Up-Regulation of Heme Oxygenase Provides Vascular Protection in an Animal Model of Diabetes through Its Antioxidant and Antiapoptotic Effects


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ABSTRACT

Heme oxygenase (HO) plays a critical role in the regulation of cellular oxidative stress. The effects of the reactive oxygen species scavenger ebselen and the HO inducers cobalt protoporphyrin and stannous chloride (SnCl₂) on HO protein levels and activity, indices of oxidative stress, and the progression of diabetes were examined in the Zucker rat model of type 2 diabetes. The onset of diabetes coincided with an increase in HO-1 protein levels and a paradoxical decrease in HO activity, which was restored by administration of ebselen. Up-regulation of HO-1 expressed in the early development of diabetes produced a decrease in oxidative/nitrosative stress as manifested by decreased levels of 3-nitrotyrosine, superoxide, and cellular heme content. This was accompanied by a decrease in endothelial cell sloughing and reduced blood pressure. Increased HO activity was also associated with a significant increase in the antiapoptotic signaling molecules Bcl-xl and phosphorylation of p38-mitogen-activated protein kinase but no significant increases in Bcl-2 or BAD proteins. In conclusion, 3-nitrotyrosine, cellular heme, and superoxide, promoters of vascular damage, are reduced by HO-1 induction, thereby preserving vascular integrity and protecting cardiac function involving an increase in antiapoptotic proteins.

Diabetes mellitus is a ubiquitous clinical problem promoting major increases in cardiovascular complications and mortality. Hyperglycemia-induced formation of reactive oxygen species (ROS), including superoxide (O₂⁻), H₂O₂, and peroxynitrite (ONOO⁻), is involved in the pathogenesis of vascular complications (Kruger et al., 2005). Diabetes and other conditions associated with elevated oxidative stress have been shown to be accompanied by an increase in endothelial cell sloughing and fragmentation as well as in the rate of cellular apoptosis (Zou et al., 2002; Abraham et al., 2004). Type 2 diabetic patients demonstrate impaired arterial function (Okon et al., 2005), which may be due to circulating endothelial cells (CECs) (Kruger et al., 2005) and the reduced bioavailability of NO (Endemann and Schiffrin, 2004). Increased CECs are seen in other cardiovascular diseases as well, due in part to decreased antioxidant gene expression (Kruger et al., 2005).

The heme oxygenase (HO) system (HO-1/HO-2) is the rate-limiting enzymatic step that catalyzes the breakdown of heme into equimolar amounts of biliverdin, an antioxidant rapidly converted to bilirubin, and carbon monoxide (CO), an antiapoptotic vasodilator, with the release of its iron moiety (Abraham and Kappas, 2005). This has led to detailed examination of the mechanism and regulation of this enzyme (Platt and Nath, 1998). Heme, the prosthetic moiety of multiple proteins, has prooxidant and proapoptotic properties that can be diminished by HO-1 induction, thereby preserving vascular integrity and protecting cardiac function involving an increase in antiapoptotic proteins.
by up-regulation of HO-1 (Gonzalez-Michaca et al., 2004). Up-regulation of HO-1 decreases oxidative stress, attenuates endothelial cell sloughing and fragmentation, and restores endothelial cell function in experimental diabetes (Abraham et al., 2004; Kruger et al., 2005). HO-1 attenuates the increased O$_2^•$ in diabetes, improves NO bioavailability, and prevents ONOO$^-$ formation via an increase in extracellular superoxide dismutase (Kruger et al., 2005; Turkseven et al., 2005). The protective actions of HO-1 extend widely to such disease processes as the inflammation associated with atherosclerosis, ischemia/reperfusion injury, and renal disease (Ishikawa et al., 1997; Laniado-Schwartzman et al., 1997; Hayashi et al., 1999; Haugen et al., 2000; Li et al., 2004; Kruger et al., 2005).

Peroxynitrite is a potent cytotoxic oxidant, which has been shown to decrease the activity of several important enzymes (Zou et al., 2002). In endothelial nitric-oxide synthase (eNOS), peroxynitrite has been shown to oxidize the cofactor tetrahydrobiopterin into inactive molecules, such as dihydrobiopterin. This results in a preferential increase in O$_2^•$ production over NO production (Milstien and Katusic, 1999). Hyperglycemia-mediated oxidative stress has been associated with elevated levels of iNOS and reduced levels of eNOS in both rats and humans (Okon et al., 2005; Turkseven et al., 2005). The increased generation of ROS causes endothelial injury, resulting in an accelerated rate of apoptosis and endothelial cell sloughing (Abraham et al., 2003a; Kruger et al., 2005).

The antiapoptotic effect of HO has become well established, and important clues are emerging as to the mechanisms involved. Increased HO activity decreases cell death as a result of increased heme turnover (Gonzalez-Michaca et al., 2004). In addition, HO generates CO, which has antiapoptotic effects (Zhang et al., 2003; Reiter and Demple, 2005). It has been demonstrated that the antiapoptotic mechanism of CO involves increases in antiapoptotic proteins, including Bcl-2 (Zhang et al., 2003) and p38-MAPK (Reiter and Demple, 2005). Up-regulation of the HO gene has also been shown to increase Bcl-xl, an important part of the antiapoptotic pathway (Ke et al., 2002). In addition, gene profiling analysis has shown that up-regulation of HO-1 prevents growth arrest by increasing positive cell cycle regulators and decreasing caspase 2, caspase 8, and p27 (Abraham et al., 2003b; Sacerdoti et al., 2005).

The present study was designed to examine basal HO protein levels and activity and vascular dysfunction as measured by the levels of iNOS, eNOS, and 3-NT in the Zucker rat model (Etgen and Oldham, 2000) of type 2 diabetes (T2DM). Our approach used two known HO-1 inducers, cobalt protoporphyrin IX (CoPP) (Turkseven et al., 2005) and stannous chloride (SnCl$_2$) (Sacerdoti et al., 1989), to examine the influence of HO induction on vascular function (through quantification of endothelial cell sloughing). We also used ebselen, a nonspecific ONOO$^-$ scavenger (Fang et al., 2005), to investigate potential HO inactivation in the setting of diabetes-induced oxidative stress. Our results demonstrate that up-regulated HO activity increased eNOS expression, attenuated oxidative and nitrosative stress function, improved hypertension, and protected vascular endothelial cells from apoptosis through an increase in the levels of Bcl-xl and phosphorylation of p38-MAPK, antiapoptotic signaling molecules.

Materials and Methods

**Animal Protocols.** Male Zucker rats (Charles River Laboratories, Inc., Wilmington, MA) were maintained on standard rat diet and tap water ad libitum. In the first protocol, we used 8-week-old (prediabetic) and 22-week-old (diabetic) Zucker diabetic fat rats (ZDF) and age-matched Zucker lean rats (ZL) controls (n = 6 animals/group). Additional ZDF and ZL (n = 6/group) rats were also treated with ebselen (5 mg/kg b.wt., twice daily via gavage) from age 12 weeks until sacrifice at age 22 weeks. In the second protocol, we examined the effects of HO-1 preconditioning, using twice weekly injections of CoPP (0.5 mg/100 g b.wt. s.c.) or SnCl$_2$ (5 mg/100 g b.wt. s.c.), from the onset of diabetes (age 12 weeks), on the expression of HO-1, endothelial cell sloughing, and the vascular phenotype (n = 6/group). Control rats were injected with an equal volume of vehicle (sodium citrate buffer). Animals were sacrificed at age 22 weeks, 2 days after the last injection.

Body weight and blood glucose levels measured in ZL and ZDF at 8 weeks of age did not differ significantly. At age 22 weeks, ZDF demonstrated elevated levels of glucose (127.3 ± 5.2 versus 100.7 ± 4.4 mg/dl) and increased body weight (764.3 ± 18.7 versus 429.7 ± 14.9 g) compared with ZL (P < 0.01 and P < 0.0001, respectively). Treatment with ebselen did not have a significant affect on either blood glucose or body weight (data not shown). CoPP therapy did result in a significant reduction in both blood glucose (107.6 ± 4.8 mg/dl; P < 0.02) and decreased body weight (596.4 ± 18.2 g; P < 0.05) compared with untreated ZDF. SnCl$_2$ administration did not result in significant changes in blood glucose or body weight (data not shown), and SnCl$_2$ has been used for long-term study as an inducer of HO-1. The Animal Care and Use Committee of New York Medical College approved all experiments.

**Detection and Quantification of Circulating Endothelial Cells.** For immunomagnetic isolation and quantification of endothelial cells, we used monodispersed magnetizable particles (Dynabeads CELLection Pan Mouse IgG kit) obtained from Invitrogen (Carlsbad, CA) according to our previously published technique (Kruger et al., 2005). Peripheral blood was obtained using typical venipuncture techniques. Quantification of endothelial cell sloughing, using rat endothelial cell antibody (RECA-1)-coated immunomagnetic beads, has been used previously and has shown the specificity of this antibody for vascular endothelial cells. Beads were coated (10 µg/ml) with RECA-1 antibody (Novus Biologicals, Littleton, CO) and then incubated with target cells for 1.5 h at 4°C on a rotator. Rosette cells were recovered in a 150-µl solution of acridine orange (a vital fluorescent dye at final concentration of 5 µg/ml in PBS) to confirm endothelial origin. Quantification was undertaken using a hemacytometer under both brightfield and fluorescence microscopy (IX81 F; Olympus, Melville, NY).

**Tissue Preparation for Western Blot.** HO Activity, Heme Content, and O$_2^•$ Levels. At the time of animal sacrifice, thoracotomy was performed, and the thoracic aorta was removed, drained of blood, and flash frozen in liquid nitrogen. Specimens were maintained at ~80°C until needed. Frozen aorta segments or renal tissues were pulverized and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Tergitol, pH 7.5). Homogenates were centrifuged at 27,000g for 10 min at 4°C. The supernatant was isolated, and protein levels were assayed (Bradford method). The supernatant was used for measurement of HO-1, HO-2, extracellular-SOD, and Cu$^{2+}$/Zn$^{2+}$-SOD (Stressgen Biotechnologies Corp., Victoria, BC, Canada), 3-NT (Upstate Cell Signaling Solutions, Chicago, IL), and eNOS and iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cellular mediators of anti- and proapoptosis signaling proteins phosphorylated p-p38-MAPK (p-p38-MAPK) (p-p38-MAPK), Bad, Bcl-2, and Bcl-xl were from Cell Signaling Technology Inc. (Beverly, MA). Proteins were visualized by immunoblotting with antibodies against each specific rat protein. Actin was used to ensure adequate sample loading for all Western blots. Antibodies were prepared in...
the following dilutions: HO-1 and HO-2 (1:1000), 3-NT (1:1000) (Upstate Cell Signaling Solutions), iNOS/eNOS (1:5000) (Santa Cruz Biototechnology, Inc.), and cellular apoptotic mediators (1:1000) (Cell Signaling Technology, Inc.). In brief, 20 µg of lysate supernatant was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) with a semidy transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 10% milk in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 30 min at 4°C. After these were washed with Tris-buffered saline/Tween 20, the membranes were incubated with anti-HO-1, anti-HO-2, anti-3-NT, anti-iNOS, or anti-eNOS antibodies for 1 h at room temperature with constant shaking. The filters were washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham Biosciences). Chemiluminescence detection was performed with the Amersham Biosciences enhanced chemiluminescence detection kit, according to the manufacturer’s instructions.

Aortic HO activity was assayed as described previously (Abraham et al., 2003a) using a technique in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (dual UV/VIS beam reader) at 450 nm with an absorption coefficient of 40 M⁻¹ cm⁻¹. Under these conditions, HO activity was linear with protein concentration, time-dependent, and substrate-dependent (Abraham et al., 2003b). For in vitro studies using peroxynitrite and H₂O₂, kidneys were prepared using the technique described above for preparation of the blood vessels. Supernatant was incubated with either peroxynitrite or H₂O₂ (250 µM) for 1 min at 37°C. The reaction was stopped using 10 µl of PBS, pH 7.4, and HO activity was assayed as described above. Determination of heme content was done using the pyridine hemochromogen method as published previously (Abraham et al., 2004). The absorbance difference between 5557 and 5530 nm was used to calculate heme content with an extinction coefficient of 20.7 M⁻¹ cm⁻¹.

NADPH Oxidase Assay. Superoxide production rates were determined as described previously (Bonnevier et al., 2004) with minor modifications. In brief, fresh aortic tissue from 22-week-old ZL and ZDF homogenates (3000-rpm supernatant) used at a concentration of 100 µg ml⁻¹ and incubated in glass tubes containing PBS, 1 mM CaCl₂, 1 mM MgCl₂, and 10 µl of SOD (final concentration 250 U/ml) was added to one tube, and the other tube had 10 µl of water and was incubated for 2 min before the addition of 50 µl of 30 mg/ml cytochrome c. The mixture was then incubated at 37°C in a shaking water bath for 15 min, and the reaction was stopped by placing the tubes on ice. Reduced cytochrome c was measured in a dual-beam PerkinElmer dual UV/VIS beam spectrophotometer lambda 25 and scanned between 570 and 530 nm (maximum difference is at 550), using SOD-inhabitable samples as the reference. The amount of O₂⁻ produced was calculated by the molar extinction coefficient 21,000 M⁻¹ cm⁻¹.

Blood Pressure Measurement. Rats were placed in a 40°C warming unit for 10 min to dilate the tail artery. Immediately after removal from the warming unit, animals were restrained, and a tail cuff was positioned on the proximal tail. The distal tail was immobilized in a foam-lined block with a photoelectric sensor unit. Systolic blood pressure (SBP) was obtained by tail-cuff plethysmography using a Natsume KN-210 manometer and tachometer (Peninsula Laboratories, San Carlos, CA). The process was repeated over several days to acclimate the animals to the procedure and apparatus before data were recorded. Once animals were acclimated, three readings were taken daily over consecutive days, and the results were averaged.

Statistical Analyses. Data are presented as mean ± S.E. for the number of experiments. Statistical significance (P < 0.05) between experimental groups was determined by the Fisher method of 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 Onset of Diabetes on the Heme Oxygenase System. The ZDF represents a well-characterized model of the metabolic syndrome, developed more than three decades ago (Zucker, 1965). The evolution of the metabolic syndrome in the ZDF is associated with the development and progression of hyperglycemia as of 8 weeks, nephropathy, insulin resistance, and hyperlipidemia by 22 weeks (Tanaka et al., 1999; Etgen and Oldham, 2000). In protocol 1, we compared the levels of HO-1 and HO-2 proteins and HO activity as a function of the development of diabetes. At 8 weeks of age, ZDF demonstrated an HO-1/actin ratio (0.75 ± 0.01) that was comparable with ZL (1.01 ± 0.06). As seen in Fig. 1A, at 22 weeks of age ZDF exhibited a markedly elevated HO-1/actin ratio (3.99 ± 0.69) compared with ZL (0.99 ± 0.10) of the same age (P < 0.001). Between 8 and 22 weeks of age, there was a significant increase in HO-1 levels in ZDF (P < 0.05), whereas levels in ZL remained unchanged. This suggests that the onset of diabetes, and not aging, was responsible for the increase in HO-1 protein levels. Levels of HO-2 in ZDF and ZL were not significantly changed during this time period (Fig. 1A).

Since HO-catalyzed breakdown of heme results in the formation of equimolar amounts of bilirubin and CO (Abraham et al., 2004), we measured HO activity by the formation of bilirubin in vascular tissue (Fig. 1B). Despite the large increase in HO-1 protein content, HO activity was decreased in 22-week-old ZDF (0.13 ± 0.01 nmol bilirubin formed/mg protein) compared with ZL (0.16 ± 0.01 nmol bilirubin formed/mg protein; P < 0.05). HO activity in 22-week-old ZL was essentially unchanged compared with age 8 weeks (0.17 ± 0.01 nmol bilirubin formed/mg protein). HO activity in 8-week-old ZDF was already lower, although not significantly, than that of ZL (0.15 ± 0.05 nmol bilirubin formed/mg protein).

To examine the effects of changes in HO activity on vascular function, the levels of iNOS and eNOS were assayed via Western blot, and the mean optical density is expressed as a ratio to actin. Twenty-two-week-old ZDF had roughly double (1.12 ± 0.02 versus 0.52 ± 0.05) the level of iNOS/actin compared with ZL (P < 0.05; Fig. 1C). In contrast, the level of eNOS in ZDF was dramatically reduced compared with ZL (0.48 ± 0.15 versus 5.86 ± 2.88; P < 0.05; Fig. 1D). In fact, at 8 weeks of age ZDF already demonstrated a significant decrease in the level of eNOS compared with ZL (1.13 ± 0.22 versus 3.41 ± 1.01; P < 0.05). Both the increased iNOS and decreased eNOS levels in 22-week-old ZDF were significantly different from the levels at age 8 weeks (P < 0.01 and P < 0.05, respectively), whereas no significant changes occurred in ZL. Increased phosphorylation of eNOS at threonine 485 in ZDF, compared with ZL, indicates decreased activity in association with the decreased expression (data not shown), although eNOS activity was not measured directly.

Differential Effect of Peroxynitrite and H₂O₂ on HO Activity. The disparity between HO protein and activity led to the hypothesis that HO was being inactivated in a setting of increased formation of ROS due to diabetes. We performed
an in vitro comparison of HO activity in aortic vascular tissue in response to 250 μM peroxynitrite or 250 μM H2O2 as potential candidates for HO inactivation. We performed dose-response studies of HO activity for both ONOO− and H2O2, and the 250 μM dose gave the maximum effect for each (data not shown). As seen in Fig. 2A, the basal levels of microsomal HO activity were diminished from 0.27 ± 0.009 nmol bilirubin formed/mg protein in control to 0.135 ± 0.008 nmol bilirubin formed/mg protein as a result of incubation with peroxynitrite (P < 0.001). However, following microsomal incubation with H2O2, HO activity increased to 0.415 ± 0.020 nmol bilirubin formed/mg protein (P < 0.01). To further test our hypothesis, kidney homogenates were HO-1 immunoprecipitated following exposure to or absence of ONOO−. Although the total level of HO-1 was unchanged, the anti-nitrotyrosine antibody revealed an increase in nitration of immunoprecipitated HO-1 following the addition of ONOO− to the microsomal fraction (Fig. 2B).

Based on the results of these in vitro studies, we extended our first protocol to assess HO activity in the aorta following treatment with the ROS scavenger ebselen. Ebselen therapy decreased the amount of HO-1 protein in 22-week-old ZDF 10-fold (P < 0.02), whereas the level in ZL was unchanged (Fig. 3A). Despite the diminished level of HO-1 protein in ZDF, there was a restoration of HO activity to normal levels seen in ZL but modest increases compared with ZDF untreated from 0.13 ± 0.01 (ZDF untreated) to 0.18 ± 0.01 nmol bilirubin formed/mg protein (ZDF + ebselen) (P < 0.005; Fig. 3B). This restoration of HO activity to normal levels in ZDF by ebselen did not change cellular heme content.
To further investigate the involvement of ROS inactivation of HO in the aorta, we measured the levels of 3-NT, which is formed by peroxynitrite nitration of tyrosine, via Western blot analysis (Fig. 3C). Densitometry analysis was performed, and the ratio of 3-NT to actin is expressed as mean ± S.E.M. In 8-week-old ZL and ZDF, there were equivalent amounts of 3-NT. At 22 weeks, the 3-NT level did not change in ZL, but it increased significantly in ZDF from 0.13 ± 0.03 to 0.35 ± 0.04 (P < 0.05). Following treatment with ebselen, the level of 3-NT in ZDF was reduced to 0.11 ± 0.001 (P < 0.05).

Effects of HO-1 Gene Expression on Vascular eNOS, iNOS, and 3-NT. Since ROS scavenging by ebselen is non-specific, we conducted further experiments to assess the effects of HO induction on oxidative and nitrosative stress. Measurement of HO activity in the aorta was repeated following treatment with either CoPP or SnCl₂ for 2 weeks. As seen in Fig. 4A, increased HO activity was seen in ZDF following treatment with both CoPP (0.33 ± 0.05 nmol bilirubin formed/mg protein; P < 0.0005) and SnCl₂ (0.45 ± 0.05 nmol bilirubin formed/mg protein; P < 0.0005) compared with control (0.13 ± 0.01). HO induction also increased activity in ZL from 0.16 ± 0.01 to 0.69 ± 0.05 nmol bilirubin formed/mg protein (P < 0.00005) and to 0.75 ± 0.06 nmol bilirubin formed/mg protein (P < 0.00005) using CoPP and SnCl₂, respectively. The increased HO activity in ZDF treated with either CoPP (P < 0.0005) or SnCl₂ (P < 0.0005) achieved a level significantly above that seen in ZL controls.

The effects of increased HO activity on oxidative/nitrosative stress were assessed through an examination of 3-NT and eNOS levels following treatment with CoPP and SnCl₂. Induction of HO resulted in a robust decrease in 3-NT/actin ratio using either CoPP (0.27 ± 0.10; P < 0.05) or SnCl₂.
in ZL and ZDF. Metalloporphyrins, such as CoPP, may (Fig. 5B) in aortic tissue from 22-week-old in ZDF to 1.96 levels were 4.26 nmol/mg in ZL (0.191 ± 0.021 nmol/mg; P < 0.01 versus untreated ZL). Treatment with ebenselen did not significantly affect heme levels (data not shown) in ZL (0.267 ± 0.029 nmol/mg) or in ZDF (0.396 ± 0.009 nmol/mg). O2 levels were 4.26 ± 0.16 μmol/mg in ZDF compared with 3.05 ± 0.09 μmol/mg in ZL (P < 0.005). HO induction significantly reduced levels of O2 in ZDF to 1.96 ± 0.27 μmol/mg (P < 0.05), whereas an observed decrease in ZL (2.676 ± 0.31 μmol/mg) was not statistically significant. ZDF treated with SnCl2 demonstrated O2 levels equivalent to 8-week old control ZL, whereas heme levels were reduced to levels significantly below 8-week-old ZL, ZDF and ZDF not shown.

Effect of HO-1 on Vascular Cellular Heme and O2 Levels in Diabetes. To demonstrate the antioxidant effects of HO induction, we measured the levels of cellular heme and O2 in ZL and ZDF. Metalloporphyrins, such as CoPP, may have antioxidant effects beyond their ability to induce HO-1 (Tauskela et al., 2005), and since CoPP and SnCl2 yielded similar changes in HO activity and 3-NT levels, further experiments were repeated using only SnCl2 to more selectively demonstrate the effects of HO activity. Levels of both heme (Fig. 5A) and O2 (Fig. 5B) in aortic tissue from 22-week-old ZDF were significantly elevated compared with ZL and were reduced by the induction of HO. Cellular heme levels in ZDF were 0.386 ± 0.006 nmol/mg compared with 0.287 ± 0.007 nmol/mg in ZL (P < 0.0005). Treatment with SnCl2 reduced cellular heme in ZDF 0.196 ± 0.004 nmol/mg (P < 0.00001 versus untreated ZDF) and in ZL (0.191 ± 0.021 nmol/mg; P < 0.005 versus ZL control).

Effect of HO Induction on the Vasculature. As an assessment of the potential clinical benefits of HO-1 induction, we measured SBP and EC sloughing following treatment with SnCl2. Systolic blood pressure was significantly lower in control ZL (148.4 ± 4.5 mm Hg) than in ZDF (171.2 ± 4.4 mm Hg), demonstrating the link between T2DM and hypertension (P < 0.001; Fig. 6). Following HO induction with SnCl2, SBP was decreased to 136.1 ± 3.3 mm Hg in ZL (P < 0.05) and to 134.0 ± 4.2 mm Hg in ZDF (P < 0.000001). In fact, SnCl2 was able to lower SBP in ZDF to a level significantly below that in the ZL control (P < 0.05).

To further assess vascular damage in response to HO induction, we isolated endothelial cells sloughed from the vascular walls, a well established method for studying EC dysfunction (Quan et al., 2004; Kruger et al., 2005). Endothelial cells typically have a round to oval shape and are 20 to 50 μm (Fig. 7A). The number of CECs was assessed using 22-week-old ZL and ZDF (Fig. 7B). The number of sloughed cells in untreated ZDF (21.1 ± 4.5 cells/ml) was significantly increased compared with ZL (5.5 ± 1.7 cells/ml; P < 0.005), whereas induction with SnCl2 reduced EC sloughing in ZDF to 8.33 ± 1.7 cells/ml (P < 0.05).

Effect of HO on Cellular Mediators of Apoptosis. To investigate the possibility that differential activation of anti- or proapoptotic signaling molecules could account for decreased EC sloughing in ZDF, we assessed the levels of Bcl-xl, Bcl-2, p38-MAPK, and Bad. As seen in Fig. 8, ZDF demonstrated a significant reduction in the amounts of the antiapoptotic proteins Bcl-xl and Bcl-2 and decreased phosphorylation of p38-MAPK, a signaling mediator in the antiapoptotic pathway. In contrast, the level of the proapoptotic protein Bad was not significantly increased in ZDF compared with ZL. These changes demonstrate a proapoptotic environment associated with diabetes, suggesting the rats of pro-

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** A, cellular heme content in aortic tissue from 22-week-old ZL and ZDF (mean ± S.E.; n = 4). *P < 0.0005 versus ZL control; **P < 0.01 versus ZL control; ***P < 0.0001 versus ZDF control. B, superoxide levels in aortic tissue from 22-week-old ZL and ZDF (mean ± S.E.; n = 4). *P < 0.005 versus ZL control; **P < 0.05 versus ZDF control.

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Systolic blood pressure measurements from 22-week-old ZL and ZDF. *P < 0.001 versus ZL control; **P < 0.05 versus ZL control; and ***P < 0.000001 versus ZDF control and P < 0.05 versus ZL control.
apoptotic signaling may be a contributing to the increase in endothelial cells sloughing and death. Up-regulation of HO activity by SnCl$_2$ resulted in a significant increase in the antiapoptotic proteins and an increase in p38-MAPK phosphorylation to levels greater than those seen in ZL. In contrast, the proapoptotic Bad seemed unaffected by HO induction (Fig. 8). Densitometry analysis (Fig. 8, B–D) demonstrated the following reductions in protein levels relative to control ($n=3$): Bcl-xl, $45.9 \pm 8.3\%$ ($P<0.05$); Bcl-2, $52.1 \pm 5.1\%$ ($P<0.05$); and p-p38-MAPK, $34.3 \pm 5.5\%$ ($P<0.005$).

The response of these proteins following HO induction by SnCl$_2$ was also examined. Administration of HO-1 inducer (SnCl$_2$), as of 8 weeks old, caused significant increases in Bcl-xl, $186.4 \pm 6.0\%$ ($P<0.005$; Fig. 8B) but no significant increases in Bcl-2, $73.5 \pm 19\%$ (Fig. 8C). In contrast to Bcl-2, p-p38-MAPK increased by $142.2 \pm 11.6\%$ ($P<0.005$; Fig. 8D). Induction of HO-1 in ZDF did not change Bad proteins levels.

**Discussion**

This study demonstrates, for the first time, that up-regulation of HO-1 gene expression during the development of diabetes decreased oxidant levels, prevented endothelial cell sloughing and fragmentation, increased eNOS, and normalized blood pressure in type 2 diabetes. Administration of the ONOO$^-$ scavenger ebselen decreased oxidants and modestly increased HO activity, although the increase was not sufficient to decrease cellular heme levels. Increased heme degradation and generation of CO and bilirubin are important regulators of vascular function (Ryter and Otterbein, 2004). Bilirubin is an important antioxidant in humans, and its increase prevents cardiovascular disease as seen in Gilbert’s disease (Vitek et al., 2002). Therefore, up-regulation of HO-1 has a dual effect by increasing the levels of CO and also bilirubin. Our findings that ONOO$^-$ generates 3-NT within an HO-1 immunoprecipitate and decreases HO activity offers a potential mechanism for our hypothesis of HO inactivation by ONOO$^-$.

Further experiments that demonstrate HO nitration in vivo would provide additional support for this hypothesis. The increased HO activity with ebselen may be related to its removal of ROS other than ONOO$^-$ (Fang et al., 2005). Since ebselen is not a specific scavenger for ONOO$^-$, a direct cause and effect linking HO nitration and inactivation has not been proven; other mechanisms may contribute to HO inactivation in addition to or in place of nitration. Since protein kinase phosphatidylinositol 3-kinase/Akt phosphorylates HO-1, and increased HO activity results, a decrease in activated Akt in diabetes may be considered as an additional mechanism for the observed decrease in HO activity (Di Noia et al., 2006). In type I diabetes, a decreases in activated Akt is seen with inhibition of HO activity using stannous mesoporphyrin, and activated Akt was restored by up-regulation of HO-1 using pharmacological inducers (Di Noia et al., 2006). Our findings strongly support, however, that measurement of HO activity, and not simply HO-1 protein level, is a far more accurate assessment for studying the beneficial effects of HO induction in pathological conditions.

Decreased HO activity is associated with type II diabetes, as seen in both the Zucker diabetic rat model as well as in human disease (da-Silva et al., 1997; Bruce et al., 2003). Decreased HO activity results in increased heme, cyclooxygenase-2, iNOS, and other oxidants (Kruger et al., 2005; Turkseven et al., 2005). Normalization of blood glucose levels in human diabetes, using a hyperinsulinemic clamp, has been shown to restore HO-1 levels (Bruce et al., 2003). Absence of HO activity has been shown to exacerbate myocardial ischemia in diabetic mice (Liu et al., 2005), whereas the complete deletion of HO-1 in humans is lethal (Abraham and Kappas, 2005). Induction of HO activity, using CoPP or SnCl$_2$, prevented hyperglycemia-mediated oxidative stress indicated as decreased levels of cellular heme and superoxide. Reduction of O$_2^-$ through induction of HO has been shown to provide vascular protection in previous studies (Abraham et al., 2003a, 2004; Turkseven et al., 2005). Heme is normally protein-bound and present in only moderate quantities, but it can be toxic at elevated levels through increased oxidative stress and inflammation (Nath et al., 1992). Excess heme has been shown to be a major instigator of renal dysfunction (Agarwal et al., 1995), which can be prevented by selective HO-1 gene expression (Nath et al., 1992; Agarwal et al., 1995; Kruger et al., 2005). Decreased HO-1 expression accelerates endothelial cell apoptosis through an increase in cellular heme levels both in vitro and in vivo (Abraham et al., 2003a, 2004). Likewise, the increased heme associated with decreased HO activity has been implicated in contributing to the development of hypertension (Sacerdoti et al., 1989). Our experiments further suggested that up-regulation of HO-1 is efficacious in preventing superoxide formation, which can preserve renal function and attenuate hypertension.

Our finding of increased CECs in T2DM may suggest that loss of ECs may be an early warning for EC dysfunction and may lead to the development of atherosclerosis or other vascular diseases. The ability of HO inducers, as pharmacological agents, to prevent sloughing and apoptosis of ECs in the diabetic ZDF may have clinical potential in the prevention of cardiovascular disease. Diabetes has previously been shown to cause endothelial cell dysfunction in both rats and humans (Abraham et al., 2004; Kruger et al., 2005; McClung et al.,...
In this study, increased HO activity attenuated EC injury, preventing sloughing. We have previously shown that the elevated levels of EC sloughing in diabetes correlated with a loss of vascular function (Kruger et al., 2005). We believe that the ability of HO to reverse hyperglycemia-mediated increase in the rate of apoptosis by decrease in cellular heme can be attributed to effects of its products CO and/or bilirubin. Inhibition of HO activity by stannous mesoporphyrin increased endothelial cell apoptosis and sloughing (data not shown), confirming that inhibition of HO activity enhances cell fragmentation (Kruger et al., 2005). We demonstrated that enhanced HO activity, probably via the generation of CO, increased the levels of the antiapoptotic Bcl-xl and Bcl-2, the phosphorylation of p38-MAPK (p-p38-MAPK), and the proapoptotic mediator Bad in the aorta from ZDF treated with SnCl₂. Densitometry analysis is shown normalized to actin for each protein studied. Graphs are optical density relative to control expressed as mean ± S.E. (n = 3). B, Bcl-xl decreased with ZDF but was restored with SnCl₂, *P < 0.05 versus control; †, P < 0.001 versus ZDF untreated. C, Bcl-2 decreased in ZDF, but SnCl₂ only partially restored its level. *, P < 0.05 versus control. D, phosphorylation of p38-MAPK (p-p38-MAPK) decreased in ZDF and was restored with treatment with SnCl₂, *, P < 0.005 versus control; †, P < 0.0001 versus ZDF untreated.

In summary, SnCl₂, a potent inducer of HO-1, decreases endothelial cell sloughing and fragmentation and normalizes blood pressure to control levels, presumably by a mechanism involving increased eNOS, Bcl-xl, and p38-MAPK in an anti-

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Fig. 8. Aorta lysate from 22-week-old untreated (ZL and ZDF) rats and SnCl₂-pretreated (from age 12 to 22 weeks) ZDF was examined. A, representative Western blots are shown for the antiapoptotic mediators Bcl-xl and Bcl-2, the phosphorylation of p38-MAPK (p-p38-MAPK), and the proapoptotic mediator Bad in the aorta from ZDF treated with SnCl₂. Densitometry analysis is shown normalized to actin for each protein studied. Graphs are optical density relative to control expressed as mean ± S.E. (n = 3). B, Bcl-xl decreased with ZDF but was restored with SnCl₂, *P < 0.05 versus control; †, P < 0.001 versus ZDF untreated. C, Bcl-2 decreased in ZDF, but SnCl₂ only partially restored its level. *, P < 0.05 versus control. D, phosphorylation of p38-MAPK (p-p38-MAPK) decreased in ZDF and was restored with treatment with SnCl₂, *, P < 0.005 versus control; †, P < 0.0001 versus ZDF untreated.


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**References**


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