Heme Oxygenase-1 Attenuates Glucose-Mediated Cell Growth Arrest and Apoptosis in Human Microvessel Endothelial Cells

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Abstract—Heme oxygenase-1 (HO-1) is a stress protein that has been suggested to participate in defense mechanisms against agents that may induce oxidative injury, such as heme and inflammatory molecules. Incubation of endothelial cells in a high-glucose (33 mmol/L) medium for 7 days resulted in a decrease of HO activity by 34% and a decrease in HO-1 and HO-2 proteins compared with cells exposed to low glucose (5 mmol/L) (P<0.05) or cells exposed to mannitol (33 mmol/L). Overexpression of HO-1 was coupled with an increase in HO activity and carbon monoxide synthesis, decreased cellular heme, and acceleration in all phases of the cell cycle (P<0.001). The rate of cell cycle or cell birth rate was increased by 29% (P<0.05) in cells overexpressing HO-1 but decreased by 23% (P<0.05) in cells underexpressing HO-1 compared with control cells. Exposure to high glucose significantly decreased cell-cycle progression in control cells and in cells underexpressing HO-1 but did not decrease cell-cycle progression in cells overexpressing HO-1. High glucose induced p21 and p27 in control cells but not in cells overexpressing HO-1. The addition of tin-mesoporphyrin (SnMP), an inhibitor of HO activity, reversed the HO-1–mediated decrease of p21 and p27 in cells overexpressing HO-1. These findings identify a novel effect of HO-1 on endothelial cell growth and indicate that heme metabolism and HO-1 expression regulate signaling systems in cells exposed to high glucose, which controls cell-cycle progression. (Circ Res. 2003;93:507-514.)

Key Words: cell cycle ■ oxidative stress ■ superoxide anion production ■ gene transfer ■ heme oxygenase

Exposure of endothelial cells to elevated glucose levels causes glucose oxidation, resulting in the generation of excess reactive oxygen species (ROS) in endothelial cells. A reduction in antioxidant reserves has been attributed to endothelial cell dysfunction in diabetes, even in patients with well-controlled glucose levels. Hyperglycemia-mediated local formation of ROS is considered to be the major contributing factor to endothelial dysfunction, including abnormalities in cell cycling and delayed replication, and these abnormalities can be reversed by antioxidant agents and an increased expression of antioxidant enzymes. Du et al have demonstrated that hyperglycemia stimulates the induction of apoptosis in endothelial cells by a mechanism that involves the generation of ROS and superoxide anion formation. Moreover, high glucose conditions facilitated the susceptibility of various serum proteins to oxidation, which contributes to the inhibition of endothelial cell proliferation. Wolf et al have reported that high glucose stimulates mitogen-activated protein kinase, which was associated with an enhancement in p27 Kip1 protein and growth arrest.

We have previously shown that overexpression of the human heme oxygenase-1 (HO-1) gene in rabbit and rat endothelial cells renders the cells resistant to oxidative stress–causing agents and enhances cell growth and angiogenesis, which highlights the important metabolic and cytoprotective role of the HO-1 gene. Inhibition of HO activity has been shown to exacerbate the inflammatory response in the arterial wall in animal models of atherosclerosis model. HO-1 is expressed, under basal conditions, at low levels in endothelial cells and can be induced in these cells in response to oxidants, including heme, H2O2, and tumor necrosis factor. It is conceivable, then, that upregulation of HO activity could function to attenuate the glucose-mediated inhibition of cell-cycle progression.

The objectives of this study were to determine the effects of glucose on HO activity and the expression of HO-1 and HO-2 proteins and DNA distribution and to examine the role of heme metabolism by HO on cell-cycle progression. We also examined the effect of overexpression and underexpression of human HO-1 using retrovirus vectors to enhance or suppress HO activity in endothelial cells and assessed the effect of this gene on cell-cycle progression and on cyclin kinase inhibitors, possible target genes for glucose-mediated decrease in cell growth.
Our data demonstrate that endothelial cells exposed to high glucose showed decreased HO activity, HO-1 protein, and cell-cycle progression. Significant abnormalities in DNA distribution were also observed. Our data also demonstrate that overexpression of HO-1 is associated with a decrease in cellular heme and p21 and p27 levels in glucose-exposed cells. These results provide direct evidence that inhibition of HO activity and an increase in cellular heme are potential contributing factors to glucose-mediated DNA distribution abnormalities, apoptosis, and endothelial cell dysfunction. These abnormalities in DNA distribution can be reversed by upregulation of HO-1 gene expression.

Materials and Methods

Cell Culture Conditions

Human dermal microvessel endothelial cells were grown in MCDB131 medium (GIBCO-BRL) supplemented with 10% FBS, 10 ng/mL epithelial growth factor (Sigma), and 1 μg/mL hydrocortisone (Sigma). The cells were incubated at 37°C in a 5% CO2 humidified atmosphere and maintained at subconfluency by passage with Trypsin-EDTA (GIBCO-BRL). For the pattern of DNA distribution, growing endothelial cells (40% to 50% confluent) were stained as described.22

Construction and Propagation of Retroviral Vectors

The retroviral vector–mediated delivery of HO-1 in sense and antisense orientation was constructed as described previously.13 Endothelial cells were infected by the supernatants of the retroviral packaging cells (LSN-human HO-1 and LSN-human HO-1-antisense) to obtain the endothelial cells expressing human HO-1 and the endothelial cells underexpressing human HO-1, respectively.

Stathmokinetic Assay of the Cell-Cycle Progression Rate, Cell Proliferation, and Apoptosis Analysis

Control endothelial cells and cells transduced with HO-1 sense and HO-1 antisense were harvested, permeabilized, and stained with DAPI as previously described.24 The rate of cell-cycle progression was estimated in the stathmokinetic assay by measuring the rate of cell entry into mitosis.25 The mitotic spindle disrupting drug vincristine (Sigma Aldrich) was added to cultures at a concentration of 50 ng/mL. The cell-cycle assay was performed after 1.5, 3, and 6 hours of drug administration. The percentage of cells in G2/M was estimated for each time point. The increase in frequency of G2/M cells was then plotted as a function of time to estimate the kinetics of cell entry into mitosis. Cell proliferation was determined using a cell counting kit (Dojindo Molecular Technologies, Inc) using 5×10⁴ cells and a 96-well microtiter plate.

Western Blot Analysis and HO Activity

Cells were harvested using cell lysis buffer as previously described.26 HO activity was assayed in cell homogenates as described previously.27

![Figure 1](http://circres.ahajournals.org/content/93/3/508/F1.png)

**Figure 1.** Effect of glucose (33 mmol/L) and mannitol (33 mmol/L) on HO activity and HO-1 and HO-2 protein expression. A, HO-1 and HO-2 protein levels in endothelial cells treated with glucose (33 mmol/L) or mannitol (33 mmol/L) for 7 days. HO-1 and HO-2 protein levels were determined by Western blot. B, Cell lysate HO activity was assayed by measuring bilirubin using the difference in absorbency from 460 to 530 nm with an absorption coefficient of 40 mmol/L cm-¹. HO activity (nmol/mg per hour bilirubin) is expressed as the mean±SE of 3 experiments. Statistical analyses were performed by t test; *P<0.05. C, Cell proliferation was measured after 7 days, during which cells were harvested after 5 days and seeded in 96-well plates for an additional 48 hours in the presence of glucose. Cell proliferation was determined as described in Materials and Methods; n=4; †P<0.05, glucose vs untreated cells. D, DNA distributions of control or cells treated with glucose or mannitol. Data are representative of 3 independent experiments. Representative DNA distributions are shown.
Determination of Microsomal Heme and CO Production
Cellular heme was determined as the pyridine hemochromogen method. Carbon monoxide (CO) production was analyzed using an HP589A mass spectrometer interfaced to a HP5890 gas chromatograph.

Superoxide Anion (O$_2^-$) Production
The O$_2^-$ production was assayed by the spectrophotometric measurement of ferricytochrome c reduction. Cells were harvested 7 days after glucose treatment. Cells were washed and incubated with 0.5 mL of reaction mixture consisting of Krebs Ringer phosphate buffer containing 80 μmol/L cytochrome c and 2 mmol/L NaN$_3$. After 1 hour of incubation at 37°C, the supernatants were collected and used to assay the amount of reduced cytochrome c by the difference in absorbance at 550 to 468 nm using the extinction coefficient micrometer per liter.

Statistical Analyses
The data are presented as mean±SD for the number of experiments. Statistical significance (P<0.05) between the experimental groups was determined by Fisher methods of analysis of multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor ANOVA for multiple groups or unpaired t test for two groups.

Results
Effect of High Glucose Exposure and Mannitol on HO Activity and HO-1 and HO-2 Protein Expression
HO activity and HO-1 and HO-2 protein expression were evaluated in control endothelial cells and after 7 days of exposure to either high glucose or mannitol (33 mmol/L). Because HO activity is compromised by total HO-1 and HO-2 activity, we assessed HO-1 and HO-2 proteins levels in cells exposed to glucose and mannitol. Exposure of endothelial cells to high glucose for 7 days decreased HO-1 protein without significant effect on HO-2 compared with control cells (Figure 1A). As shown in Figure 1A, endothelial cells exposed to high glucose, but not mannitol, showed a significant decrease in HO activity (Figure 1B). Because cells exposed to mannitol did not change in HO activity, this excludes the possibility of involvement of an osmotic effect of glucose on HO activity in endothelial cells. The decrease in HO-1 protein and HO activity as a result of glucose exposure was associated with an increase in superoxide anion production. Glucose exposure for 7 days increased O$_2^-$ production from 0.75±0.12 μmol/L O$_2^-$/60 min per mg protein in control cells to 1.35±0.09 μmol/L O$_2^-$/60 min per mg protein in cells exposed to 33 mmol/L glucose. Exposure of endothelial cells to mannitol did not change O$_2^-$ levels from the control value. We additionally assessed the effect of changes in HO activity and HO-1 protein on cell proliferation and DNA distribution. As shown in Figures 1C and 1D, addition of glucose (33 mmol/L) to endothelial cells caused a significant inhibition of cell proliferation and caused abnormalities in DNA distributions, including increase in apoptotic cell number compared with control cells (P<0.05) (Figure 1D). In contrast, the addition of mannitol to endothelial cells did not change cell proliferation or cause any abnormalities in DNA distribution (Figures 1C and 1D).

Functional Expression of Human HO-1 Gene Transfer: Effect of HO Activity and Cell-Cycle Progression Using Retrovirus Vector
Initially, the effect of HO-1 overexpression and underexpression on HO activity and HO-1 protein were assessed using a retrovirus vector to deliver human HO-1 gene in the sense and antisense orientation, respectively. As shown in Figure 2A, compared with control cells, HO-1 protein was markedly increased in cells transduced with the retrovirus-mediated HO-1 sense constructs and was significantly decreased (P<0.05) in cells transduced with HO-1 antisense. Transduction of endothelial cells with a naked retrovirus vector does not change HO-1 and HO-2 protein or cell number (data not shown). As seen in Figure 2B, total HO activity in endothelial cells overexpressing HO-1 increased by 3-fold compared with control endothelial cells (P<0.001). Conversely, HO activity was decreased by 40% in cells underexpressing HO-1 via transduction of the HO-1 gene in the antisense orientation. Compared with control cells, cellular heme content significantly decreased in cells transduced with HO-1 sense (P<0.05) and significantly increased in cells transduced with HO-1 antisense (P<0.05) (Figure 2C). These results indicate that endothelial cells transduced with HO-1 sense metabolize heme at a faster rate than control cells, whereas heme level is significantly impaired in cells transduced with HO-1 antisense.

To assess the functional expression of human HO-1 gene transfer, we measured the levels of CO production in cells transduced with human HO-1 in sense and antisense orientations after exposure to heme. As shown in Figure 2D, CO
levels in the control cells amounted to 52.7 ± 8.0 whereas CO levels were 60% higher and 50% lower, respectively, in cells transduced with HO-1 sense and HO-1 antisense. These results indicate that CO production was increased by HO-1 overexpression and diminished by HO-1 underexpression.

**Effect of HO-1 Overexpression on Cell Proliferation and DNA Distribution**

Cell proliferation rates were measured at 12, 24, and 36 hours after seeding. As shown in Figure 3, cell proliferation significantly increased in cells transduced with HO-1 sense compared with control endothelial cells (24 hours, \( P < 0.01 \); 36 hours, \( P < 0.05 \)). In contrast, cell proliferation significantly decreased in cells transduced with HO-1 antisense compared with control endothelial cells (24 hours, \( P < 0.01 \); 36 hours, \( P < 0.05 \)).

To evaluate the effect of HO-1 overexpression and underexpression on cell cycle progression, DNA distribution was analyzed. As shown in the Table, flow cytometry analysis revealed that the G1 phase significantly increased in cells transduced with HO-1 antisense compared with control endothelial cells or cells transduced with HO-1 sense (\( P < 0.05 \)). Furthermore, cells in the S phase were significantly decreased in cells underexpressing HO-1 compared with control endothelial cells (\( P < 0.05 \)). Compared with control cells, the only significant (\( P < 0.05 \)) change in DNA content was seen in the G2/M phase of cells transduced with HO-1 sense increased significantly compared with control cells (\( P < 0.05 \)). This finding suggests that HO-1 overexpression protects endothelial cells from the cytotoxic effect of glucose.

**Effect of Glucose on Endothelial Cell Proliferation**

Cells were harvested and used for seeding in 96-well plates. The effect of glucose on cell proliferation was assessed using a cell-counting kit for an additional 48 hours, with a final glucose exposure of 7 days. As shown in Figure 5, 33 mmol/L glucose caused a significant decrease in cell proliferation compared with cells treated with the vehicle. We next examined the proliferative effect of glucose on cells transduced with the retroviral HO-1 construct in sense and antisense orientations. As seen in Figure 5, at 33 mmol/L glucose was ineffective in decreasing cell proliferation in cells expressing HO-1 in the sense orientation. In contrast, 33 mmol/L glucose produced an additional decrease in cell proliferation in cells expressing HO-1 in the antisense orientation compared with control cells (\( P < 0.05 \)). This finding suggests that HO-1 overexpression protects endothelial cells from the cytotoxic effect of glucose.

**Effect of High Glucose on the Levels of Cyclin-Dependent Kinase Inhibitors p21 and p27**

The protein levels of the cyclin-dependent kinase inhibitors p21 and p27 were greatly affected by the level of expression of HO-1. As seen in Figure 7, the basal and glucose-induced...
p21 levels were decreased by 45% and 160%, respectively, in endothelial cell HO-1 sense (EC-HO-1S) cells compared with control cells \( (n = 3, \ P < 0.05) \). In contrast, in endothelial cell HO-1 antisense (EC-HO-1AS), basal and glucose-induced p21 levels were increased by 4- and 3-fold, respectively \( (n = 3, \ P < 0.01) \). Likewise, basal and glucose-induced p27 levels were significantly reduced by in EC-HO-1S and increased in EC-HO-1AS cells compared with control cells \( (n = 3, \ P < 0.05) \). The decreased levels of these proteins in cells overexpressing HO-1 may underlie, in part, the mechanisms by which HO-1 overexpression and HO activity promote cell-cycle progression.30

**Discussion**

This study demonstrates that expression of the HO-1 gene participates in the regulation of cell-cycle progression in high-glucose exposed cells, presumably through cellular heme metabolism and alteration in the levels of bilirubin, an antioxidant, and CO, a vasoactive molecule. Three key findings substantiate this conclusion. First, glucose was observed to inhibit HO activity, decrease HO-1 protein, and, under these conditions, decrease cell-cycle progression (Figure 1). In our study, glucose, which is known to increase superoxide anion levels in endothelial cells,31 did not result in an increase of HO activity. This contradicts the conventional known effect of oxidants, which increase HO-1 gene expression as an adaptive response to ROS.16,21 In agreement with the present data, glucose deprivation, associated with an elevation in ROS, resulted in an increase in HO-1 protein, and this increase can be reversed by the addition of glucose.32 The glucose-mediated decrease in HO activity is not limited to endothelial cells but has also been shown to occur in the liver and kidneys of diabetic rats induced by streptozotocin.33 The mechanisms by which glucose decreased HO activity remain to be determined. The suppressive effect of glucose on the observed decreased in HO activity can result from several possible explanations. Glucose may result in deactivation of HO-1 proteins via ROS generated during glucose oxidation, similar to that seen in other enzyme proteins.1,34,35 Zou et al1 have demonstrated that high glucose via peroxynitrite causes inactivation of prostacyclin synthase in human aortic endothelial cells. High glucose may also cause activation of transcriptional factors and act as negative regulator of the HO-1 promoter and an inhibitor of HO-1 gene expression (authors’ unpublished observation, 2003). Recently, Kitamura et al36 have demonstrated that Bach 1, a heme-regulated transcriptional repressor, functions as a hypoxia-inducible repressor for the HO-1 gene. It is possible that such a factor is upregulated by glucose, thus contributing to decreased HO-1 expression. We have shown that the HO-1 promoter may be also regulated by hormonal levels and dexamethasone; the latter can act as a negative regulators by silencing HO-1 gene expression at the nuclear factor-κB/STAT3 cis-acting elements.37

The second key observation is that upregulation of HO-1 attenuated glucose-mediated abnormalities in DNA distribution and cell-cycle progression. We measured the rate of cell entry to mitosis using vinblastine, because adding vinblastine to the cultures arrests cells in mitosis. In the presence of this agent, the percentage of cells entering mitosis increases with time, and the rate of the increase reflects the kinetics of cell-cycle progression.31 As described in Figure 6, the rate of

![Figure 4](image-url). Effect of HO-1 overexpression and suppression on DNA distribution in control and vinblastine. A, Control endothelial cells (ECs) and EC-HO-1S or EC-HO-1AS were treated with vinblastine for 1.5, 3, and 6 hours, stained with DAPI, and analyzed by flow cytometry. B, Representative DNA distributions are presented for each group.

![Figure 5](image-url). Effect of high glucose on cell proliferation. Control endothelial cells (ECs) and EC-HO-1S or EC-HO-1AS were exposed to high glucose (33 mmol/L) for 5 days and then washed. Cell proliferation was measured as described in Materials and Methods. Cell number is expressed as mean±SD of 3 experiments for each group. *P<0.05, control vs transduced with HO-1 or HO-1 antisense; †P<0.05, nonexposed cells vs cells exposed to glucose.

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cells entering into mitosis can be estimated from the rate of increase in the percentage of G2/M cells. At 6 hours, after the induction of stathmokinesis, the percentage of G2/M cells increased from 24% to 45% in control cultures, which gives the rate of cells entering mitosis as 3.5% of the cells per hour (h−1). During the same time period, cells with the HO-1 gene transcript in sense orientation were entering mitosis at a faster rate (4.5% h−1). In contrast, the rate of entry into mitosis of cells with antisense HO-1 was much slower. Only 1.3% of the cells were entering this phase per hour. The increase in rate of cell entry into mitosis seen in cells overexpressing HO-1 gene may explain the advantageous effect of upregulation of HO-1 on cells exposed to high glucose. Upregulation of HO-1 will also enhance utilization of NADPH needed for HO activity, because the degradation of every heme molecule required two molecules of NADPH.33 Enhanced utilization of NADPH may alter redox or substrate availability for energy metabolism in a manner that enhances cell-cycle progression.

The present data define a novel function for human HO-1 in endothelial cell proliferation and protection against glucose-mediated DNA abnormalities by diminishing p21 and p27 levels (Figures 5 through 7). High glucose–mediated oxidative stress has been shown to contribute to an elevation of p27 and a decrease in cell-cycle progression.11 Overexpression of the HO-1 gene mediated an increase in cell proliferation and may be attributable to the decreased p21 and p27. Other studies have shown that HO-1 overexpression attenuates cell death by oxidants such as H2O2 and tumor necrosis factor,38,39 an effect attributed to CO. A product of HO activity, CO has been shown to play an important role in controlling cell-cycle progression.22,38,40

The third key finding is that inhibition of HO activity by SnMP in cells overexpressing HO-1 reversed the protective effect against glucose-mediated abnormalities in DNA distribution and cell-cycle progression. This effect seems to be independent of HO-2. HO-2 is constitutively expressed in the blood vessels and endothelium, and its levels are unaffected by glucose or factors known to act as inducers of HO-1.41,42 Other studies have shown that with a decrease in HO-1 expression or HO activity in mice or in a humans lacking functional HO-1, the levels of oxidants and oxidative stress—

Figure 6. Effect of high glucose on DNA distribution. A, Control cells, nonexposed cells, glucose-exposed control cells, and EC-HO-1S or EC-HO-1AS were stained with DAPI and analyzed by flow cytometry. Representative DNA distributions are shown (n=4 for each group). B, Cells transduced with HO-1 and treated with SnMP while exposed to high glucose were stained with DAPI and analyzed by flow cytometry. Representative DNA distributions are shown (n=4). EC indicates control endothelial cell.

Figure 7. Effect of high glucose on p21 and p27 protein levels in EC-HO-1S and EC-HO-1AS. Cells were exposed to 33 mmol/L glucose for 7 days. The levels of p21 and p27 were visualized by immunoblotting with antibodies against p21 and p27. Representative blots are shown (n=3). EC indicates control endothelial cell.
mediated cell injury were significantly increased, providing strong support for the concept that this enzyme confers protection against oxidative stress.\textsuperscript{13,14} Indeed, inhibition of HO by the addition of SnMP or in cells underexpressing HO-1 resulted in increased cellular heme and decreased generation of the antioxidant, bilirubin, and oxidative stress.\textsuperscript{13,41} Mazza et al\textsuperscript{44} have shown that a decrease in HO-1 proteins increased DNA degradation. Thus, HO-1 expression could have a major influence on the biological effect of oxidative stress. In addition to glucose-mediated increases in oxidative stress, high glucose enhances the levels of angiotensin II, and the latter also enhances ROS.\textsuperscript{45,46} Angiotensin II alone resulted in an increase in HO-1 as a protective mechanism against ROS in vitro and in vivo.\textsuperscript{47,49} Because glucose causes suppression of HO-1 expression, this may exacerbate the effect of ROS generated directly or indirectly by glucose.

In summary, this study demonstrated that enhanced heme oxygenase activity, brought about by retroviral-mediated human HO-1 gene transfer, attenuated glucose-mediated abnormalities in cycle progression. Conversely, the deficiency of HO-1 exacerbated glucose-mediated endothelial cell dysfunction. Furthermore, an increase in HO-1 activity, elicited by gene transfer, may be beneficial in enhancing bilirubin and CO production. Both of these substances play a significant role in regulating both cell-cycle progression and blood flow. The findings of studies such as this may have important clinical as well as experimental relevance.

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