Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes

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Abraham, Nader G., Rita Rezzani, Luigi Rodella, Adam Kruger, Derek Taller, Giovanni Li Volti, Alvin I. Goodman, and Attallah Kappas. Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes. Am J Physiol Heart Circ Physiol 287: H2468–H2477, 2004. First published July 29, 2004; doi:10.1152/ajpheart.01187.2003.—Heme oxygenase (HO)-1 represents a key defense mechanism against oxidative injury. Hyperglycemia produces oxidative stress and various perturbations of cell physiology. The effect of streptozotocin (STZ)-induced diabetes on aortic HO activity, heme content, the number of circulating endothelial cells, and urinary 8-epi-isoprostane PGF2α (8-Epi) levels in control rats and rats overexpressing or underexpressing HO-1 was measured. HO activity was decreased in hyperglycemic rats. Hyperglycemia increased urinary 8-Epi, and this increase was augmented in rats underexpressing HO-1 and diminished in rats overexpressing HO-1. The number of detached endothelial cells and O2 formation increased in diabetic rats and in hyperglycemic animals underexpressing HO-1 and decreased in diabetic animals overexpressing HO-1 compared with controls. These data demonstrate that HO-1 gene transfer in hyperglycemic rats brings about a reduction in O2 production and a decrease in endothelial cell sloughing. Upregulation of HO-1 decreases oxidant production and endothelial cell damage and shedding and may attenuate vascular complications in diabetes.

HEMEmOXYGENASE (HO)-1 gene expression exerts antioxidant actions, which participate in defense mechanisms against agents that induce oxidative injury, such as heme and other inflammatory molecules (3). These antioxidant actions originate in the capacity of HO-1 to degrade heme released from oxidant-distabilized heme proteins (50) and the production of bilirubin, which has potent antioxidant effects (65) as well as the generation of the vasodepressor gas carbon monoxide (CO) (34, 69, 73), which, by itself, can bind to the heme moiety of heme proteins causing either enzyme activation or inhibition (3). HO-derived CO has antiapoptotic properties and provides cellular cytoprotection against oxidants and inflammatory molecules (41, 53, 54). HO-1 also recruits iron binding proteins, which lower intracellular iron levels (25, 57).

Exposure of endothelial cells to elevated blood glucose levels leads to the generation of excess reactive oxygen species (ROS) in the cells (5). Hyperglycemia-mediated local formation of ROS is considered to be a major contributing factor to endothelial dysfunction, including abnormalities in cell cycling (9) and delayed replication (75); these abnormalities can be reversed by antioxidant agents (18) or by increased expression of antioxidant enzymes (14). Du et al. (23) demonstrated that hyperglycemia stimulates the induction of apoptosis in endothelial cells by a mechanism that involves the generation of ROS and O2. A reduction in antioxidant reserves has been related to endothelial cell dysfunction in diabetes, even in patients with well-controlled glucose levels (10, 39). In a recent study (5), we demonstrated that increased expression of HO-1 attenuates glucose-mediated cell growth arrest and reduces apoptosis in human microvessel endothelial cells. The protective effect of the HO-1 signaling mechanism, which attenuated glucose-mediated endothelial cell apoptosis, was maintained through suppression of p21 and p27 cyclin kinase inhibitors (5) or, perhaps, through its powerful antioxidant properties, and the increase in selective expression of HO-1 in renal epithelial cells indicates that there is an inextricable link between upregulation of HO-1 and GSH levels (59).

In earlier studies, we and others demonstrated that overexpression of the HO-1 gene in human, rabbit, and rat endothelial cells not only renders the cells resistant to agents that elicit oxidative stress (3, 5, 27, 41) but also enhances cell growth (58, 60) and angiogenesis (20). These effects highlight the important metabolic and cytoprotective role of the HO-1 system (6, 30, 68, 74). Inhibition of HO activity has been shown to exacerbate the inflammatory response in the arterial wall, increasing the expression of adhesion molecules and enhancing endothelial cell death in animal models of atherosclerosis (33).

The objectives of this study were to determine the effects of hyperglycemia on overall HO activity and expression of the specific HO-1 and -2 proteins in vivo and to examine the effects of a nonpharmacological approach to increasing or decreasing HO-1 on endothelial cell shedding, oxidant production, and heme levels in the experimental diabetic state. Transgenic rats, in which HO-1 overexpression (60) and HO-1 underexpression (72) were generated, were used to assess the relationship of HO-1 gene expression to these parameters.

Our results demonstrate that hyperglycemia decreases vascular HO activity and increases the number of endothelial cells released into the circulation. Human HO-1 gene transfer provided vascular protection and attenuated the hyperglycemia-mediated increase in circulating endothelial cells, O2 formation, and urinary 8-epi-isoprostane PGF2α (8-Epi) output, whereas underexpression of rat HO-1 magnified these effects, substantiating a significant role for HO-1 as part of the cellular antioxidant defense system against oxidant-mediated damage in vascular endothelium. These findings also suggest that there is an inverse relationship between the HO system and the
vascular complications observed in the experimental model of diabetes examined in these studies.

MATERIALS AND METHODS

Development of HO-1 transgenic rats. HO-1 transgenic rats were generated by retroviral gene transfer of human HO-1 sense or rat HO-1 antisense (AS) oligonucleotides to newborns. Twelve pregnant Sprague-Dawley (SD) rats (Charles River Laboratory; Wilmington, MA) were required to deliver about 120 littersmates. Male rats (about 50%) were separated and used for viral delivery. The concentrated retroviruses (3–5 × 10⁹ colony-forming units/ml) were prepared as previously described (58, 60) and injected into newborns twice intraventricularly at day 5 (20 μl) and day 12 (40 μl). After the injection, rats were allowed to recover and were returned to their cages with the appropriate mothers for continued weaning. Transgenic rats overexpressing and underexpressing the HO-1 gene were kept in a pathogen-free environment. The animals were weaned at 21 days and housed in their own cages. Experiments were conducted in 12–13wk-old male rats (350–375 g body wt), which were divided into two groups: one overexpressing HO-1 and one underexpressing HO-1. At different time points, rats were taken to measure the expression of human or rat HO-1 mRNA and protein in various tissues (28, 60). RT-PCR analysis demonstrated that human HO-1 mRNA was detected in all tissues, including the kidney, aorta, heart, femoral artery, lung, and liver (data not shown). Rats transduced with the AS construct LSN-rat HO-1 AS were treated with either heme (10 mg/kg iv for 24 h) or vehicle solution (Trisma buffer, pH 7.8), and the aortas were taken to measure HO-1 and HO activity.

Development of diabetes in SD and transgenic rats. Hyperglycemia was induced by the administration (once intraperitoneally) of streptozotocin (STZ; Sigma) dissolved in 0.05 mol/l citrate buffer (pH 4.5). SD rats received empty vector (LSXN), and age-matched transgenic rats were used. STZ-treated rats transduced with the AS construct LSN-rat HO-1 AS and control SD rats developed hyperglycemia within 3–5 days as expected, with glucose levels reaching 580 ± 68 and 475 ± 65 mg/dl, respectively. In contrast, rats transduced with the sense LSN-human HO-1 viral construct (LSN-HHO-1) did not develop hyperglycemia to the same levels as the controls; therefore, they were injected a second time after 3 days with STZ (65 mg/kg) to obtain the same degree of hyperglycemia as controls. Glucose levels were 212 ± 49 mg/day after the first injection and reached 405 ± 51 mg/day after the second injection. Insulin was administered 3 times/wk [neutral protamine Hagedorn (NPH), 2–3 U·day⁻¹·300 g body wt⁻¹] to maintain glucose levels below 300 mg/dl (35) in all diabetic rats. The basis for the resistance against the development of hyperglycemia in rats transduced with human HO-1 may be due to an alteration in STZ metabolism or possibly islet β-cell resistance to STZ-mediated destruction when HO-1 is elevated. Numerous reports have shown that upregulation of HO-1 protects islet β-cells and stimulates insulin release (29, 43, 44). Six rats were used in each group, and the experiments were repeated twice. Blood was obtained weekly from the tail vein via capillary tube and used for glucose determinations. All specimens were obtained after an overnight fast.

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Alternatively, the supernatant was then centrifuged at 105,000 × g for 1 h at 4°C. The supernatant was then centrifuged at 105,000 × g for 1 h at 4°C. The supernatant was then centrifuged at 105,000 × g for 1 h at 4°C. The supernatant was then centrifuged at 105,000 × g for 1 h at 4°C. The supernatant was then centrifuged at 105,000 × g for 1 h at 4°C. The supernatant was then centrifuged at 105,000 × g for 1 h at 4°C.

Preparation of tissues for immunohistochemistry. The fixed vessels were prepared for immunohistochemistry as previously described (2). Briefly, vessels were fixed in 4% paraformaldehyde, cut into small pieces, processed according to standard procedures, embedded in paraffin, and sectioned at 5 μm by microtome. The sections were then stained by hematoxylin-eosin and by HO-1/2 immunohistochemistry. Immunostaining was carried out using anti-HO-1/2 antibodies (Santa Cruz Biotechnology; Santa Cruz, CA). Serial sections were immersed in 3% hydrogen peroxide and diluted in methanol for 30 min to block endogenous peroxidase activity. The sections were then washed in TBS (0.1 M, pH 7.4), incubated with biotinylated goat anti-rabbit immunoglobulin and avidin-biotin-horseradish peroxidase complex, and stained by immersing the slides in a solution of 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide. All slides were counterstained with hematoxylin-eosin, dehydrated, and mounted. Negative controls for nonspecific binding, incubated with a secondary antibody only or TBS, were processed and revealed no signal. On the basis of the intensity and distribution of HO staining, the scores for HO-1 were assessed semiquantitatively by the intensity of immunohistochemical staining.

O₂⁻ production. O₂⁻ production was assayed by the spectrophotometric measurement of ferricytochrome c reduction. Tissue homogenates from diabetic and nondiabetic rats were frozen at −80°C until use. Tissue homogenates were incubated with 0.5 ml reaction mixture, consisting of Krebs-Ringer phosphate buffer containing 80 μM cytochrome c and 2 mM NaN₃. After 1 h 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 wavelengths of 550–688 nm using the extinction coefficient (in μM/l) as previously described (5).

Western blot analysis of HO-1 and -2. Homogenates were used as a source for the measurement of HO-1 and -2 proteins as previously described (5). Tissues were lysed in a buffer (lysis buffer B) consisting of 0.5% Nonidet P-40, 0.5% deoxycholate, and 10 mM EDTA in TBS (20 mM Tris and 150 mM NaCl, pH 7.4) containing 10 μg/ml each of leupeptin, antipain, and pepstatin and 1 mM PMSF. Unbroken cells or cell debris was removed by centrifugation at 300 g for 10 min. Protein from the supernatants was precipitated by adding 5 volumes of

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methanol, and the resulting pellet was then dissolved in sample buffer and separated on a 10% SDS-polyacrylamide gel. For immunoblotting, the separated proteins (20 μg total protein) were electrophoretically transferred to membranes after 2 h with 100 V. The membranes were blocked with % milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBS-T) buffer at 4°C overnight. After being washed with TBS-T, the membranes were incubated with a 1:2,000 dilution of anti-HO-1 or anti-HO-2 antibodies (Stressgen Biotechnologies; Victoria, British Columbia, Canada) for 1 h at room temperature with constant shaking. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham; Piscataway, NJ) at a dilution of 1:2,000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer’s instructions.

Detection and quantification of circulating endothelial cells. One milliliter of rat whole blood was diluted with Hanks’ balanced solution without calcium and with 1 mM EDTA and 1% FBS. Diluted blood was layered on one-half volume of Histopaque 1077 (Sigma; St. Louis, MO) and centrifuged at 250 g for 30 min. The interface layers were centrifuged at 2,000 g for 10 min. The resulting buffy coat (pellet) was resuspended and transferred to microslides. To further identify endothelial cells, we used two different methods and compared the results: one by using ulex, a lectin that specifically binds to endothelial cells (15), and the other using immunomagnetic beads. For the ulex method, 1 ml cell suspension was incubated with 10 μl ulex (1:1,000) for 2 h at room temperature. An aliquot of this suspension was placed on a coverslipped slide, and endothelial cells were visualized by fluorescence microscopy (Olympus IX81 F). For immunomagnetic isolation and quantitation, we used monodispersed magnetizable particles (Dynabeads CELLlection Pan Mouse IgG kit) obtained from Dynal (Lake Success, NY). The 4.5-μm-diameter polystyrene beads are coated with affinity-purified pan-anti-mouse immunoglobulin G1 covalently bound to the surface. The beads were washed according to the manufacturer, using a strong magnet (MPC6, Dynal) to remove sodium azide. Typically, 100 μl of bead suspension were noncovalently coated with 10 μg/ml of RECA-1 (Novus Biologicals; Littleton, CO), a pan-rat endothelial cell-specific monoclonal antibody diluted 1:10 in PBS-0.1% BSA by overnight incubation at 4°C with head-over-head agitation. After being washed three times with PBS-BSA, to remove excess antibodies, the beads were resuspended in buffer until use. RECA-uncovered particles were used as a negative control. If the beads were stored for a long period of time, 0.1% sodium azide was added to the buffer. Beads and target cells were incubated for 1.5 h at 4°C on a rotator. The amount of beads (4 × 10^5 beads/ml) was calculated to be in great excess of target cells (>2,000 beads/target cell). Separation of beads and rosetted cells from the blood samples required a minimum of a 1-min exposure to the magnet. Three washes were performed in this device to completely remove nonrosetted cells. After the third wash, rosetted cells were recovered in a 150-μl solution of acridine orange (a vital fluorescent dye at final concentration of 5 μg/ml in PBS), and observations were made in a hemacytometer under both white and fluorescent blue excitation using fluorescence microscopy. Because both methods yielded similar results, we used the ulex method for quantitation in the present report.

Statistical analysis. Data are presented as means ± SE for the number of experiments. Statistical significance (P < 0.05) between the experimental groups was determined by the Fishers method of analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by single-factor ANOVA for multiple groups or an unpaired t-test for two groups.

RESULTS

Effect of hyperglycemia on the heme-HO system. We compared the effects of oxidants that are known to cause the induction of HO-1 and compared them with those from the induction by glucose. We assessed the basal levels of HO-1 and -2 proteins in the aorta and femoral artery isolated from rats treated with heme (10 mg/kg) and the HO-1 inducer SnCl2 (50 mg/kg) (37) for 24 h. Figure 1, A and B, shows that both the aorta and femoral artery expressed HO-1 and -2 and responded in the same manner to heme and SnCl2. In both types of vascular tissues, heme and SnCl2 markedly induced the levels of HO-1 protein without affecting the basal levels of HO-2. In a second set of experiments, we compared the effect of hyperglycemia on the levels of HO-1 and -2 in the aorta. Rats were injected with STZ or vehicle and killed at 1 mo. Blood glucose levels were 119 ± 8 mg/dl and reached 475 ± 65 mg/dl in
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Vehicle- and STZ-injected rats after 3 days, respectively. To maintain the blood glucose levels between 270 and 325 mg/dl, diabetic rats were administered insulin (NPH, 2–3 U/day, 300 g body wt−1, 3 times/wk) to maintain glucose levels below 300 mg/dl (35). Figure 1C shows a representative Western blot depicting HO-1 and -2 expression in control and hyperglycemic rats. The basal levels of HO-1 protein were not increased compared with control rats, which is unexpected in view of the known effects of diabetes in increasing oxidative stress. Hyperglycemia did not affect HO-2 protein levels.

**HO-1 and -2 immunohistochemistry in aortas from control and diabetic rats.** We compared the basal levels of HO-1 and -2 proteins and the effect of heme on these levels in aortas from control and hyperglycemic rats using immunohistochemistry. We observed moderate HO-1 immunoreactivity in the intima and in the media and adventitia of the aorta from control rats (Fig. 2A). Heme administration (10 mg/kg for 24 h) resulted in strong immunoreactivity in the intima (thin arrow) and in the media (thick arrow), as depicted in Fig. 2B. In hyperglycemic rats, HO-1 immunoreactivity was reduced (nonsignificantly) in the intima but not in the media or adventitia. In contrast to control rats, HO-1 immunoreactivity in the intima and in the media was less intense in hyperglycemic rats treated with heme, as depicted in Fig. 2, C and D. HO-2 immunoreactivity was evident in the intima and in the media and adventitia and was not affected by heme or hyperglycemia (data not shown).

**Effect of hyperglycemia on HO activity and heme content in aortas.** To further define the effect of hyperglycemia on vascular HO-1 protein, we measured HO activity and the levels of cellular heme in aortas from control and hyperglycemic rats after administration of heme or cobalt protoporphyrin, a potent inducer of HO-1 expression (36). As seen in Fig. 3A, hyperglycemia decreased HO activity in both untreated and heme-treated rats compared with controls. HO activity was 0.31 ± 0.07 nmol bilirubin·mg⁻¹·30 min⁻¹ in control rats compared with 0.21 ± 0.05 nmol bilirubin·mg⁻¹·30 min⁻¹ in hyperglycemic rats. Although HO activity was not significantly decreased, it was surprisingly not increased in the face of oxidative stress generated by hyperglycemia (9, 14, 18, 39). In hyperglycemic rats treated with heme, HO activity was induced, but to a lesser degree, compared with nonhyperglycemic control rats. HO activity increased to 0.65 ± 0.14 nmol bilirubin·mg⁻¹·30 min⁻¹ in hyperglycemic rats compared with 0.94 ± 0.09 nmol bilirubin·mg⁻¹·30 min⁻¹ in control rats (n = 4, P < 0.05).

Cellular heme content was significantly increased in aortas of hyperglycemic animals compared with control aortas. The basal levels of microsomal heme were 0.55 ± 0.12 nmol heme/mg protein in control aortas and were significantly (n = 4, P < 0.05) increased to 0.77 ± 0.19 nmol heme/mg protein in hyperglycemic aortas (Fig. 3B). Although the administration of heme resulted in increased HO activity, total microsomal heme content was also increased due to the inability of HO-1 to metabolize fully the administered heme (Fig. 3B). The increase in HO-1 activity in hyperglycemic rats was consistent with the effect of hyperglycemia on the formation of O₂⁻, which increased from 1.47 ± 0.13 μmol/mg protein in control rat aortas to 2.25 ± 0.19 μmol/mg protein in hyperglycemic rat aortas (P < 0.05).

**Effect of expression of human HO-1 and rat HO-1 AS on HO-1 protein and HO activity.** The functional expression of human HO-1 gene transfer was determined by the levels of human HO-1 protein in aortas of rats receiving LSN-HHO-1 compared with LXSN control rats. Western blot analysis of aortas obtained from LSN-HHO-1 rats showed human HO-1 protein expression (Fig. 4A). In contrast, no human HO-1 protein was expressed in aortas from rats injected with the control, LXSN. These data confirmed the functional expression of the human HO-1 gene on HO-1 protein levels after intracardiac delivery of LSN-HHO-1. More importantly, neither rat HO-2 nor actin was affected by expression of the human HO-1 gene as indicated by the comparable expression of HO-2 and actin in human HO-1 gene transfer-treated rats and LXSN rats. Expression of the transgenes human HO-1 and neo⁰ genes lasted up to 4–5 mo (the duration of the experiments).
of the difficulties in delivering the retroviral preparation into the left cardiac ventricle of a newborn, the rats not expressing human HO-1 were excluded from the study.

Functional expression of the rat HO-1 AS gene on HO-1 protein and HO activity was assessed by parallel determination of rat HO-1 protein and HO activity. Because the basal levels of HO-1 are barely detectable under normal conditions, we assessed the rat HO-1 AS response to HO-1 inducers and inhibitors. Both basal and heme-induced HO activity in rats injected with LSN-rat HO-1 AS were significantly lower than basal and heme-induced HO activity in control LXSN-transduced rats (Fig. 4B). Although rats injected with LSN-rat HO-1 AS expressed lower HO activity in response to heme, the decrease in HO activity was not significant. SnMP, a known inhibitor of HO activity, caused a 70% decrease in HO activity in both control and LSN-rat HO-1 AS rats (Fig. 4B).

Effect of HO-1 overexpression and underexpression on HO activity and 8-Epi in hyperglycemic rats. We assessed the effect of hyperglycemia on HO activity in rats overexpressing (LSN-HHO-1) and underexpressing HO-1 (LSN-rat HO-1 AS). Blood glucose levels in LXSN, LSN-rat HO-1 AS, and LSN-HHO-1 rats 3 days after the injection of STZ were 475 ± 65, 580 ± 68, and 405 ± 51 mg/dl, respectively (n = 6), and maintained between 275 and 325 mg/dl for 4 wk by administration of NPH insulin. As seen in Fig. 5A, STZ-induced hyperglycemia resulted in significantly reduced HO activity in both control rats, LXSN rats, and rats transduced with LSN-rat HO-1 AS. Hyperglycemia also reduced the overall HO activity in LSN-HHO-1 rats, but basal HO activity levels were maintained at about
and O₂ production (5), we examined the effect of HO overexpression and underexpression on the generation of 8-Epi, which is considered as a reliable approach to assess oxidative stress status in vivo in normal and hyperglycemic rats. As seen in Fig. 5B, urinary excretion of 8-Epi was increased in hyperglycemic rats compared with control rats (P < 0.05). LSN-HHO-1 rats showed a significant decrease in urinary excretion of 8-Epi (n = 5, P < 0.05). In contrast, rats underexpressing HO-1 excreted higher levels of 8-Epi, which were further increased as a result of hyperglycemia. Underexpression of HO-1 exacerbated the excretion of 8-Epi, increasing its output from 120 pg/ml in control rats to 184 ± 21 pg/ml in LSN-rat HO-1 AS rats. To investigate the mechanism by which human HO-1 gene transfer into rats exerts antioxidant effects, we measured O₂⁻ in control and diabetic rat vessels (aortas) overexpressing and underexpressing HO activity. As seen in Fig. 5C, production of O₂⁻ was increased by about twofold in diabetic rats compared with control rats. We also examined whether the prophylactic vascular protection induced by human HO-1 gene transfer would decrease vascular O₂⁻. As shown in Fig. 5C, HO-1 gene transfer attenuated the hyperglycemia-mediated increase in O₂⁻. In contrast, diminished HO-1 expression potentiated the hyperglycemia-mediated increase in O₂⁻ seen in transgenic LSN-rat HO-1 AS rats. These results provide additional evidence that delivery of the HO-1 gene to the vascular system provides prophylactic vascular protection and subserves an antioxidant defense mechanism against hyperglycemia-induced oxidative stress by decreasing O₂⁻ formation.

**HO-1 and circulating endothelial cells.** We examined the effect of hyperglycemia on endothelial cell damage and sloshing into the circulation in control and hyperglycemic rats overexpressing or underexpressing HO-1. For these experiments, we developed, as noted above, a method for isolating circulating endothelial cells using immunomagnetic beads coated with RECA-1 (specific antibodies for endothelial cells) and ulex, a fluorescence lectin that specifically binds to endothelial cells. These cells yield a positive staining for anti-von Willebrand’s factor VIII antibody (15), a specific factor for endothelial cells. Figure 6 depicts endothelial cells isolated by immunomagnetic beads and stained with acridine (A and C) and endothelial cells in whole blood identified by ulex staining (B and D). Both methods gave similar findings. The results of experiments in which rats overexpressing and underexpressing HO-1 were made hyperglycemic by the injection of STZ are depicted in Fig. 6B. The basal levels of circulating endothelial cells in HO-1 transgenic and control rats before the onset of diabetes were not significantly different but were significantly elevated in transgenic LSN-rat HO-1 AS rats as a result of diminished HO activity (P < 0.05). The number of circulating endothelial cells in blood obtained from control rats was very low. A 10-fold increase in the number of circulating endothelial cells was observed in blood obtained from hyperglycemic rats. This number was further increased (~30%, P < 0.05) in hyperglycemic rats underexpressing HO-1. The mean (±SE) numbers of circulating endothelial cells in normal rats, diabetic rats receiving LXSX, diabetic rats receiving LSN-HHO-1, and diabetic rats receiving LSN-rat HO-1 AS were 5.5 ± 2.1, 48 ± 12, 16.0 ± 4.3, and 63 ± 8 endothelial cells/ml whole blood, respectively. Taken together, these results demonstrate that the vascular system may develop an adaptive response to hyperglycemia-mediated stress as a result of transduction of the HO-1 gene and decreased detachment of endothelial cells. In
contrast, the number of circulating endothelial cells in hyperglycemic rats underexpressing HO-1 was significantly higher than that in control hyperglycemic rats \((P < 0.05)\), suggesting that the extent and severity of the increase in circulating endothelial cells was not predetermined at the onset of diabetes but could be modified by the level of HO-1 gene expression.

**DISCUSSION**

To date, most approaches to reduce oxidative stress in human diabetes have met with little success, and it is now evident that alternative approaches are required \((10, 14, 18, 39, 75)\). This study provides evidence supporting the concept that the heme-HO system may play a significant role in counteracting hyperglycemia-mediated oxidative stress and endothelial cell sloughing and enhances endogenous antioxidant properties by decreasing \(O_2^\cdot\) formation. Presumably, the system acts by influencing the rate of production of the antioxidant bilirubin and by enhancing the formation of CO, a known cytoprotective end product of heme metabolism. Three of the main findings reported are consistent with this conclusion.

First, hyperglycemia was shown to suppress HO-1 activity and to increase \(O_2^\cdot\) production. Similarly, immunohistochemi-
istry data showed that, in the aorta of hyperglycemic rats, there was a reduction in HO-1 immunostaining and that this reduction was limited to the intima, whereas immunostaining was not similarly affected in the media and adventitia. The selective decrease of HO-1 expression within the intima not only indicates that endothelial HO-1 is responsive to hyperglycemia but also suggests that suppression of HO-1 activity weakens an important defense system against hyperglycemia-induced oxidative damage in vascular endothelium.

Second, the underexpression of HO-1 increased heme content and enhanced the production of 8-Epi, effects that were reversed by overexpression of HO-1. Isoprostanes, products generated from the nonenzymatic oxidation of arachidonic acid, are a sensitive marker for in vivo lipid peroxidation and have been used as an indicator of oxidative stress in vivo (47) as well as an index of oxidant levels (19, 55). Thus overexpression of the human HO-1 gene associated with an increase in bilirubin production and a decrease in cellular heme content may together contribute to the reduction of the hyperglycemia-mediated generation of isoprostanes, which we observed. Others have shown that STZ increased HO-1 in the heart (24). However, in our study, STZ did not increase HO-1 after 1 mo of treatment, suggesting a differential effect of diabetes on HO-1 expression in the heart compared with vessels.

Another oxidant-generating system that can contribute to increased oxidative stress, and may be regulated by the level of HO-1 expression, is the NADPH oxidase system. Heme is an essential component for NADPH oxidase and thus for the generation of O$_2^\cdot$ (11, 56, 63). Heme levels are critical for the synthesis and binding of gp91$^{phox}$ to the p22 subunit of NADPH oxidase necessary for its activity (11, 26, 45). Therefore, upregulation of HO-1 gene expression may decrease the binding of the gp91 subunit necessary for NADPH oxidase activity and the generation of O$_2^\cdot$. In contrast, a decrease in HO-1 may magnify the heme-mediated activation of NADPH oxidase and O$_2^\cdot$ generation in diabetes.

Third, the hyperglycemia-mediated increase in circulating endothelial cells was prevented in transduced rats expressing the human HO-1 gene. In contrast, underexpression of HO-1 and diminished HO activity, observed in rats transduced with the construct LSN-rat HO-1 AS, significantly increased hyperglycemia-mediated endothelial cell sloughing. This effect was independent of HO-2, which is constitutively expressed in blood vessels and endothelium. The increase in endothelial cell debris in diabetic rats suggests that the increase in circulating endothelial cells may be a result of increased progenitor endothelial cells in the circulation as well as an increase in endothelial cell detachment or both; in either circumstance, the presence of high levels of circulatory endothelial cells is a marker for vascular injury not seen in diabetic rats overexpressing the HO-1 gene, suggesting that HO-1 plays an important physiological function in vascular endothelium. The potential clinical relevance of these findings is underscored by observations in human HO-1 deficiency wherein end-organ injury, especially to the vascular system, has been observed (71) and by our findings in human endothelial cells, in which suppression of HO-1 resulted in increased cellular content of the oxidant heme and O$_2^\cdot$ (5).

Furthermore, a decrease in HO-1 expression has been shown to enhance endothelial cell death in response to a high glucose level, an effect that can be prevented by overexpression of HO-1 and that presumably involves a decrease in p21 and p27 cyclin kinase inhibitors, which are associated with cell growth arrest (5) and growth-promoting activity (7). It should be noted that rats overexpressing HO-1 were resistant to STZ-induced hyperglycemia but developed hyperglycemia only after a second dose of STZ. This suggests that HO-1 gene transfer may, in some way, moderate the diabetogenic action of STZ. Oxidative-stress-mediated cell injury and an increase in circulating endothelial cells described in our diabetic rat model also occur in other conditions involving vascular injury (15, 48, 67, 70) as well as in sickle cell anemia (64).

Hyperglycemia, which is known to increase the levels of oxidants such as O$_2^\cdot$ via mechanisms that include an increase in protein kinase C (32), lipoxygenase (12), or cellular heme (5), did not result in an increase in HO-1 activity in this study. The hyperglycemia-mediated increase in oxidant levels was clearly shown to be related to the levels of 8-Epi in cardiovascular complications, and 8-Epi levels have been used as a sensitive marker and indicator of oxidative stress (19, 55). One might expect that the increase in oxidant levels, which is generally viewed as an immediate response to oxidative stress in many tissues (3), would proceed with induction of HO-1. However, this response may be limited in time, and early development of hyperglycemia causes a reduction in HO-1 induction (vide infra). To this end, our data are in agreement with other reports showing that glucose deprivation was associated with an elevation in HO-1 protein and that the increase in HO-1 was reversed by the supplementation of glucose (8, 16).

HO-1 gene expression is crucial for homeostasis and counters pathological conditions in vitro, ex vivo, and in vivo (13, 66). The beneficial effect of HO-1 upregulation on the vascular system probably stems from the combined properties of the HO-1 activity-mediated catabolic products of heme. One product, CO, is a vasoactive molecule, an inhibitor of platelet aggregation and adhesion molecule production (30, 68), and has antiapoptotic properties (53). Another product of HO-1 activity, bilirubin, is a potent antioxidant, which has been shown to attenuate oxidative-mediated DNA damage (46) and glucose-mediated apoptosis (5). Upregulation of HO-1 activity by retroviral-mediated human HO-1 gene transfer results in the elevation of bilirubin levels and attenuated heme levels as well as angiotensin II-mediated increases in cyclooxygenase-2 and isoprostane production (1). Bilirubin scavenges ROS (17, 22, 51) and inhibits activation of NADPH oxidase (42), which has been shown to be involved in oxidant-induced vascular injury (32, 40, 49). In humans, bilirubin levels have been shown to be inversely related to cardiovascular disease (21, 31, 62, 62a). Therefore, failure of glucose to enhance vascular HO-1 gene expression contributes to the observed increase in endothelial cell sloughing and supports the idea that the heme-HO-1 system plays an important role in the mediation of hyperglycemic vascular complications. High glucose has been shown to inactivate heme-dependent proteins such as prostacyclin synthase and nitric oxide synthase in human aortic endothelial cells (52, 75). Such mechanisms may also apply to the observed decrease in HO-1 activity.

In summary, the present report demonstrates that the decrease in HO-1 gene expression observed with hyperglycemia in experimental diabetes is associated with an increase in shedding of endothelial cells into the circulation, presumably.
reflecting cell damage, and an elevation in 8-Epi output, an index of oxidant production in vivo. These findings were reversed by human HO-1 gene transfer. Thus the heme-HO-1 system appears to be significantly involved in vascular endothelial cell physiology.

The changes in levels of circulating endothelial cells and O2

108. formation after alterations in HO-1 gene expression may prove useful as an index of vascular injury in diabetes and raises the possibility of prophylactic approach involving regulation of the heme-HO-1 systems and HO-1 gene transfer might attenuate the vascular complications associated with the diabetic state. The findings of this study support the existence of an inverse relationship between the activity of the HO-1 system and hyperglycemic vascular complications and suggest that further exploration of this relationship have clinical pathological relevance.

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REFERENCES


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