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A Significant Role for the Heme Oxygenase-1 Gene in Endothelial Cell Cycle Progression

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Heme oxygenase (HO) catalyzes the conversion of heme to biliverdin with the release of iron and carbon monoxide. HO-1 is inducible by inflammatory conditions, which cause oxidative stress in endothelial cells. Overexpression of human HO-1 in endothelial cells may have the potential to provide protection against a variety of agents that cause oxidative stress. We investigated the physiological significance of human HO-1 overexpression, using a retroviral vector, on cell cycle progression in the presence and absence of pyrrolidine dithiocarbamate (PDTC). The addition of PDTC (25 and 50 μM) to human microvessel endothelial cells over 24 h resulted in significant ($P < 0.05$) abnormalities in DNA distribution and cell cycle progression compared to cells overexpressing the HO-1 gene. The addition of PDTC resulted in a significantly decreased G_1 phase and an increased G_2/M phase in the control cells, but not in cells transduced with the human HO-1 gene ($P < 0.05$). Further, PDTC had a potent effect on DNA distribution abnormalities in exponentially grown cells compared to subconfluent cells. Upregulation of HO activity in endothelial cells, as a result of overexpressing human HO-1, prevented PDTC-mediated abnormalities in DNA distribution. Inhibition of HO activity by tin-mesoporphyrin (SnMP) (30 μM) resulted in enhancement of PDTC-mediated abnormalities in cell cycle progression. Bilirubin or iron did not mediate DNA distribution. We conclude that an increase in endothelial cell HO-1 activity with subsequent generation of carbon monoxide, elicited by gene transfer, reversed the PDTC-mediated abnormalities in cell cycle progression and is thus a potential therapeutic means for attenuating the effects of oxidative stress-causing agents.

Key Words: **cell cycle; oxidative stress; gene transfer; heme oxygenase; carbon monoxide; retrovirus.**

Heme oxygenase (HO) isoforms catalyze the conversion of heme to carbon monoxide and bilirubin with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration (1, 2). This is the sole physiological pathway of heme degradation and, consequently, plays a critical role in the regulation of endothelial heme levels (1). Heme functions as a prosthetic group in hemoprotein enzymes, e.g., nitric oxide synthase, soluble guanylate cyclase, cytochrome P450, peroxidase, and catalase. To date, three HO isoforms have been characterized, each encoded by a different gene (1, 3–5). HO-1 and -2 are catalytically very active, whereas HO-3 activity appears to be less than that of other isozymes (5). HO-1 is expressed, under basal conditions, at low levels in endothelial cells (6–10), as well as the kidney (11, 12), liver and spleen, and can be induced in these cells and in other tissues by oxidative stress causing agents, including hyperthermia (15), oxidized lipoproteins (16), inflammatory cytokines (17) and hypoxia (18, 19), nitric oxide and heavy metals (1, 3, 13). HO-2 is constitutively expressed in blood vessels, endothelium, testis and most other tissues and its levels are relatively unaffected by factors inducing HO-1 (1, 3). HO-3 is constitutively expressed at low levels and is not active in heme metabolism (5). All heme oxygenase isoforms may be inhibited by certain synthetic heme analogs in which the central iron atom is replaced by other metals (14).

HO-1 is considered one of the most sensitive and reliable indicators of cellular oxidative stress. The enzyme is normally difficult to detect in cells other than macrophages and is found to be upregulated in disease states such as ischemia (20, 21) and Alzheimer's disease (22). Overexpression of human HO-1 in rabbit and rat endothelial cells renders the cells resistant to hemoglobin toxicity and highlights the important metabolic and cytoprotective role of the heme oxygenase gene (7, 23, 24).We have shown previously that upregulation of HO-1 in endothelial cells, via delivery of the human HO-1 gene, enhances cell proliferation and an-

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giogenesis (23). More recently, we have shown that delivery of human HO-1 into spontaneously hypertensive rats lowers blood pressure and promotes growth and that this effect maybe attributed to endogenous elevation of carbon monoxide (CO) (25). Recently, it has been reported that pyrrolidine dithiocarbamate (PDTC) induced apoptosis in various cells. It has also been reported that PDTC induced cell death in rat thymocytes (26), and induces apoptosis in vascular smooth muscle cells and endothelial cells (27, 28).

The objective of this study was to examine the feasibility of utilizing a retrovirus-mediated transfer of human HO-1, under the control of the human HO-1 promoter, to regulate endogenous HO-1 expression in order to understand the mechanism by which HO-1 expression may regulate cell cycle progression in the presence and absence of PDTC.

Our data demonstrate, for the first time, that selective delivery of the human HO-1 gene into human endothelial cells results in an attenuation of PDTC-mediated abnormalities in DNA distribution and apoptosis by increasing the rate of CO synthesis and that this effect is brought about without altering endogenous HO-2 protein. Thus, by use of retroviral-mediated human HO-1 gene expression, it is possible to envisage the prolonged attenuation of stress-mediated modulation in cell cycle progression in clinical or experimental circumstances where this might prove useful.

MATERIALS AND METHODS

Cell culture conditions. Human dermal microvessel endothelial cells (HMEC) were a kind gift from Dr. Michael Dillon (National Center for Infectious Diseases, Atlanta, GA) and grown in MCDB131 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml EGF (Sigma, St. Louis, MO) and 1 μg/ml hydrocortisone (Sigma). The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere, and maintained at subconfluency by passaging with trypsin–EDTA (GIBCO-BRL).

Development of recombinant retroviral vectors and viral propagation. The LSN-HOP-human HO-1 was constructed as previously described (29). Briefly, the retroviral vector pGEM-HOP was constructed by cloning a 1519-bp (+19 to −1500) human HO-1 transcriptional-regulated sequence (HOP) from the plasmid A-CAT at the *Xho*I and *Hin*dIII sites of the plasmid pGEM-7zf (+) (Promega, Madison, WI). The retroviral vector LSN-HOP was constructed by cloning the *Xho*I–*Eco*RI HOP sequence of the pGEM-HOP at the *Eco*RI and *Xho*I sites of the retroviral vector LXSN. The 987 bp (−63 to +924 bp) *Hin*dIII human HO-1 cDNA fragment from the pRc-CMV-human HO-1 (23) was end-blunted and inserted at the end-blunted *Bam*HI site of the LSN-HOP. After clone selection, the transcription-oriented construct was designated as LSN-HOP-human HO-1.

The amphotropic retroviral packaging cell line PT67 (Clontech Laboratories, Inc., Palo Alto, CA) was used for generation of replication-deficient recombinant retroviruses. The PT67 cells were grown in Dulbecco's modified Eagle medium (DMEM; GIBCO-BRL) supplemented with 10% heat-inactivated FBS and transfected with the retroviral vector (LSN-HOP-human HO-1) using Lipofectamine reagent (Life Technologies, Inc., Grand Island, NY). HMEC were infected by supernatants of the retroviral packaging cells (PT67/LSN-HOP-human HO-1) and HMEC-HO-1 (expressing human HO-1) were obtained after clone selection with G418.

Cell preparation and DNA distribution. The HMEC and HMEC-HO-1 were cultured at 40–50% (exponentially grown cells) and 80–90% confluence (subconfluent cells), respectively. To investigate the effect of PDTC (Sigma) on DNA fragmentation of HMEC and HMEC-HO-1, these cells were treated with 25 μM and 50 μM PDTC for 24 h and stained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Inc., Eugene, OR). They were then analyzed by an EPICS flow cytometry (Beckman Coulter, Inc., Miami, FL). Furthermore, to investigate the effect of HO-1 on DNA distribution after the addition of PDTC, HMEC, and HMEC-HO-1 were treated with PDTC in the presence and absence of tin mesoporphyrin (SnMP; 30 μM; Porphyrin Products, Inc., Logan, UT). Twenty-four hours after treatment, the cells were analyzed by flow cytometry following staining with DAPI. To evaluate the effect of biliverdin and iron on DNA distributions, HMEC and HMEC-HO-1 were treated with biliverdin (10 μM) and ferrous ammonium sulfate (10 μM) for 24 h.

Western blot analysis. Cells were harvested using cell lysis buffer, as previously described (30). The lysate was collected for Western blot analysis and protein levels were visualized by immunoblotting with antibodies against human HO-1 or HO-2 (Stressgen Biotechnologies Corp., Victoria, BC, Canada). Briefly, 30 μg of lysate supernatant was separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Inc., Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 5% milk in 10 mM Tris–HCl (pH 7.4) 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated with a 1:2000 dilution of anti-HO-1 or anti-HO-2 antibodies for 1 h at room temperature with constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) at a dilution of 1:2000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions.

Measurement of HO activity. Microsomal HO activity was assayed as previously described (31). In brief, bilirubin, which is the product of HO degradation, was extracted with chloroform and its concentration was determined spectrophotometrically using the difference in absorbance from λ_{460} to λ_{530} nm with an absorption coefficient of 40 mM^{-1} cm^{-1}.

Statistical analyses. Data are presented as means ± SE for the number of experiments. Statistical significance ($P < 0.05$) between the experimental groups was determined by means of ANOVA and Student's *t* test.

RESULTS

Differential Effect of PDTC on DNA Distribution in Cell Cycle and Functional Impact of Human HO-1 Gene

We compared DNA cell cycle distributions in control HMEC and cells overexpressing human HO-1 (HMEC-HO-1). The results demonstrated that the percentages of DNA distributions in G_1, S and G_2/M cell cycle phases in the HMEC were 25.5 ± 3.3, 49.7 ± 6.3, and 24.8 ± 4.9, respectively, whereas, the percentages in the control HMEC-HO-1 were 30.4 ± 3.8, 45.9 ± 4.5, and 23.7 ± 1.9, respectively. Consequently, the G_1 phase in the control HMEC-HO-1 was significantly increased compared with control HMEC ($P < 0.05$). To investigate the influence of PDTC on DNA distribution

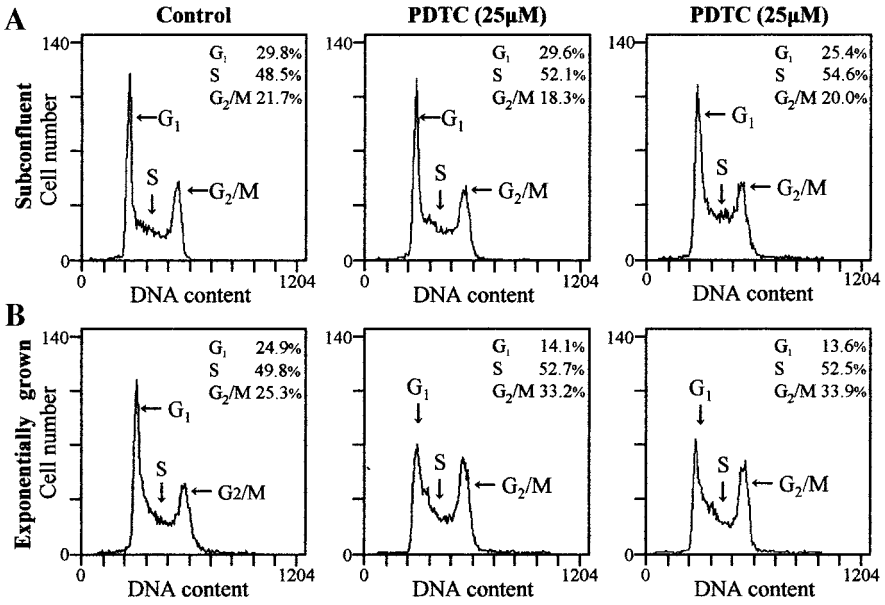

FIG. 1. DNA distributions of HMEC treated with PDTC. Exponentially grown and subconfluent HMEC were treated with 25 and 50 μM PDTC, stained with DAPI, and analyzed by flow cytometry as described under Materials and Methods. Data are representative of three independent experiments. Representative DNA distributions are shown.

in exponentially grown (40% confluent) and subconfluent HMEC (80% confluent) were treated with PDTC (25 and 50 μM) and DNA distributions were analyzed by flow cytometry. As shown in Fig. 1A, when the subconfluent cells were treated with PDTC, the percentages of DNA distributions in G_1, S, and G_2/M phases were not significantly changed from untreated cells. In contrast, when the exponentially grown cells were treated with PDTC, marked changes in the percentages of DNA distributions in G_1, S, and G_2/M phases were found. The most dramatic effects were seen in the G_1 phase, which was significantly decreased ($P < 0.05$) from 24.9% in control cells to 13.6% after by treatment with PDTC 50 μM. In contrast, the G_2/M phase was significantly ($P < 0.05$) increased from 25.3 to 33.9% after PDTC 50 μM treatments (Fig. 1B).

To investigate the influence of overexpression of HO-1 on DNA distribution, we analyzed DNA distributions in HMEC-HO-1 after treatments with PDTC (25 and 50 μM). When the subconfluent (Fig. 2A) or exponentially grown HMEC-HO-1 cells (Fig. 2B) were treated with PDTC (25 or 50 μM), the percentages of DNA distribution in G_1, S, and G_2/M phases were not significantly changed. These result showed that endothelial cells transduced with human HO-1 are resistant to PDTC, unlike nontransduced cells which were vulnerable to PDTC mediated changes in the normal levels of DNA distributions and cell cycle phases.

Effect of Suppression of HO Activity on DNA Distribution

To investigate the significance of selective upregulation of HO-1 gene transfer and HO activity, we evaluated the effect of SnMP on DNA distributions in HMEC/HMEC-HO-1 with or without PDTC. As shown in Figs. 3A and 3B, the addition of SnMP and PDTC to either HMEC or HMEC-HO-1, resulted in a significant decrease in the G_1 phase and an increase in the G_2/M phase ($P < 0.05$). Moreover, dramatic changes in DNA distribution by the treatment of SnMP alone were found in both HMEC-HO-1 and HMEC. SnMP modulated cell cycle phases in the HMEC-HO-1 and resulted in a significant decrease in the G_1 phase and an increase in the S phase ($P < 0.05$).

Effect of Heme Oxygenase Products Biliverdin and Iron on DNA Distribution

We next examined the relative contributions of bilirubin and iron on DNA distribution. Exponentially grown and subconfluent cells were treated with bilirubin and iron, as described under Materials and Methods, and DNA distribution was measured after 24 h. The basal DNA distribution in G_1, S, and G_2/M phase in endothelial cells was not changed by the addition of either bilirubin or iron to either untreated cells (Fig. 4) or cells treated with PDTC (25 μM) treated cells (data not shown).

Effect of Biliverdin, Iron, and SnMP on HO-1 and HO-2 Proteins

The effects of biliverdin, iron and SnMP on HO-1 and HO-2 protein expression in the exponentially grown HMEC were evaluated by Western blot analysis. As shown in Fig. 5A, treatments of HMEC with biliverdin and iron did not alter HO-1 protein. In contrast, the

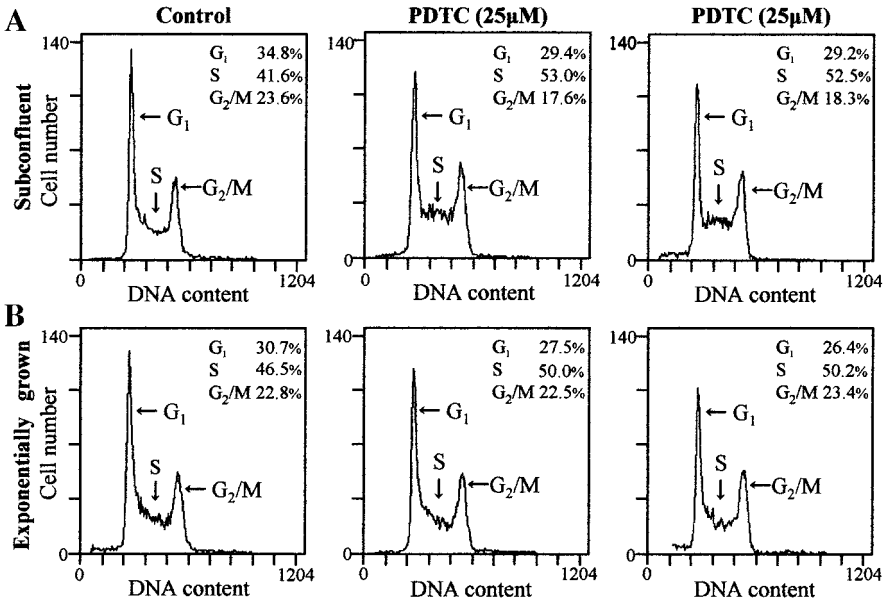

FIG. 2. DNA distributions of HMEC-HO-1 treated with PDTC. Exponentially grown and subconfluent HMEC-HO-1 were treated with 25 and 50 μM PDTC, stained with DAPI, and analyzed by flow cytometry as described under Materials and Methods. Data are representative of six independent experiments. Representative DNA distributions are shown.

level of HO-1 protein in HMEC treated with SnMP increased compared with control HMEC due to the activation of HO gene expression. Although SnMP is a potent inhibitor of HO activity and is used clinically in the control of hyperbilirubinemia in newborns (32, 33), it acts concurrently as an inducer of HO-1 protein due to its structural similarity to heme. However, the inhibitor action of SnMP exceeds its inducing action on HO. Further, biliverdin, iron and SnMP did not change the basal levels of HO-2 protein. In fact, the level of HO-2 protein treated with SnMP slightly decreased compared with the control HMEC. In another set of experiments, we evaluated the levels of HO-1 and HO-2 protein expression in HMEC and HMEC-HO-1.

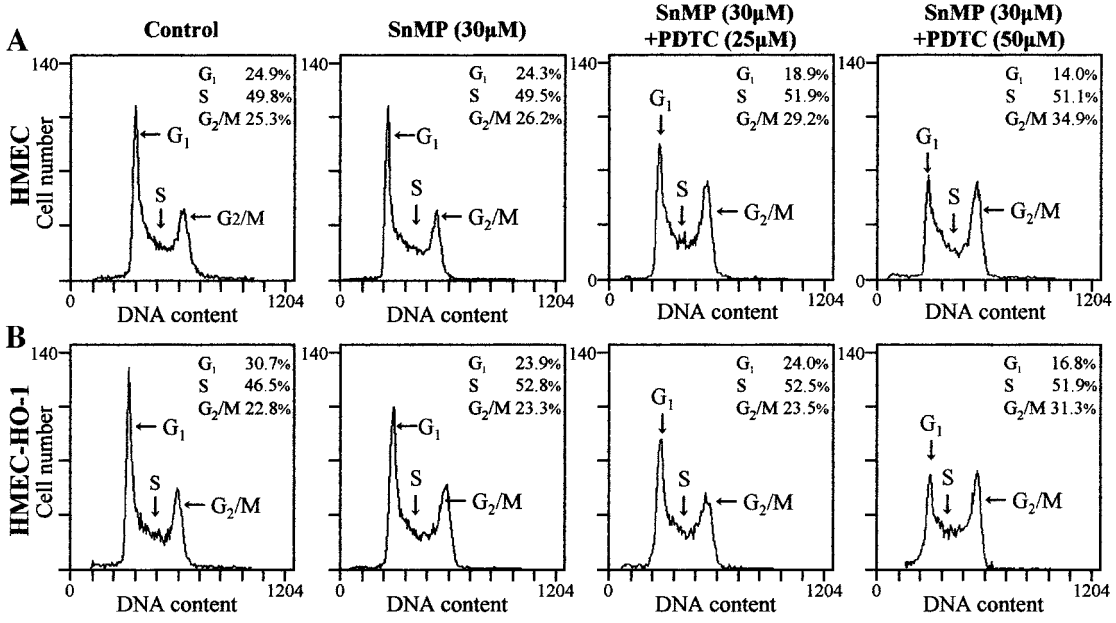

FIG. 3. DNA distributions of HMEC and HMEC-HO-1 treated with SnMP plus PDTC. Exponentially grown HMEC and HMEC-HO-1, pretreated with SnMP (30 μM) for 30 min, were treated with PDTC (25 and 50 μM), stained with DAPI, and analyzed by flow cytometry. Representative DNA distributions are shown.

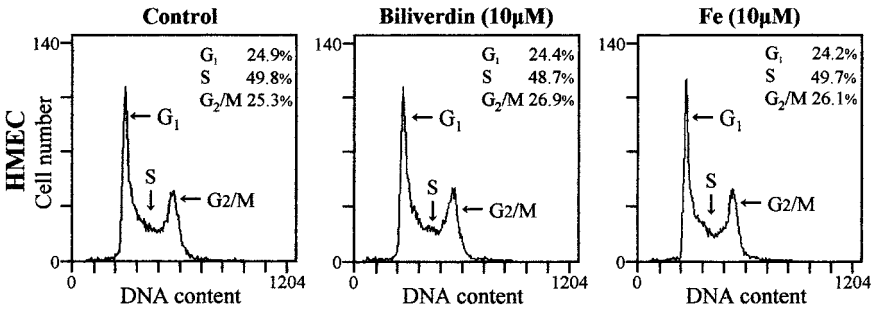

FIG. 4. DNA distributions of HMEC treated with biliverdin and iron. Exponentially grown HMEC were stained with DAPI and analyzed by flow cytometry. Representative DNA distributions are shown for HMEC-treated with biliverdin (10 μM) and iron (10 μM).

The results of the Western blot analysis, as shown in Fig. 5B, demonstrate that the levels of HO-1 protein in HMEC-HO-1 increased several-fold compared to the levels in HMEC, whereas the levels of HO-2 protein were not changed.

Suppression of HO Activity by Gene Transfer and SnMP

We investigated the effects of SnMP on HO activity in HMEC cells and cells transduced with the human HO-1 gene. As shown in Fig. 6, the level of the HO activity in control HMEC was 0.62 ± 0.1 nmol bilirubin/mg protein/h, which was decreased to 0.31 ± 0.15 after the addition of SnMP ($P < 0.05$). The level of HO activity in HMEC-HO-1 was 1.88 ± 0.17 nmol bilirubin/mg protein/hr and decreased to 0.54 ± 0.01 nmol bilirubin/mg protein/h ($P < 0.05$). As seen in Table 1, the addition of biliverdin and iron at a similar concentration (10 μM) to the substrate, did not change HO activity. For comparison, the addition of SnMP, a competitive inhibitor of HO activity (14), decreased HO activity by at least 50%.

DISCUSSION

We describe, in the present study, the physiological effect of functional expression of human HO-1 delivery to endothelial cells on DNA distribution and cell cycle via a retrovirus vector. Cells transduced with a retroviral-mediated human HO-1 gene displayed enhanced CO and bilirubin formation in cell cultures to which a heme substrate of HO activity was added. Overexpression of the human HO-1 gene in endothelial cells also provided protection against PDTC-mediated abnormalities in DNA distribution and cell cycle progression.

PDTC causes a rapid deterioration in DNA distribution in nontransduced endothelial cells. These changes in cell cycle progression following PDTC exposure were more dramatic in exponentially growing cells when DNA synthesis is essential and less dramatic when cells are in the G_1 phase. Although the mechanism by

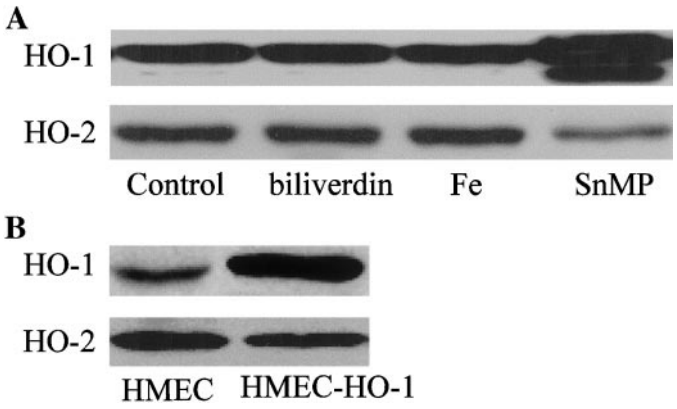

FIG. 5. Western blot. (A) HO-1 and HO-2 protein levels in HMEC treated with bilirubin (10 μM), iron (10 μM), and SnMP (30 μM) were visualized by immunoblotting with antibodies against human HO-1 or HO-2. (B) HO-1 and HO-2 protein levels in HMEC and HMEC-HO-1 were visualized by immunoblotting with antibodies against human HO-1 or HO-2. These cells were incubated with bilirubin, iron or SnMP for 24 h. Blots shown are representative of Western blot analysis from six separate experiments.

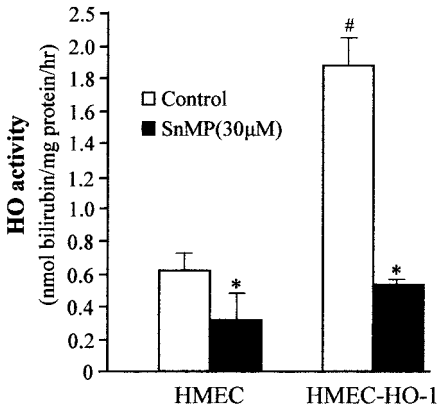

FIG. 6. Measurement of HO activity. Microsomal HO activity was assayed by bilirubin using the difference in absorbency from λ_{460} to λ_{530} nm with an absorption coefficient of 40 mM^{-1} cm^{-1}. HO activity (nmol bilirubin/mg protein) is expressed as the mean ± SE of three experiments, respectively. Statistical analyses were performed by *t* test; *$P < 0.05$, control versus SnMP-treated, and #$P < 0.05$, HMEC versus HMEC-sense.

TABLE 1

HO Activity in HMEC

Treatment[a]	HO activity (nmol/bilirubin/mg protein)
Control	0.62 ± 0.10
SnMP	0.31 ± 0.15*
Biliverdin	0.58 ± 0.07
Fe	0.75 ± 0.03

[a] HMEC-1 were treated with SnMP (30 μM), biliverdin (10 μM), and Fe (10 μM) and expressed as means ± SD of three experiments, respectively.

* Statistical analyses were performed by *t* test; $P < 0.05$, control versus SnMP-, biliverdin-, or Fe-treated.

which human HO-1 gene transfer prevents PDTC-mediated cell cycle progression abnormalities is still unclear, HO activity and its products, bilirubin and CO may play an important role. PDTC causes activation of AP-1 and upregulation of HO-1 gene expression (34). The reduction of CO formation by the inhibitor of HO activity, SnMP (32, 35), reversed the protective effect of human HO-1 gene transfer against PDTC. Cells treated with magnesium mesoporphyrin, which is not an effective inhibitor of HO activity, did not reverse PDTC-mediated DNA distribution abnormalities (data not shown). Roles for ferritin and bilirubin synthesis, which are associated with upregulation of HO activity, in the phenomenon observed are not excluded. 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 iron, thus rendering this potential cellular oxidant inactive (2). Bilirubin and biliverdin both act as antioxidants *in vitro* and *in vivo* (36) and their increased local concentrations, after HO induction, may be beneficial in protecting endothelial cells from injury. However, the addition of bilirubin did not modulate DNA distribution and cell cycle progression, but inhibition of CO by SnMP did modulate DNA distribution and cell cycle progression.

More recently, we reported that elevation of CO in endothelial cells enhances cell proliferation (29), signifying the important role of this gas in cell growth. Others have shown that when an inhibitor of heme oxygenase block heme oxygenase activity or the action of carbon monoxide is inhibited by hemoglobin, heme oxygenase activity no longer prevents endothelial cell apoptosis (37). Elevated levels of human HO-1 mRNA, protein and activity were also expressed in rapidly grown renal cancer tissue compared to normal tissue (38).

Our study defines a novel function of human HO-1 in endothelial cell proliferation and protection against PDTC-mediated DNA abnormalities and supports the notion that induction of HO-1 and formation of bilirubin, ferritin and CO are central features of this anti-oxidative mechanism. The results of studies in mice and in a child lacking functional HO-1 have provided strong support to the concept that this enzyme confers protection against oxidative stress (9, 39). Lack of HO-1 in mice exacerbates oxidative stress mediated injury in a renal ischemia model (40). Further, lack of HO-2 in mice also creates a setting that promotes oxidative stress-related disturbance (41). We have reported that H_2O_2 or heme elicits cell death and this effect can be reversed by elevation of HO-derived bilirubin levels (42). In contrast, HO inhibitors enhance cell death, an effect that can be prevented by pre-elevation of endogenous bilirubin (42). The capacity of vascular endothelial cells to generate CO via the heme-heme oxygenase system is of potential clinical importance. Collectively, our findings suggest that the heme oxygenase system and products generated through its catalytic activity and formation of bilirubin and/or CO attenuate PDTC-mediated oxidative injury and cell growth arrest via effects on DNA distribution and perhaps on other key elements in the cell cycle.

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