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Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity

(gene transfer/oxidative stress/vascular endothelium/hemorrhagic toxicity)

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ABSTRACT **Heme oxygenase (HO) is a stress protein and has been suggested to participate in defense mechanisms against agents that may induce oxidative injury such as metals, endotoxin, heme/hemoglobin, and various cytokines. Overexpression of HO in cells might therefore protect against oxidative stress produced by certain of these agents, specifically heme and hemoglobin, by catalyzing their degradation to bilirubin, which itself has antioxidant properties. We report here the successful *in vitro* transfection of rabbit coronary microvessel endothelial cells with a functioning gene encoding the human HO enzyme. A plasmid containing the cytomegalovirus promoter and the human HO cDNA complexed to cationic liposomes (Lipofectin) was used to transfect rabbit endothelial cells. Cells transfected with human HO exhibited an ≈3.0-fold increase in enzyme activity and expressed a severalfold induction of human HO mRNA as compared with endogenous rabbit HO mRNA. Transfected and nontransfected cells expressed factor VIII antigen and exhibited similar acetylated low-density lipoprotein uptake (two important features that characterize endothelial cells) with >85% of cells staining positive for each marker. Moreover, cells transfected with the human HO gene acquired substantial resistance to toxicity produced by exposure to recombinant hemoglobin and heme as compared with nontransfected cells. The protective effect of HO overexpression against heme/hemoglobin toxicity in endothelial cells shown in these studies provides direct evidence that the inductive response of human HO to such injurious stimuli represents an important tissue adaptive mechanism for moderating the severity of cell damage produced by these blood components.**

A variety of oxidative stress-inducing agents, such as metals, UV light, heme, and hemoglobin have been implicated in the pathogenesis of the inflammatory process. The cellular response to such agents involves the production of a number of soluble mediators including acute-phase proteins, eicosanoids, and various cytokines.

The rate-limiting enzyme in heme catabolism, heme oxygenase (HO; refers to HO-1 isozyme unless otherwise specified), is a stress-response protein, and its induction has been suggested to represent an important cellular protective response against oxidative damage produced by free heme and hemoglobin (1–5). Induction of HO may specifically decrease cellular heme (pro-oxidant) and elevate bilirubin (antioxidant) levels (5–9). Two HO isozymes, the products of distinct genes, have been described (10, 11). HO-1, which is ubiquitously distributed in mammalian tissues, is strongly and rapidly induced by many compounds that elicit cell injury; the natural substrate of HO, heme, is itself a potent inducer of the enzyme (10, 11). HO-2, which is believed to be constitutively expressed, is present in high concentrations in such tissues as the brain and testis and is believed to be noninducible (10).

Endotoxin, interleukin 1, and other stress agents cause a rapid (within 5–10 min) activation of the HO gene and a subsequent accumulation of HO mRNA (12–14). This process involves transcriptional activation of several regulatory sites in the HO promoter region. AP-1 and interleukin 6 responsive elements are found in the promoter region of this gene (13, 15). A recent study from this laboratory demonstrated that the proximal promoter region of the human HO gene also contains NF-κB- and AP-2-binding sequences (16). The finding of AP-2- and NF-κB-binding sites on the HO promoter suggests the importance of HO in stress/injury responses, when these transcriptional factors are known to be activated (16).

Our goal in these studies was to augment HO activity by transfecting rabbit coronary microvessel endothelial (RCME) cells with the human cDNA for HO, so as to distinguish expression of the transfected HO from that of rabbit HO and to determine whether the transfected human HO gene could functionally protect the endothelial cells against the toxic effects of free heme and hemoglobin. We used plasmid DNA complexed to a cationic liposome preparation, Lipofectin, for transfection of the human HO-encoding gene; other investigators have also used these cationic liposomes with success in *in vivo* and *in vitro* DNA delivery systems (17–21).

Our data demonstrate that it is possible to transfect rabbit coronary endothelial cells with human HO cDNA and to achieve selective overexpression of the human HO mRNA to levels significantly higher than those of the endogenous rabbit HO mRNA. The overexpression of the human HO in the rabbit cells was not associated with phenotypic changes in the cells or alterations of endothelial cell markers such as acetylated low density lipoprotein (Ac-LDC) uptake or factor VIII antigen. Our study also demonstrates that augmentation of human HO activity in the transfected cells confers substantial protection against cellular toxicity produced by free heme/hemoglobin. These data thus directly demonstrate the protective effect of human HO against the cellular toxic effects of these blood components that are known to participate in inflammatory reactions at sites of hemorrhage, thrombosis, and trauma.

MATERIALS AND METHODS

RCME Cell Culture. Cells were isolated from the midportion of the rabbit myocardium by collagenase digestion, filtra-

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Abbreviations: HO, heme oxygenase (refers to HO-1 isozyme unless otherwise specified); Ac-LDL, acetylated low density lipoprotein; RCME, rabbit coronary microvessel endothelial; CMV, cytomegalovirus; rHb, recombinant human hemoglobin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

tion, homogenization, and centrifugation as described by Gerritsen *et al.* (22). The cells were seeded onto fibronectin-coated six-well culture plates and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 20% plasma-derived serum, endothelial cell growth factor at 100 μg/ml, and 2 mM L-glutamine at 37°C in a standard humidified incubator. After 2 hr nonadherent cells were removed. Endothelial cell colonies appeared in 2–5 days and were initially characterized by their morphology (i.e., closely apposed cells with a polygonal morphology). Cultures free of pericytes and smooth muscle cells were subcultured. Homogeneity of RCME cell cultures was assessed by Ac-LDL labeling followed by visual fluorescence microscopy and fluorescence-activated cell sorting. Cells used in the experiments (passages 12–25) were cultured in DMEM/10% fetal calf serum supplemented with 20 mM Hepes, L-glutamine (0.35 mg/ml), and gentamicin (0.06 mg/ml).

Factor VIII Antigen Immunofluorescence. Fibronectin-coated Lab-Tek tissue culture chamber slides (Fisher) were used to culture RCME cells. At confluence, the medium was removed, and cells were washed with Earle's balanced salt solution (EBSS). The cells were fixed with 100% methanol (−20°C) for 3 min and then washed three times with EBSS. A 1:80 dilution of fluoresceinated anti-human factor VIII (Incstar, Stillwater, MN) was added, and the cells were incubated for 60 min and then washed with EBSS. The slides were mounted in 50% glycerol/phosphate-buffered saline and viewed and photographed with a phase microscope equipped with an epifluorescent light source.

Uptake of Ac-LDL. Preconfluent cultures (1 T-75 flask per sort) of cells were incubated with 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled Ac-LDL (10 μg/ml; Molecular Probes) in growth medium for 4 hr at 37°C. Cells were washed several times with probe-free medium and visualized by fluorescence microscopy.

Transfection of Human HO-cDNA into Rabbit Endothelial Cells. The plasmid used was a human HO construct, pRC/CMV-human HO, containing the entire protein-coding region, the *Xho* I/*Xba* I fragment (−63/924) of pH HO, in a correct orientation under the promoter/enhancer of cytomegalovirus (CMV). The plasmid was constructed as follows: both ends of the *Xho* I/*Xba* I fragment were converted to blunt ends with Klenow enzyme, ligated to the *Hin*dIII linker, and then cloned into the *Hin*dIII site of pRC/CMV (Invitrogen). The same plasmid without the HO cDNA was used as a control.

For stable transfection ≈1 million cells were seeded in a 75-cm^2 flask using standard culture medium (see above) at 37°C in 5% CO_2. Four hours later the growth medium was changed to DMEM/0.5% fetal calf serum, and cells were transfected with 20 μg of plasmid DNA–Lipofectin complex using the mammalian transfection kit (GIBCO/BRL). Cells were selected for neomycin resistance in medium containing G418 (500 μg/ml). Positive colonies were pooled and subcultured and used in all subsequent studies. CYP4A1 cDNA (2.1 kb) cloned in a pBluescript SK(+/−) vector was cut out by digestion with *Xba* I and *Xho* I and inserted into the *Xba* I and *Xho* I sites of the pBK-CMV vector (Stratagene, 4512 bp). RCME cells were transfected with the CYP4A1-CMV vector using the lipofectamine method and selected with neomycin. Total RNA was extracted and hybridized with the CYP4A1 cDNA probe. Only transfected cells expressed CYP4A1 mRNA and CYP4A1 protein as measured by Northern and immuno-hybridization, respectively (data not shown).

RNA Extraction and Northern Blot Analysis. Confluent cultures of transfected and nontransfected cells were used for Northern blot analysis and probed with either human HO cDNA or rat HO cDNA because rat HO cDNA is able to hybridize with rabbit HO mRNA. Total RNA was isolated by lysis of the cells in 4 M guanidium isothiocyanate and quantitated by spectrophotometry. Total RNA (10 μg) was denatured and size-separated by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. To verify the integrity of the samples, gels were stained for 5 min with ethidium bromide at 0.5 μg/ml in diethylpyrocarbonate-treated water, destained, and photographed. RNA was transferred to nylon membranes (GeneScreenPlus membranes, NEN) using 20× standard saline citrate (SSC; 1× SCC is 0.15 M sodium chloride/0.015 M sodium citrate) buffer as recommended by the manufacturer. The membranes were then baked for 2 hr at 80°C. Blots were hybridized at 60°C overnight and washed as described (16). The hybridization mixture consisted of 1% bovine serum albumin/7% SDS/1 mM EDTA/3× SSC, pH 7.0. Hybridized blots were washed at 60°C in a solution containing 0.5% bovine serum albumin, 5% SDS, and 1 mM EDTA in 0.3× SSC followed by a second wash with 0.1× SSC/1% SDS/1 mM EDTA. Filters were exposed to autoradiography film (DuPont/NEN) at 70°C. All filters were reprobed with cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech) to ensure that equal amounts of RNA were loaded onto each lane.

Measurement of HO Enzyme Activity. Enzyme activity was assessed using microsomes (6xT-175 flasks) from nontransfected and transfected endothelial cells. Cells were harvested and microsomes were prepared after cell homogenization and centrifugation (100,000 × *g*). Microsomes (1 mg of protein) were incubated with heme (50 μM), rat liver cytosol (2 mg/ml), $MgCl_2$ (1 mM), glucose-6-phosphate dehydrogenase (3 units), glucose 6-phosphate (1 mM), and $NADP^+$ (2 mM) in 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, for 30 min at 37°C. The reaction was stopped by placing the tubes on dry ice, and bilirubin was extracted with chloroform as described (14). The amount of bilirubin generated was estimated by using a scanning spectrophotometer and defined as the difference between 463 and 520 nm (14). Results are expressed as pmol per 5 × 10^6 cells per 30 min.

Cell Viability. Cells seeded in 12-well culture plates (1 × 10^5 cells per well) were grown for 24 hr in standard culture medium. Recombinant human hemoglobin (rHb) was obtained from Somatogen (Boulder, CO) and prepared in phosphate-buffered saline. Heme was prepared as described (23). Heme (1–200 μM) or rHb (1–400 μM) was added to the cells for 24 hr. Control cultures received the appropriate vehicles. Experiments were done in triplicate. Cell viability was measured by trypan blue exclusion. Briefly, cell suspensions were prepared by trypsinization followed by washing and resuspension in 1 ml of Hanks' balanced salt solution (HBSS). Cell suspensions (0.2 ml) were mixed with 0.5 ml of 0.5% trypan blue and 0.3 ml of HBSS and allowed to stand for 10 min at room temperature. Cells were counted by using a hemocytometer. Cell viability was calculated as the percentage viable (unstained) cells of total (stained and unstained) cells. Statistical analysis was done by using a Kruskal–Wallis ANOVA followed by a Dunn's comparison.

RESULTS

Effect of HO Gene Transfection on Factor VIII Levels and Ac-LDL Uptake. Factor VIII antigen and Ac-LDL uptake are widely used as specific markers for the characterization of the endothelial cell phenotype. As seen in Fig. 1 *A* and *B* both cell types, transfected as well as control endothelial cells, expressed factor VIII antigen; Ac-LDL uptake, as a marker to aid in identifying possible effects of human HO on the endothelial cell phenotype, was also assessed. Both transfected and nontransfected cells exhibited a similar degree of Ac-LDL uptake (Fig. 1 *C* and *D*). In both cases—i.e., factor XIII antigen and Ac-LDL uptake—85–90% of transfected and control cells stained positive. The presence of factor VIII antigen and Ac-LDL uptake supports the view that transfection of endothelial cells with the human HO gene did not significantly alter the cell phenotype.

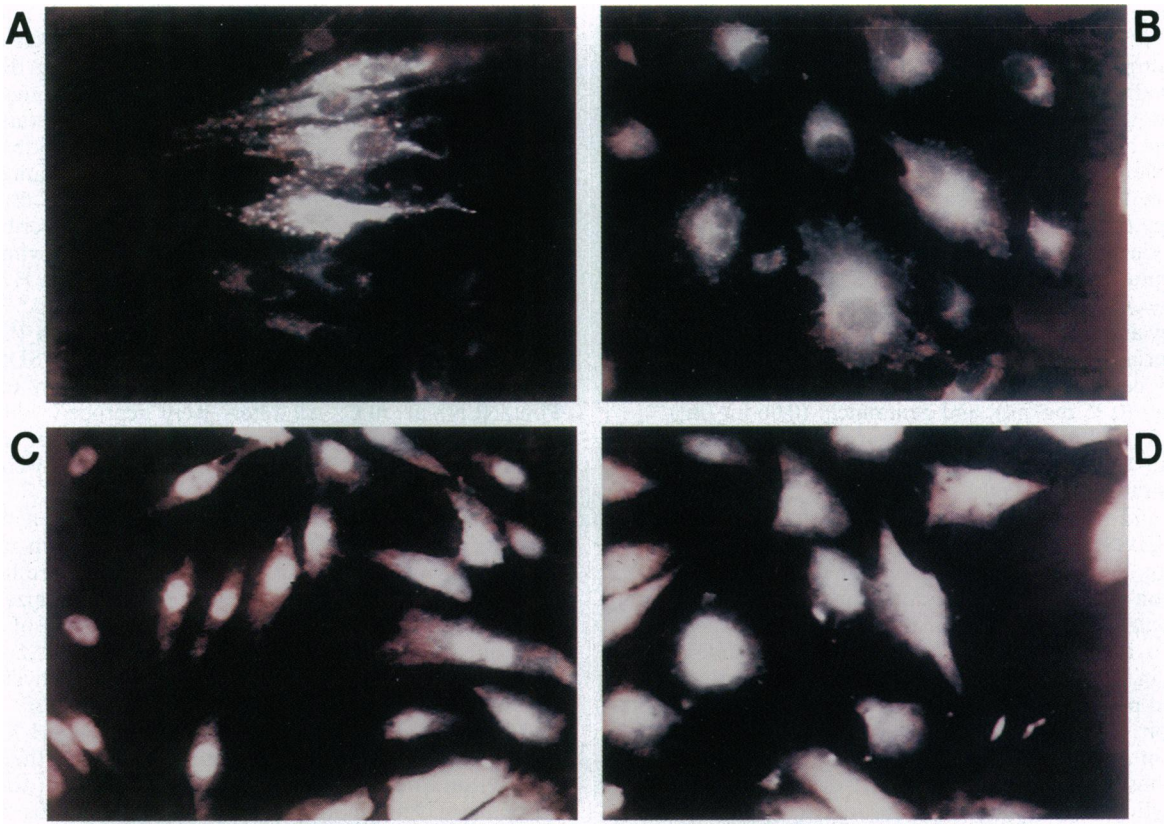

FIG. 1. Effect of human HO gene transfection on endothelial cell factor VIII antigen and Ac-LDL uptake in endothelial cells. Immunofluorescent staining of control (*A*) and HO-transfected endothelial cells (*B*) with factor VIII antibodies. Fluorescent Ac-LDL uptake in the endothelial cells of control (*C*) and HO-transfected cells (*D*), respectively.

Expression of Human HO mRNA in Rabbit Endothelial Cells. Transfection of endothelial cells with the human HO gene was assessed by parallel determinations of endogenous rabbit HO mRNA and the expressed human HO mRNA in control cells, cells transfected with human HO plasmid DNA, and cells transfected with control plasmid pRC/CMV (Invitrogen). As seen in Fig. 2, endothelial cells transfected with the human HO cDNA–Lipofectin complex significantly expressed human HO mRNA (lane 3). Endothelial cells transfected with the control plasmid did not show detectable human HO mRNA (lane 2). Similarly, control endothelial cell RNA did not hybridize with human cDNA, indicating the absence of a hybridizable band similar to human HO mRNA (lane 1). To examine the effect of the control plasmid on endogenous rabbit HO, we performed Northern blot analysis on total RNA from nontransfected and transfected cells and probed with the rat HO cDNA. Rat HO cDNA hybridizes to rabbit HO mRNA, whereas human HO cDNA does not. The presence of a hybridizable rabbit HO mRNA is seen in all three cell types; cells transfected with control plasmid; cells transfected with the human HO plasmid; and untransfected cells (Fig. 2 *Middle*). As seen in Fig. 2, there were no differences in endogenous HO mRNA levels in all cell types after transfection with control plasmid. To assure the integrity and quantity of transferred RNA, nitrocellulose filters were washed and rehybridized with radiolabeled G3PDH cDNA. As seen in Fig. 2 (*Bottom*) signals corresponding to G3PDH confirmed the integrity and transfer of equal amounts of RNA to the filters in each lane of the paired experiments.

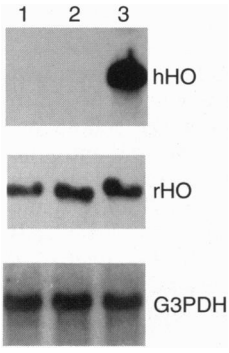

FIG. 2. Northern blot analysis of RNA from control endothelial cells (lane 1), cells transfected with a control plasmid (lane 2), and cells transfected with the human HO gene (lane 3). Analysis of total RNA (10 μg per lane) with a human HO (hHO) cDNA (*Top*), rat HO (rHO) cDNA (*Middle*), and G3PDH probe (*Bottom*). The human HO-specific probe does not detect rabbit HO mRNA transcripts, and rat HO cDNA does not detect human HO mRNA.

Enhancement of HO Activity in Endothelial Cells Transfected with Human HO. We examined HO activity in untransfected endothelial cells, cells transfected with control plasmid (pRC/CMV), and plasmid containing human HO; results are depicted in Table 1. The basal level of HO activity in endothelial cells was 160 ± 17 pmol of bilirubin formed per 5×10^6 cells per 30 min as compared with 482 ± 77 pmol of bilirubin

Table 1. Effect of Sn-mesoporphyrin on control and transfected endothelial cell HO activity

Conditions	HO activity, mean (±SEM) pmol per 5×10^6 cells per 30 min
Control cells	160 (±14)
Control cells + Sn-mesoporphyrin	14 (±4)
Cells transfected with control plasmid	152 (±27)
Transfected cells with human HO gene	482 (±77)
Transfected cells with human HO gene + Sn-mesoporphyrin	120 (±12)

HO was assayed as described. Sn-mesoporphyrin was added at a final concentration of 5 μM in the incubation mixture. Data are presented as the means ± SEMs.

formed per 5×10^6 cells per 30 min after human HO cDNA transfection ($P < 0.001$). In contrast, transfection of endothelial cells with control plasmid did not increase the endogenous rabbit HO activity (Table 1). To further ascertain the characteristics of the expressed HO protein, we tested the effect of Sn-mesoporphyrin, a potent inhibitor of HO activity, on microsomal preparations from both nontransfected and transfected cells. Addition of 5 μM Sn-mesoporphyrin to cell preparations inhibited the enzyme activity by ≈90% and ≈75% ($P < 0.001$) in both control and transfected cells, respectively (Table 1), as has been demonstrated with the normal and induced mammalian HO (24–28).

Effect of rHb and Heme on Transfected and Nontransfected Endothelial Cells. Because released hemoglobin from circulating, damaged erythrocytes results in the generation of free radicals leading to oxidative stress that can damage vascular endothelial cells (29), we examined the effect of exposure to rHb and free heme on endothelial cells transfected with the human HO gene. Both transfected and nontransfected cells were exposed to sublethal and lethal doses of rHb and heme and evaluated for cell viability after 24 hr. Control or transfected endothelial cells, not exposed to rHb, were >98% viable as measured by trypan blue exclusion. Exposure of nontransfected endothelial cells to 200 μM rHb resulted in 30% cell death within 24 hr (Fig. 3*A*). Endothelial cells transfected with the human HO gene were remarkably well preserved and resistant to hemoglobin toxicity with survival rates increased to 95% at 200 μM rHb. Exposure of endothelial cells to free heme resulted in more toxicity than was produced by rHb (Fig. 3*B*). For example, heme at concentrations of 100 μM and 200 μM caused 25% and 44% cell death, as compared to 16% and 30% after exposure to 100 μM and 200 μM rHb, respectively ($P < 0.05$). As in the case of rHb, cells transfected with the HO gene were significantly resistant to heme toxicity as demonstrated by a 35–55% increase in cell survival over control cells (Fig. 3*B*). In contrast, transfection of these cells with a plasmid containing the CYP4A1 cDNA, which resulted in high expression of CYP4A1 mRNA and protein did not confer protection against hemoglobin/heme toxicity (Fig. 3). Likewise, cells transfected with the control plasmid (sham-infected cells) did not display enhanced HO expression or a cytoprotective effect (data not shown). These results confirm that transfected endothelial cells with the HO gene have a better survival rate compared with untransfected cells after exposure to high concentrations of free heme or hemoglobin and further substantiate the specificity of HO in mediating this protective effect.

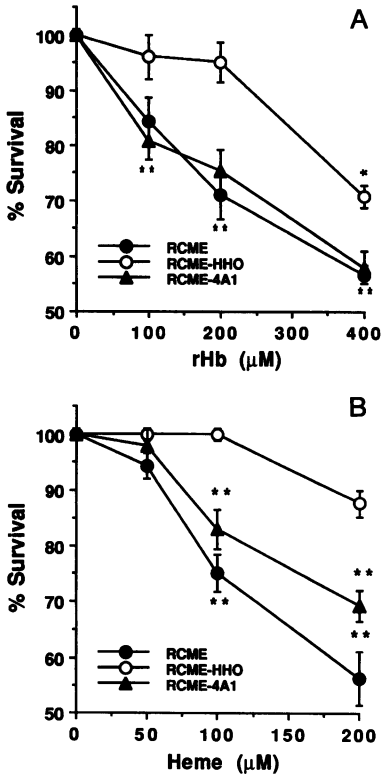

FIG. 3. Effects of heme and hemoglobin (rHb) on endothelial cell viability. Control (RCME), HO gene-transfected (RCME-HHO), and CYP4A1-transfected (RCME-4A1) cells were treated with and without rHb (1–400 μM) (*A*) and heme (1–200 μM) (*B*) for 24 hr. Cell viability was assessed by trypan blue exclusion as described. Data are from four different determinations in triplicate and are means ± SEMs. *, $P < 0.01$ (significant from untreated cells, without rHb or heme); **, $P < 0.01$ (significant from RCME-HHO cells and from untreated cells, without rHb or heme).

DISCUSSION

Induction of HO has been suggested to be an adaptive response to oxidative stress agents (4, 6, 7, 30); for example, hemoglobin released from damaged erythrocytes at tissue sites of hemorrhage or other injury, is a major pro-oxidant and a potential source of free radicals (29). Furthermore, heme moieties released from heme proteins and hemoglobin have been shown to promote the formation of oxygen radicals, generating reactive species toxic to endothelial cells (8, 29). Oxidant-mediated tissue damage may contribute significantly to the pathogenesis of diseases such as atherosclerosis and to reperfusion injury after myocardial ischemia and strokes. Thus, overexpression of HO may offer a means of cellular protection against heme/hemoglobin oxidative injury by enhancing the degradation of these pro-oxidants to bile pigments, which themselves have antioxidant properties (6, 7).

The iron release resulting from HO activity is believed to be the cause of the increased expression of ferritin synthesis, which serves to sequester the metal, thus rendering this potential cellular oxidant inactive (30, 31). Bilirubin and biliverdin both act as antioxidants *in vitro* and *in vivo* (6), and their increased local concentrations after HO induction may be beneficial in the protection of endothelial cells from injury. Recently, Neuzil and Stocker (32) have demonstrated that free and albumin-bound bilirubin efficiently inhibits lipid oxidation and that this antioxidant activity is likely due to an interaction of bilirubin with the antioxidant α-tocopherol and lipoprotein (32). Finally, CO, a by-product of heme degradation, may mimic NO and thus also serve as a modulator of endothelial cell functions following hemorrhagic/oxidative injury. CO is also a vasodilator; it may, therefore, together with other vasodilator substances, counteract the vasoconstrictive properties of hemoglobin and heme (33).

Pertinent to the role of HO in hemorrhagic injury is the study of Nath *et al.* (7); these investigators demonstrated that *in vivo* induction of HO prevents renal failure and drastically reduces mortality after glycerol-induced rhabdomyolysis in the rat, a condition that is characterized by an increased release of myoglobin and hemoglobin into the extracellular renal space, resulting in irreversible tissue injury, renal failure, and death. These investigators also showed the coupling of ferritin synthesis to enhanced HO activity *in vivo* (7). Similarly, Balla and coworkers (29) demonstrated that endothelial cells *in vitro* respond to heme/hemoglobin by induction of HO and the production of large amounts of ferritin, and they suggested that both HO and ferritin are major intracellular factors assisting cells in resisting oxidative damage. Skin fibroblasts also respond to oxidative injury such as that produced by UV light in a similar manner—i.e., rapid induction of HO and accumulation of ferritin (34). In these and other cell types, induction of

HO before exposure to oxidative assaults is thought to provide cell protection and an increase in cell viability (34, 35).

In the present study we were able to successfully transfect human HO cDNA into rabbit coronary endothelial cells using Lipofectin as a means of gene delivery. The transfected DNA directed increased expression of the human HO gene without affecting expression of the endogenous rabbit HO gene and without altering, morphologically or biochemically, the endothelial cell phenotype. Transfection of the human HO gene caused a significant increase in HO activity, indicating a successful and functional gene transfer. Overexpression of the human HO gene considerably enhanced resistance of the endothelial cells to oxidative injury produced by concomitant exposure to free heme or hemoglobin. The protective effect was dose-related and substantial, up to concentrations of 200 μM heme/hemoglobin. Cellular protection against the damaging consequences of H_2O_2 exposure by catalase gene transfer into endothelial cells has been demonstrated (36). Although catalase is important in the dissipation of H_2O_2, the enzyme does not appear to be a stress protein and, in any case, does not act physiologically to degrade heme moieties—a physiological function specifically reserved to HO. Thus, HO activity can assist other antioxidant systems in diminishing the overall production of reactive oxygen species and can thereby contribute to cellular resistance to such injury, as this study demonstrates. It can also be postulated that under conditions in which glutathione levels are reduced (37) or catalase and superoxide dismutase activities are compromised (38), induction of HO may be further beneficial.

The ability to transfect the human HO gene into endothelial cells and to demonstrate its overexpression in such cells offers a wide range of experimental possibilities for studying the role of this human enzyme in tissue protective mechanisms against oxidative injury. The direct evidence provided in this study that human HO overexpression in coronary endothelial cells provides these cells with significant protection against heme/hemoglobin-induced toxicity suggests that this enzyme could play an important role in moderating tissue damage associated with many pathologic processes in humans, as, for example, trauma, hemorrhage, thrombosis, and reperfusion injury. Pharmacological manipulation of HO to further enhance its synthesis and activity may thus have important therapeutic potential in these clinical circumstances.

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