Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes

Saadet Turkseven, Adam Kruger, Christopher J. Mingone, Pawel Kaminski, Muneo Inaba, Luigi F. Rodella, Susumu Ikehara, Michael S. Wolin, and Nader G. Abraham. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. Am J Physiol Heart Circ Physiol 289: H701–H707, 2005. First published April 8, 2005; doi:10.1152/ajpheart.00024.2005.—Increased heme oxygenase (HO)-1 activity attenuates endothelial cell apoptosis and decreases superoxide anion (O$_2^-$) formation in experimental diabetes by unknown mechanisms. We examined the effect of HO-1 protein and HO activity on extracellular SOD (EC-SOD), catalase, O$_2^-$, inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS) levels and vascular responses to ACh in control and diabetic rats. Vascular EC-SOD and plasma catalase activities were significantly reduced in diabetic compared with nondiabetic rats (P < 0.05). Upregulation of HO-1 expression by intermittent administration of cobalt protoporphyrin, an inducer of HO-1 protein and activity, resulted in a robust increase in EC-SOD but no significant change in Cu-Zn-SOD. Administration of tin mesoporphyrin, an inhibitor of HO-1 activity, decreased EC-SOD protein. Increased HO-1 activity in diabetic rats was associated with a decrease in iNOS but increases in eNOS and plasma catalase activity. On the other hand, aortic ring segments from diabetic rats exhibited a significant reduction in vascular relaxation to ACh, which was reversed with cobalt protoporphyrin treatment. These data demonstrate that an increase in HO-1 protein and activity, i.e., CO and bilirubin production, in diabetic rats brings about a robust increase in EC-SOD, catalase, and eNOS with a concomitant increase in endothelial relaxation and a decrease in O$_2^-$. These observations in experimental diabetes suggest that the vascular cytoprotective mechanism of HO-1 against oxidative stress requires an increase in EC-SOD and catalase.

antioxidant enzymes; extracellular superoxide dismutase; nitric oxide synthase; endothelial dysfunction; superoxide; oxidative stress

THE HEME–HEMЕ OXYGENASE (HO) system is a regulator of endothelial cell integrity and oxidative stress (2, 15). HO-1 and HO-2, two isoforms of HO, are viewed as having a major role in the formation of carbon monoxide (CO) and bilirubin and in heme breakdown. The fact that HO-1 is strongly induced by oxidant stress and its substrate heme, in conjunction with the robust ability of HO-1, to guard against oxidative insult (1, 2, 15) suggests a countervailing system to oxidative stress injury (1–3, 6, 15, 31). The antioxidant effects of HO-1 arise from its capacity to increase reduced glutathione levels and to degrade heme, as well as from the elaboration of biliverdin and bilirubin, which have potent antioxidant properties (45). CO, a product of HO, is not an antioxidant (52, 53), but it does have an antipapoptotic effect (33). Furthermore, CO is a vasodilator that has been shown to enhance endothelial function and plays an important role in regulating basal and constrictor-induced vascular tone (26, 32, 54).

Hyperglycemia-mediated local formation of reactive oxygen species (ROS) is considered to be a major contributing factor to endothelial dysfunction, including endothelial cell apoptosis (2), abnormalities in cell cycling (2), and delayed replication (55), which can be reversed by antioxidant agents or by increased expression of antioxidant enzymes (11). A reduction in antioxidant reserves has been related to endothelial cell dysfunction in diabetes (7, 55). In recent studies, we demonstrated that increased expression of HO-1 attenuated cytokine- and glucose-mediated cell growth arrest and apoptosis in vitro and decreased endothelial cell sloughing in diabetic rats in vivo (2, 24, 37, 39). Inhibition of HO activity has been shown to exacerbate inflammatory responses, thus increasing endothelial cell death in animal models of atherosclerosis (16).

Superoxide dismutase (SOD) is the antioxidant enzyme that catalyzes the dismutation of superoxide anion (O$_2^-$) into H$_2$O$_2$ and molecular oxygen (12). All mammalian tissues contain three forms of SOD: Cu-Zn-SOD, Mn-SOD, and extracellular SOD (EC-SOD). Each is a product of a distinct gene (8): Cu-Zn-SOD is localized in cytosol, Mn-SOD in mitochondria, and EC-SOD in extracellular space (35, 46). The vascular concentration of EC-SOD is a key determinant of nitric oxide (NO) bioactivity (35), decreasing the reaction of NO with superoxide, producing peroxynitrite, which is a potent cytotoxic oxidant (12).

The major objective of this study was to determine the mechanism by which HO-1 renders the vascular system tolerant to ROS in diabetes. We hypothesized that HO-1 preconditioning and an increase in HO-derived CO and bilirubin regulate the levels of antioxidant genes, such as EC-SOD and catalase, subsequently decreasing endothelial impairment in experimental diabetes. Our results demonstrate that intermittent administration of cobalt protoporphyrin (CoPP), a potent inducer of HO-1 protein and activity, increased EC-SOD and endothelial NO synthase (eNOS) levels and attenuated inducible NO synthase (iNOS) expression while increasing vascular relaxation.

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H702  HEME OXYGENASE INCREASES ANTIOXIDANT GENES

MATERIALS AND METHODS

Development of diabetes. Diabetes was induced in Sprague-Dawley rats by a single injection, via the tail vein, of streptozotocin (65 mg/kg, pH 4.5). Age-matched control rats were injected with an equal volume of vehicle (sodium citrate buffer). Rats were divided into four groups: control, diabetic, CoPP-treated diabetic, and tin mesoporphyrin (SnMP)-treated diabetic (4). CoPP (0.5 mg/100 g body wt sc) and SnMP (2 mg/100 g body wt sc) were given once a week starting from the day after development of diabetes. Glucose levels were 475 ± 65 mg/dl in diabetic rats. Before insulin treatment, the levels of glucose were maintained between 300 and 650 mg/dl. Rats were then treated with insulin (neutral protamine Hagedorn (NPH) 1–2 mg/dl in diabetic rats. Before insulin treatment, the levels of glucose were maintained below 300 mg/dl in all diabetic rats (20). Blood for glucose determination (LifeScan, Milpitas, CA) was obtained from the tail vein. All specimens were obtained after an overnight fast.

Preliminary experiments assessed time-dependent increases in HO-1 gene expression in diabetic rats compared with the control group. Rats were divided into groups, and CoCl2 (3 mg/100 g body wt), SnMP (2 mg/100 g body wt), or CoPP (0.5 mg/100 g body wt) was administered (39). These doses have been shown to be consistent with an increase in HO-1 protein (21, 22, 39) or a decrease in HO activity (21, 22). Animals were killed by pentobarbital sodium injection, and tissues were immediately excised, frozen in liquid nitrogen, and stored at −80°C. Plasma was separated and stored at −80°C until it was processed. All experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Tissue preparation, HO activity, and immunohistochemistry. Frozen thoracic aorta segments were pulverized under liquid nitrogen and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% Tergitol, pH 7.5). Homogenates were centrifuged at 27,000 g for 10 min at 4°C. supernatant was isolated, and protein levels were assayed (Bradford method).

Western blot analysis. Protein levels were visualized by immunoblotting with antibodies against rat HO-1, HO-2, EC-SOD, and Cu-Zn SOD (Stressgen Biotechnologies, Victoria, BC, Canada) and eNOS and iNOS (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 20 μg of lysate supernatant were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed as described elsewhere (2). Chemiluminescence was detected with the Amersham ECL kit according to the manufacturer’s instructions.

Measurement of plasma catalase activity. Plasma catalase activity was determined with a commercial enzyme assay kit (Cayman Chemical, Ann Arbor, MI) following the instructions provided by the manufacturer. Briefly, the enzyme assay for catalase is based on reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) as the chromogen (540 nm).

Measurement of vascular O2− levels. Vessels from control and diabetic animals were placed in plastic scintillation vials, containing 5 μM lucigenin for the detection of O2− in a final volume of 1 ml of air-equilibrated Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4), as previously described (14, 29). Lucigenin chemiluminescence was measured in a liquid scintillation counter (model LS6000IC, Beckman Instruments, San Diego, CA).

Determination of changes in force in rat aorta. Rat aortas were isolated and cleaned of surrounding tissue. The arteries were cut into 2-mm-long rings, which were mounted on wire hooks attached to a force displacement transducer (model FT03, Grass) for measurement of changes in isometric force (28). Tension was adjusted to 2 g, which is the optimal passive force for maximal contraction. Changes in force were recorded on a polygraph (model 7, Grass). Vessels were incubated in 10-mM baths (Metro Scientific, Farmingdale, NY), which were individually thermostated (37°C) in Krebs buffer (in mM: 118 NaCl, 4.7 KCl, 1.5 CaCl2, 2 H2O2, 25 NaHCO3, 1.1 MgSO4, 1.2 KH2PO4, and 5.6 glucose) gassed with 21% O2-5% CO2-balanced N2. Arteries were incubated for 1–2 h, during which passive tension was maintained at 2 g. The vessels were depolarized with Krebs solution containing 123 mM KCl (high K+), in place of NaCl, and then reequilibrated with Krebs solution before exposure to the experimental protocol. According to the experimental protocol, acetylcholine (ACh)-induced endothelium-dependent vasorelaxant responses were tested. For this purpose, arterial rings were precontracted with phenylephrine (10−7 M), and, during plateau contraction, ACh was added in a cumulative manner (10−8–10−5 M).

Statistical analyses. Values are means ± SE. Statistical significance (P < 0.05) between the experimental groups was determined by Fisher’s analysis of multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by a single-factor analysis of variance for multiple groups or unpaired t-test for two groups.

RESULTS

Effect of HO inducer on HO-1 and HO-2 expression in normal and diabetic rats. We compared the effect of two known inducers of HO activity on the aorta as a function of time. The time course of the effect of administration of CoCl2 (3 mg/100 g body wt sc), CoPP (0.5 mg/100 g body wt sc), and SnMP (2 mg/100 g body wt sc) is depicted in Fig. 1, A and B. CoCl2 caused an upregulation of HO-1 protein, which was maximal at 72 h and returned to baseline by 96 h. CoPP caused a more sustained induction of HO-1, which was maximal at 3–5 days and returned to baseline by 6 days. None of the inducers affected HO-2 protein. The last effect of other known inducers of HO-1, such as SnCl2 and heme, was less than that of CoPP (data not shown). Accordingly, we used CoPP as an inducer of HO-1 protein and HO activity in subsequent experiments.

We examined the effect of diabetes on HO-1 and HO-2 in rat aorta. Whereas 6 wk of diabetes did not stimulate HO-1 protein compared with control, CoPP administration caused significant increases in HO-1 protein and HO activity (Fig. 1C). SnMP also increased HO-1 protein. HO activity increased from 0.81 nmol·60 min−1·mg protein−1 in control aorta to 3.58 nmol·60 min−1·mg protein−1 after 4 days of CoPP administration (Fig. 1D). The structural similarities between SnMP and heme resulted in activation of the HO-1 promoter, as has been observed previously (2, 39), but inhibited HO activity (Fig. 1C). SnMP, which has been shown to inhibit HO activity in animals and humans (22), inhibited HO activity to 0.24 nmol·60 min−1·mg protein−1. Acting as inducer (CoPP) and inhibitor (SnMP), these two pharmacological agents allowed us to compare the effects of HO-1 protein alone and HO-1-associated activity.

Effect of HO-1 induction on vascular O2− levels. O2− production at low concentrations of lucigenin (5 μM) was measured using chemiluminescence in aortic tissues from the same group of rats, as previously described (14, 29, 41). Aorta from diabetic rats showed a significant increase in O2− (Fig. 2). However, the increase in HO activity, as a result of CoPP...
administration, was closely associated with a decrease in $\text{O}_2^-$.
In contrast, a decrease in HO activity, but not HO-1 protein, in rats treated with SnMP magnified $\text{O}_2^-$ production in diabetic rat vessels.

HO-1 immunohistochemistry in aortic endothelium from diabetic and nondiabetic rats pretreated with CoPP. We compared the basal levels of HO-1 and the effect of CoPP on these levels in endothelium from control and diabetic rats 6 wk after the development of diabetes. CoPP was given once a week for the duration of the experiments. HO-1 staining in the endothelium was barely detectable in control and diabetic rats (Figs. 3, A and B). Administration of CoPP to diabetic rats (Fig. 3C) produced strong immunoreactivity compared with control and untreated diabetic rats.

Effect of HO-1 expression on the antioxidant system. Because the vascular levels of $\text{O}_2^-$ are regulated by SOD (13), we examined whether HO-1 increases the level of antioxidant enzymes, such as SOD. Furthermore, HO-1 overexpression has been shown to decrease cell apoptosis, presumably by suppressing $\text{O}_2^-$ and by increasing reduced glutathione levels (18, 39, 40). Vascular EC-SOD was significantly reduced in diabetic rats compared with nondiabetic rats (Fig. 4A; $P < 0.05$). EC-SOD protein levels were significantly increased ($P < 0.001$) in diabetic rats treated with CoPP compared with untreated diabetic rats. Cu-Zn-SOD protein levels did not change in diabetic rats treated with CoPP (Fig. 4A). To evaluate whether HO-1 activity might be responsible for the increase in EC-SOD, the administration of SnMP, an inhibitor of HO activity, resulted in decreased EC-SOD protein. As seen in the densitometry analysis (Fig. 4B), EC-SOD was increased significantly in CoPP-treated diabetic rats overexpressing HO-1 compared with untreated diabetic rats ($P < 0.05$). In contrast, no significant changes in the levels of Cu-Zn-SOD protein were detected in control or diabetic rats.

Effect of HO-1 expression on catalase. Because catalase activity is considered important in regulating the levels of $\text{H}_2\text{O}_2$ generated from SOD, we measured the effect of the CoPP-mediated increase in HO-1 on catalase activities. Catalase activity was decreased by 45% in vessels from diabetic rats compared with controls (Fig. 5). In contrast, plasma catalase activity was significantly increased in aorta from
CoPP-treated diabetic rats compared with untreated diabetic and nondiabetic rats ($P < 0.01$ and $P < 0.05$, respectively). Plasma catalase activity was $35 \pm 6$ and $121 \pm 18$ mmol·min$^{-1}$·ml$^{-1}$ in diabetic rats and diabetic rats preconditioned with the HO-1 gene, respectively.

**Effect of HO-1 expression on vascular responses to ACh.** The above results suggest that the upregulation of HO-1 protein expression is associated with an increase in antioxidants, a decrease in superoxide, and, perhaps, improved vascular response. To assess the effect of HO-1 expression on vascular response, we measured ACh-induced relaxation in thoracic aorta. ACh-induced concentration-dependent relaxation was seen in all vessels precontracted with $10^{-7}$ M phenylephrine (Fig. 6). For example, ACh-induced vascular relaxation ($10^{-6}$ M) decreased in diabetic rats compared with controls: $101.10 \pm 6.83\%$ in control vs. $62.04 \pm 9.49$ in diabetic rats. However, this reduction was reversed by upregulation of HO-1 with CoPP: $62.04 \pm 9.49$ in diabetic rats vs. $107.70 \pm 4.58$ in CoPP-treated diabetic rats.

**Effect of HO-1 expression on eNOS and iNOS expression.** We examined whether the enhancement of relaxation in CoPP-treated diabetic rats is related to the levels of iNOS and eNOS. Western blot analysis demonstrated a significant decrease in iNOS in vascular tissue from CoPP-treated diabetic rats (Fig. 7A). Administration of SnMP, an inhibitor of HO activity, to diabetic rats augmented iNOS levels compared with untreated diabetic rats ($P < 0.05$). Although eNOS protein was not significantly decreased in diabetic rats compared with controls, CoPP administration to diabetic rats resulted in a significant increase in eNOS protein ($P < 0.05$) compared with untreated diabetic rats. In contrast, inhibition of HO activity, as a result of the administration of SnMP to diabetic rats, inhibited eNOS protein (Fig. 7B).

**DISCUSSION**

The vascular cytoprotective effect of HO-1 and increased cell survival in vitro and in vivo have been previously described (2, 39); however, little is known regarding the mechanism involved. In the present study, treatment of diabetic rats with CoPP, an inducer of HO-1, significantly increased EC-
SOD and catalase without affecting Cu-Zn-SOD. In addition, upregulation of HO-1 in diabetic rats increased eNOS but decreased iNOS. The effects of HO-1 on EC-SOD and catalase appear to be dependent on the generation of CO and bilirubin, because an increase in HO-1 protein without activity, as seen in rats treated with SnMP, did not enhance EC-SOD or catalase. Furthermore, the increase in EC-SOD was associated with a decrease in $O_2^-$. This novel finding demonstrated a direct effect of HO-1 on the enhancement of EC-SOD, catalase, and eNOS, leading to improvement in vascular relaxation in diabetic rats.

Recent studies have highlighted the potential importance of EC-SOD in the attenuation of ROS and peroxynitrite generated from iNOS- and eNOS-derived NO. EC-SOD is highly expressed in blood vessels, and its activity can constitute up to 70% of the SOD activity (35). EC-SOD efficiently scavenges $O_2^-$ in the extracellular matrix and, thus, controls the vascular redox processes toward removal of extracellular $O_2^-$. A decrease in $O_2^-$ decreases vascular damage and endothelial cell apoptosis in hyperglycemic conditions in vitro (2). In situations involving a severe loss of EC-SOD, such as diabetes (5), $O_2^-$ will scavenge NO and lower NO bioavailability in the vascular regions, leading to increased peroxynitrite production and inflammation. High levels of HO-1 in blood vessels may, therefore, be important in maintaining high levels of EC-SOD, thus diminishing extracellular $O_2^-$ and decreasing endothelial cell apoptosis. In support of these findings, the plasma of diabetic patients is known to contain a high concentration of inactive glycated EC-SOD (5), highlighting the importance of EC-SOD in the prevention of $O_2^-$ formation. Although rats have very low vascular levels of EC-SOD compared with most other mammalian species (12), upregulation of HO-1 in these animals increased EC-SOD protein expression.

Upregulation of HO-1 has been shown to decrease cellular heme (4) and increase reduced glutathione levels (40). This may shift the redox state to a reduced state and decrease $O_2^-$ formation. Recently, others showed that HO-1-derived CO causes activation of MAP kinase in endothelial cells and prevents apoptosis (43). Qadri et al. (38) showed that MAP kinase has a direct stimulatory effect on cellular SOD activity, which may explain the mechanism by which HO-1 increases EC-SOD (48). CO-mediated activation of soluble guanylate cyclase leads to increased cGMP production in vascular tissues (30). The soluble guanylate cyclase-cGMP pathway has been implicated in mediating the effects of CO on vascular relaxation and the inhibition of platelet aggregation and smooth muscle cell proliferation (10, 30, 34). cGMP-independent mechanisms of vasoregulation by CO have also been proposed. CO may dilate blood vessels by directly activating calcium-dependent potassium channels (50). Kaide et al. (19) showed

**Fig. 6.** Vessels from diabetic rats precontracted with phenylephrine (PE, $10^{-7}$ M) and exposed to acetylcholine (ACh) in a dose-dependent manner ($10^{-8}$–$10^{-5}$ M) exhibit attenuated relaxation, which was significantly improved with CoPP. Values are means ± SE; $n = 6$.

**Fig. 7.** Effect of HO-1 expression on inducible and endothelial nitric oxide synthase (iNOS and eNOS) in control and diabetic rats treated with CoPP or SnMP. Representative Western blots of iNOS (A) and eNOS (B) and $\alpha$-actin are shown, along with densitometry analysis of 5–6 different rats. Values are means ± SE; $n = 6$. $^*P < 0.05$ vs. corresponding control. $^*P < 0.05$ vs. STZ. $^p < 0.05$ vs. STZ + CoPP.
that decreased CO production was accompanied by a decrease in the number of open potassium channels in smooth muscle cells and an increase in vascular contractility; these effects were reversed with exogenous CO.

The second product of HO-1 activity is bilirubin, which has been shown to inhibit PKC and NADPH oxidase activities (17, 25, 42). Bilirubin-mediated inhibition of PKC and NADPH oxidase may be one mechanism by which HO-1 attenuates the diabetes-mediated generation of oxidants and uncoupling of eNOS. Glucose enhances endothelial O_2^- production, leading to increased vascular formation of the NO/superoxide reaction product peroxynitrite (23). Peroxynitrite oxidizes the active NOS cofactor tetrahydrobiopterin to cofactor inactive molecules, such as dihydrobiopterin (27). This uncouples the enzyme, which then preferentially increases O_2^- production over NO production (27, 51). Our findings suggest that functional, rather than dysfunctional uncoupled, eNOS expression was increased by HO-1 gene expression. Therefore, the HO-1 gene expression-mediated increase in EC-SOD may lead to protecting eNOS from uncoupling. Furthermore, numerous reports indicate that a higher serum bilirubin level, which was related to a decrease in lipid peroxidation, is associated with a decrease in the risk for coronary artery disease in humans (42, 49). The benefits of bilirubin as an antioxidant and cytoprotective agent have been recently reviewed (44).

Finally, upregulation of HO-1 may decrease cellular heme availability for the synthesis of gp91<sub>phox</sub> and related NOx oxidase (9). Increased NOx oxidase activity, a key factor in the progress of many aspects of vascular pathophysiology, is one of the major sources of O_2^- in the vascular system (29, 36). Recently, upregulation of HO-1 gene expression was shown to decrease the availability of the heme containing the gp91 subunit necessary for NADPH oxidase activity and the generation of O_2^- (47). In contrast, a decrease in HO-1 magnifies the heme-mediated activation of NADPH oxidase and O_2^- generation. The beneficial effect of an increase in HO-1 and a decrease in O_2^- in diabetes may be indirectly related to a decrease in cellular heme and a subsequent decrease in NADPH oxidase activity.

In summary, our data show that upregulation of HO-1 renders endothelial cells resistant to diabetes-induced oxidative stress by increasing other antioxidant genes, including EC-SOD and catalase. Increases in EC-SOD and catalase are associated with decreases in O_2^- and iNOS but an increase in eNOS, which subsequently ameliorates endothelial impairment in diabetes.

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HEM OXYGENASE INCREASES ANTIOXIDANT GENES


