HO-1/β-actin bands.

### Western Blot Analysis

Western blot analysis was performed as previously described (24, 26). After incubation of human tracheal epithelial cells with the medium containing hemin (10 μM) or IL-1β (10 ng/ml) for 0, 8, and 24 h, cells were lysed in lysis buffer with the following composition: 20 mM Tris-HCl (pH 7.4), 1% Triton X100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The cell lysates were centrifuged at 12,000 × *g* for 10 min and the supernatant obtained was quantified for protein by BCA Protein Assay Reagent (Pierce, Rockford, IL). A total of 10 μg of protein in the supernatant was mixed with loading buffer, boiled for 5 min, incubated at room temperature for 10 min, and then electrophoresed in an SDS–polyacrylamide gel (17.5%). The proteins in the gel were transferred to the membrane (Immobilon; Millipore Corp., Bedford, MA) and probed with diluted antirat HO-1 polyclonal antibody, OSA-100 (400-fold; StressGen Biotechnologies Co., Victoria, BC, Canada). The membrane was subsequently treated with the second antibody (Vectastain ABC rabbit IgG kit; Vector Laboratories, Inc., Burlingame, CA), and then subjected to the procedure of staining, which allowed visual comparison of the relative staining intensity of an individual band as an indicator of HO-1 induction.

### HO Activity

Measurements for HO activity analysis were made as described previously (20, 27). After exposing human tracheal epithelial cells to hemin (10 μM) for 0, 8, and 24 h, cells were collected and stored at −80°C until assay for HO. Thawed cells suspended in PBS were disrupted by sonication for 30 s at 160 W, followed by centrifugation at 20,000 × *g* for 15 min at 4°C. The supernatant obtained was successively centrifuged at 105,000 × *g* for 60 min. The resulting pellets (microsomes) were suspended in 50 mM potassium phosphate buffer (pH 7.4) by the sonication, and assayed for HO activity. Reaction mixtures, in a final volume of 200 μl, had the following composition: 0.1 M of potassium phosphate buffer, 500 μg/ml of microsomes, 15 μm of hemin, 100 μg/ml of bovine serum albumin (BSA), 100 μg/ml of a partially purified biliverdin reductase (28), and 420 μg/ml of nicotinamide–adenine dinucleotide phosphate. The mixtures were aerobically incubated for 20 min at 37°C. The reaction was then stopped by placement on ice, and the amount of bilirubin formed was measured with a double-beam spectrophotometer (Hitachi U-2000; Hitachi, Tokyo, Japan) as optical density 464 to 530 nm (excitation coefficient, 43.5/mM/cm for bilirubin). HO activity was expressed as picomoles of bilirubin formed per milligram of protein. The protein content was determined by the method of Lowry and associates (29).

We also studied the effects of either SnPP-9 (50 μm) or ZnPP-9 (10 μM) on hemin (10 μM)-induced increases in HO activity.

### Measurement of Bilirubin and Ferritin

To measure the content of bilirubin and ferritin in the cultured human tracheal epithelial cells, cells were preincubated with either hemin (10 μM) or the vehicle of hemin for 8 h. After 30 min of disaggregation in a trypsinizing flask on an orbital shaker (240 rpm) at room temperature, cells were suspended in a mixture of 40% Ham's F-12 medium, 40% Dulbecco's modified Eagle's medium, and 20% fetal calf serum. Cell counts were made using a hemocytometer. Cells were pelleted by centrifugation (200 × *g*, 10 min) at 22°C and washed twice with PBS. After centrifugation (200 × *g*, 10 min) at 22°C, cells were suspended in 200 μl PBS and sonicated on ice. Supernatant was then obtained by centrifugation (15,000 rpm, 20 min) at 4°C and stored at −80°C until use.

We measured bilirubin and its derivatives using enzyme-linked immunosorbent assay with an antibilirubin monoclonal antibody 24G7 as previously reported (30). Cell-associated bilirubin content is expressed in microns per $10^6$ cells.

We also measured ferritin using immunoradiometric assay with an RIA kit (Gamma·Dab Ferritin; Dade-Behring, IL) as described previously (31). Cell-associated ferritin content is expressed in picomoles per $10^6$ cells. We also studied the effects of either SnPP-9 (50 μM) or ZnPP-9 (10 μM) on hemin (10 μM)-induced increases in cell-associated ferritin content.

### Drugs

Hemin, SnPP-9, and BSA were from Sigma Chemical (St. Louis, MO), and $H_2O_2$ and ZnPP-9 were from Wako Pure Chemical (Osaka, Japan).

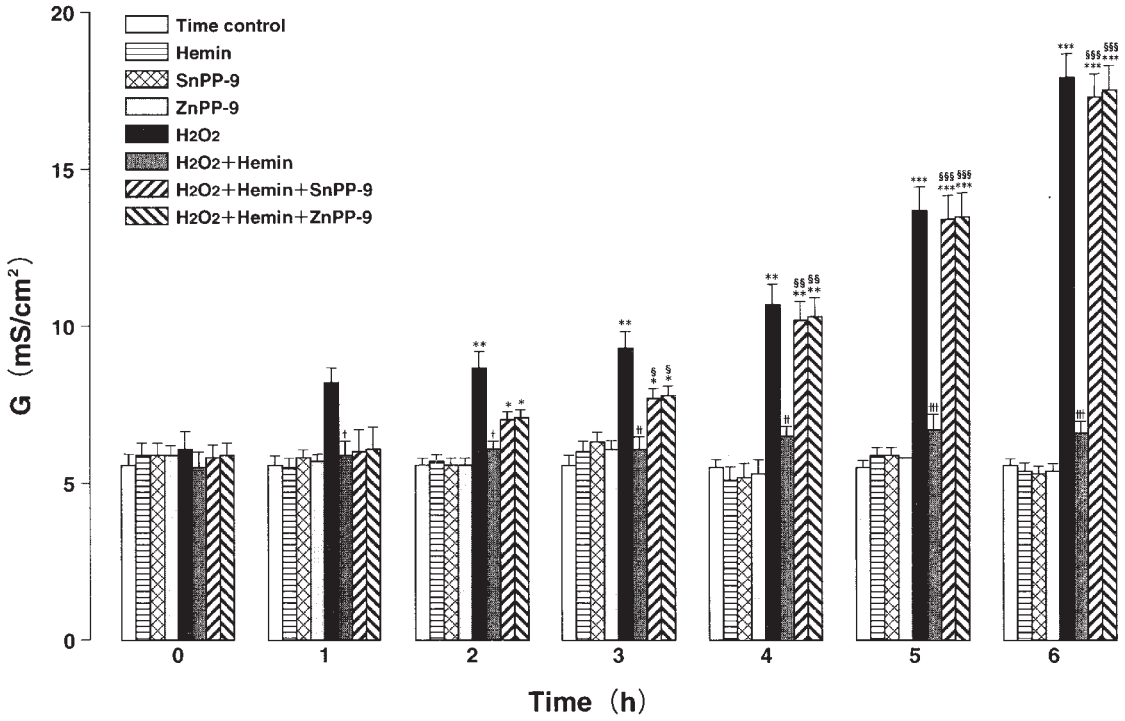


*Figure 1.* Time-course changes in transepithelial membrane conductance ($G$) in time control, hemin alone, SnPP-9 alone, ZnPP-9 alone, and those induced by $H_2O_2$ alone (1 mM), $H_2O_2$ plus hemin (10 μM), $H_2O_2$ plus hemin (10 μM) and SnPP-9 (50 μM), and $H_2O_2$ plus hemin (10 μM) and ZnPP-9 (10 μM). Results are reported as means ± SEM from seven samples. Significant differences from time control values are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant differences from $H_2O_2$ alone are indicated by $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$. Significant differences between hemin/$H_2O_2$ and hemin/$H_2O_2$/SnPP-9 or between hemin/$H_2O_2$ and hemin/$H_2O_2$/ZnPP-9 are indicated by $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, and $^{\S\S\S}P < 0.001$.

### Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance and Duncan's multiple range test. Significance was accepted at $P < 0.05$; and $n$ refers to the number of donors from which cultured epithelial cells were used.

## Results

### Effects of $H_2O_2$ on $G$

Baseline resistance of the cultured epithelial cells was 444 ± 31 Ω · cm² ($n$ = 28). Figure 1 shows the time course of $H_2O_2$ (1 mM)-induced effects on $G$ in the cultured tracheal epithelium. The baseline values of $G$, in mS/cm², were 5.6 ± 0.4 in controls, 5.7 ± 0.5 in hemin alone, 5.7 ± 0.4 in SnPP-9 alone, 5.7 ± 0.4 in ZnPP-9 alone, 6.0 ± 0.6 in $H_2O_2$ alone, 5.4 ± 0.6 in hemin plus $H_2O_2$, 5.6 ± 0.6 in hemin plus SnPP-9, and 5.6 ± 0.6 in hemin plus ZnPP-9, and were stable with time for 6 h in the control condition. $G$ increased with the addition of $H_2O_2$ (1 mM) and reached a value of 17.8 ± 0.9 mS/cm² at 6 h. Neither hemin (10 μM), SnPP-9 (50 μM), nor ZnPP-9 (10 μM) alone altered $G$. However, hemin (10 μM) significantly inhibited $H_2O_2$ (1 mM)-induced increases in $G$ for the period of 1 to 6 h after the addition of $H_2O_2$. SnPP-9 (50 μM) and ZnPP-9 (10 μM) reduced hemin (10 μM)-induced inhibitory effects (Figure 1).

Figure 2 summarizes the effects of various treatments on $H_2O_2$-induced increases in $G$. Neither DFO (5 mM) nor APO (1 μM) altered $H_2O_2$-induced increases in $G$. Likewise, DFO did not alter the inhibitory effects of hemin on

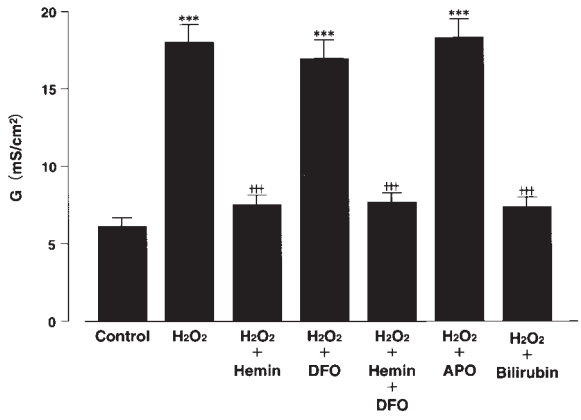


*Figure 2.* Changes in transepithelial membrane conductance ($G$) induced by $H_2O_2$ (1 mM) 6 h after administration, and the effects of $H_2O_2$ plus hemin (10 μM), DFO (5 mM), hemin and DFO, APO (1 μM), and bilirubin (1 μM). Results are reported as means ± SEM from seven samples. Significant differences from time control values are indicated by $^{***}P < 0.001$. Significant differences from $H_2O_2$ alone are indicated by $^{\dagger\dagger\dagger}P < 0.001$.

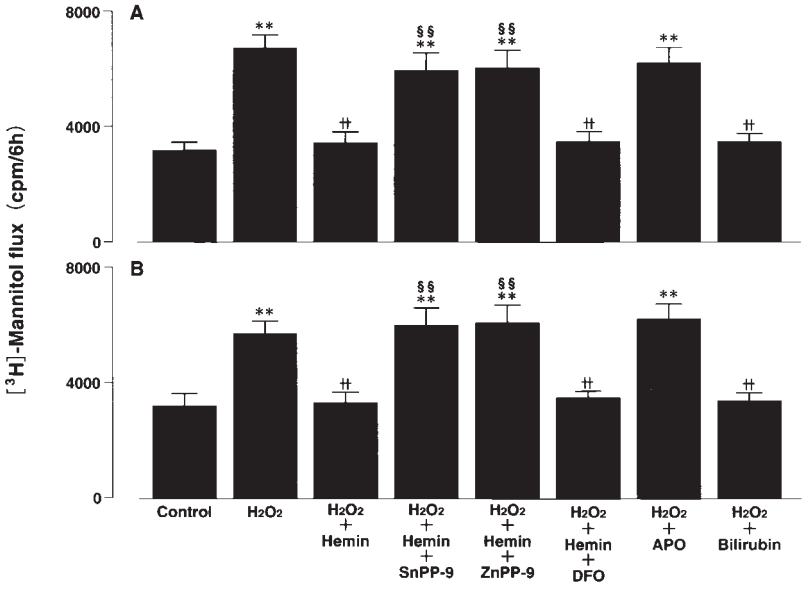


*Figure 3.* Changes in [$^3$H]mannitol flux from serosa to mucosa (*A*) and from mucosa to serosa (*B*) induced by $H_2O_2$ (1 mM) 6 h after administration, and the effects of $H_2O_2$ plus hemin (10 μM), hemin and SnPP-9 (50 μM), hemin and ZnPP-9 (10 μM), hemin and DFO (5 mM), APO (1 μM), and bilirubin (1 μM). Results are reported as means ± SEM from seven samples. Significant differences from time control values are indicated by $^{**}P < 0.01$. Significant differences from $H_2O_2$ alone are indicated by $^{\dagger\dagger}P < 0.01$. Significant differences between hemin and hemin plus SnPP-9 or between hemin and hemin plus ZnPP-9 are indicated by $^{\S\S}P < 0.01$.

$H_2O_2$-induced increases in *G*. However, hemin-induced inhibitory effects were mimicked by bilirubin (1 μm).

### Effects of $H_2O_2$ on Mannitol Flux

Mannitol flux was stable with time for 12 h in the control condition. $H_2O_2$ (1 mM) significantly increased mannitol flux from mucosa to serosa (Figure 3A) and from serosa to mucosa (Figure 3B). Increases in mannitol flux induced by $H_2O_2$ were completely inhibited by hemin (10 μM) and either SnPP-9 or ZnPP-9 reduced hemin-induced inhibitory effects. However, DFO (5 mM) failed to alter hemin-induced inhibitory effects. Although bilirubin (1 μM) mimicked hemin-induced inhibitory effects, APO (1 μM) failed to alter $H_2O_2$-induced increases in mannitol flux (Figure 3).

Similar to hemin, IL-1β (10 ng/ml) pretreatment alone altered neither the baseline *G* nor mannitol flux compared with controls (Table 1). IL-1β (10 ng/ml) pretreatment significantly inhibited $H_2O_2$ (1 mM)-induced increases in *G* and mannitol flux, and either SnPP-9 (50 μM) or ZnPP-9 (10 μM) reduced IL-1β–induced inhibitory effects (Table 1).

### Effects of Hemin and IL-1β on HO-1 mRNA Expression

Exposure of human tracheal epithelial cells to either hemin (10 μM) or IL-1β (10 ng/ml) caused increases in HO-1 mRNA. The maximal expression of HO-1 mRNA was observed at 8 h after hemin exposure and at 16 h after IL-1β exposure (Figure 4, *upper panels*). Levels of HO-1 mRNA at 8 h after hemin exposure and at 16 h after IL-1β exposure were significantly high compared with controls (Figure 4, *lower panels*).

### Effects of Hemin and IL-1β on HO-1 Protein Production

Exposure of human tracheal epithelial cells to either hemin (10 μM) or IL-1β (10 ng/ml) caused an increase in HO-1 protein synthesis detected by Western blot analysis (Figure 5).

### Effects of Hemin on HO Activity

Figure 6 shows the HO activity at 8 h after exposure of hemin (10 μM) to the cultured epithelial cells. Hemin sig-

TABLE 1

*Effects of IL-1β pretreatment on $H_2O_2$-induced increases in conductance and [$^3$H]mannitol flux*

| | Conductance ($mS/cm^2$) | [$^3$H]Mannitol Flux | |
|---|---|---|---|
| | | Mucosa to Serosa | Serosa to Mucosa |
| | | (*cpm/6 h*) | |
| Control | 6.6 ± 0.2 | 3,312 ± 213 | 3,259 ± 279 |
| IL-1β | 6.7 ± 0.2 | 3,186 ± 199 | 3,384 ± 248 |
| $H_2O_2$ | 19.2 ± 2.4$^\dagger$ | 6,244 ± 478* | 6,315 ± 552* |
| $H_2O_2$ + IL-1β | 6.9 ± 0.2 | 3,384 ± 288 | 3,231 ± 268 |
| $H_2O_2$ + IL-1β + SnPP-9 | 18.0 ± 2.1$^\dagger$ | 6,114 ± 436* | 6,217 ± 503* |
| $H_2O_2$ + IL-1β + ZnPP-9 | 18.2 ± 1.9$^\dagger$ | 6,224 ± 502* | 6,320 ± 499* |

Values are means ± SEM from seven samples.
*$P < 0.01$ from Control.
$^\dagger P < 0.001$ from Control.

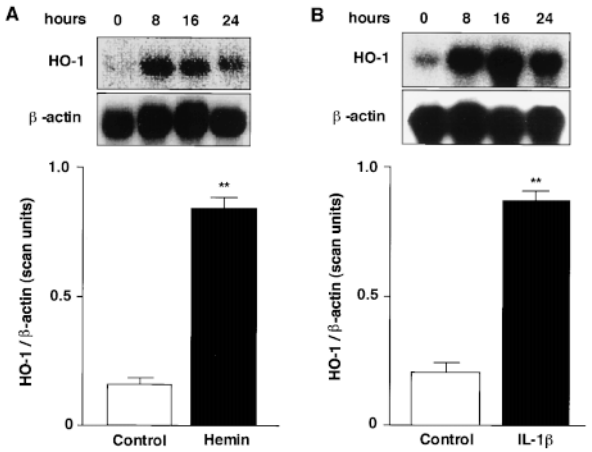


*Figure 4.* Northern blot analysis demonstrating increases in HO-1 mRNA levels of human tracheal epithelial cells after either hemin (10 μM) (*A*) or IL-1β (10 ng/ml) (*B*) treatment. β-actin was used as a housekeeping gene (*upper panels*). Expression of HO-1 mRNA in human tracheal epithelial cells 8 h after treatment with hemin or vehicle (Control) (*A*) and 16 h after treatment with IL-1β or vehicle (*B*). HO-1 mRNA is normalized to constitutive expression of β-actin mRNA (*lower panels*). Results are reported as means ± SEM from seven samples. Significant differences from control values are indicated by $^{**}P < 0.01$.

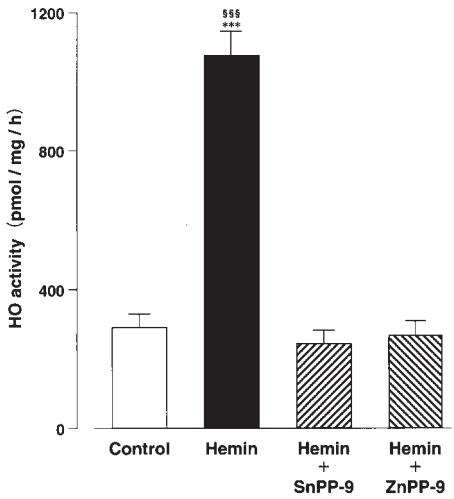


*Figure 6.* HO activity of the cultured human tracheal epithelial cells 8 h after treatment with either hemin (10 μM), hemin (10 μM) plus SnPP-9 (50 μM), hemin (10 μM) plus ZnPP-9 (10 μM), or vehicle (Control). Results are reported as means ± SEM from seven samples. Significant difference from control values is indicated by $^{***}P < 0.001$. Significant differences between hemin and hemin plus SnPP-9 or between hemin and hemin plus ZnPP-9 are indicated by $^{§§§}P < 0.001$.

nificantly increased the HO activity compared with controls, and either SnPP-9 (50 μM) or ZnPP-9 (10 μM) decreased HO activity to levels similar to controls.

### Bilirubin Content

Bilirubin content in the cells treated with hemin (10 μM) for 8 h was significantly higher than that in controls (2.8 ± 0.2 μM/$10^6$ cells versus 1.7 ± 0.1 μM/$10^6$ cells; $P < 0.01$, $n = 7$).

### Effects of Hemin on Ferritin Content

Ferritin content in the cells treated with hemin (10 μM) for 8 h was significantly higher than that in controls (2.2 ± 0.2 pmol/$10^6$ cells versus 1.3 ± 0.1 pmol/$10^6$ cells; $P < 0.01$, n = 7), and either SnPP-9 (50 μM) or ZnPP-9 (10 μM) decreased ferritin content to levels similar to control (1.4 ± 0.1 and 1.3 ± 0.1 pmol/$10^6$ cells, respectively; $P > 0.20$, n = 7).

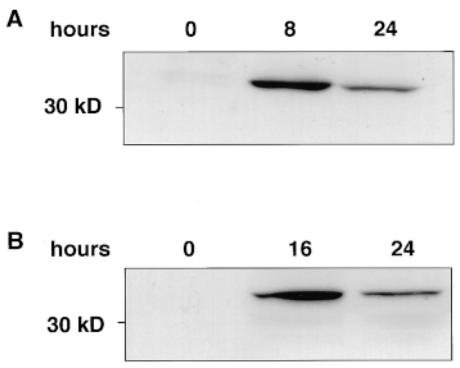


*Figure 5.* Western blot analysis demonstrating an increase in HO-1 protein level of human tracheal epithelial cells after treatment with either hemin (10 μM) (*A*) or IL-1β (*B*).

## Discussion

An alteration in the permeability of epithelial barriers is one of the consequences of acute inflammation. In the present study we found that exposure of human tracheal epithelial cells to $H_2O_2$ resulted in an increase in permeability. Increased permeability induced by $H_2O_2$ was inhibited by pretreatment of cells with either hemin or IL-1β, and inhibitory effects induced by hemin and IL-1β were reduced by SnPP-9 and ZnPP-9, competitive inhibitors of HO-1 (3, 6, 10, 17, 21). Hemin treatment increased HO-1 mRNA expression, HO-1 protein production, and HO activity. From these results, we conclude that induction of HO-1 may have protective effects on oxidant-induced injury of the cultured human airway epithelial cells.

HO-1 is induced by a variety of agents including heme, hemin, heavy metals, cytokines, endotoxin, hormones, and agents causing oxidative stress in alveolar macrophages, liver, kidney, and lung (2–6, 8, 32). Upregulation of HO-1 activity has been shown to provide protection against oxidant-induced kidney failure (7), endotoxin shock (8), and hyperoxia-induced pulmonary epithelial cell death (10). The present study suggests that increases in HO-1 activity induced by both hemin and IL-1β may have protective effects on oxidant-mediated injury of the cultured human tracheal epithelial cells.

Lung cells contain microsomal heme (18), and these heme pools may be the source substrate of HO in the lung. Although the mechanisms by which HO-1 has a protective effect against oxidant stress is still uncertain, the by-products derived from the reactions catalyzed by HO-1 have

been suggested to be responsible for the protective effects of HO-1 against oxidative stress (9, 33). The deleterious effects of reactive oxygen species such as superoxide and $H_2O_2$ are dependent on the presence of iron (33). Intracellular free iron can react with both $H_2O_2$ and superoxide via the Fenton reaction, giving rise to the toxic hydroxyl radical. Because the catalysis of heme by HO releases free iron, ferritin may be induced and may serve as a sink to prevent iron from participating in the Fenton reaction. Ferritin production is upregulated by a variety of agents including hemin, methemoglobin, $H_2O_2$, and UV irradiation in fibroblasts and endothelial cells (5, 9, 34). This accumulation of ferritin is associated with iron sequestration and protection against oxidative damage. Vile and coworkers (5) showed that the protective effects of HO-1 against UV irradiation are dependent on ferritin synthesis via HO-1. Balla and colleagues (9) have also shown that HO-1–induced protection against $H_2O_2$ in cultured endothelial cells is accompanied by increased ferritin expression. We observed that pretreatment of cells with hemin and the iron chelator DFO inhibited $H_2O_2$-induced increases in *G* and mannitol permeability, although pretreatment of cells with hemin increased ferritin content as well as HO-1 activity in the cells. Further, pretreatment of cells with exogenous APO did not alter $H_2O_2$-induced increases in *G* and mannitol permeability. These observations are in agreement with a previous report that the protection provided by hemoglobin-induced HO-1 expression against lethal endotoxemia is not dependent on ferritin induction in rats *in vivo* (8). Therefore, ferritin may not play a major role in providing protection against $H_2O_2$-induced injury of cultured human tracheal epithelial cells. However, antioxidant effects of ferritin produced by HO-1 (5, 9) cannot be ruled out.

The initial degradation of heme by microsomal HO involves the loss of iron and the formation of biliverdin, which is subsequently reduced to bilirubin by the cytosolic biliverdin reductase. Therefore, higher intracellular HO activity leads to an increased content of bilirubin, which is an efficient scavenger of reactive oxygen species as reported in an *in vitro* study by Stevens and Small (35) and Kaul and coworkers (36). Bilirubin interacts chemically with the superoxide anion (37), and its activity as chain-breaking antioxidant has also been shown (38, 39). Further, increased bilirubin levels by HO induction in various tissues have been regarded as an important cellular defense mechanism against oxidative injury (40). Accordingly, our results from cultured human tracheal epithelial cells exposed to $H_2O_2$ showed a neutralizing effect of bilirubin against oxidative injury. Although bilirubin is generally considered a toxic catabolite when accumulated at an abnormally high concentration in the tissue, the micromolar concentration used in our experiments was in a physiologic range (39).

SnPP-9 and ZnPP-9 not only are competitive HO inhibitors (3, 6, 10, 17) but also have a variety of biologic effects (21). In the present study, SnPP-9 and ZnPP-9 alone did not alter either baseline *G* or mannitol flux, suggesting that SnPP-9 and ZnPP-9 do not affect airway epithelial functions. However, metalloporphyrins are potent generators of oxygen free radicals that destroy proteins and affect epithelial permeability (21). Therefore, it is possible that inhibitory effects of SnPP-9 and ZnPP-9 may be due to oxygen free radicals induced by these inhibitors as well as inhibition of HO-1. Further study is needed to examine the precise mechanisms.

Because HO-1 induction was associated with increased bilirubin levels, it seems from our data that the observed stimulation of the HO–bilirubin pathway by hemin may, in part, make the cells more resistant to oxidative assaults. However, the antioxidant effects of carbon monoxide (CO), another catalytic product of heme catabolism by HO-1 (3, 6, 41), have not been studied. Similar to nitric oxide, CO increases cellular cyclic guanosine monophosphate (cGMP) (41), and a role for cGMP in protection against cellular oxidant injury has been suggested (15). Therefore, by-products other than bilirubin, derived from the reactions catalyzed by HO-1, may be involved in HO-1–induced protection against oxidant-induced injury of the airway epithelium.

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