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Title Page

Upregulation of heme oxygenase provides vascular protection in an animal model of diabetes through its anti-oxidant and anti-apoptotic effects.

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Running Title Page

HO-1 prevents vascular complications of diabetes.

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Abbreviations

CEC: circulating endothelial cells

CO: carbon monoxide

CoPP: cobalt protoporphyrin IX

D-4F: apolipoprotein mimetic peptide

EC endothelial cells

eNOS: endothelial nitric oxide synthetase

HO: heme oxygenase

iNOS: inducible nitric oxide synthetase

3-NT: 3-nitrotyrosine

NO: nitric oxide

NOS: nitric oxide synthetase

O_2^-: superoxide

$ONOO^-$: peroxynitrite

RECA-1: rat endothelial cell antibody

ROS: reactive oxygen species

SBP: systolic blood pressure

$SnCl_2$: stannous chloride

T2DM: type 2 diabetes mellitus

ZDF: Zucker diabetic fat rat

ZL: Zucker lean rat

Abstract

Heme oxygenase plays a critical role in the regulation of cellular oxidative stress. The effects of the reactive oxygen species scavenger, Ebselen, and the HO inducers, CoPP and $SnCl_2$, 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. Upregulation 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 anti-apoptotic signaling molecules, Bcl-xl and phosphorylation of p38-MAPK but no significant increases in Bcl-2 or BAD proteins. In conclusion, 3-NT, cellular heme and superoxide, promoters of vascular damage, are reduced by HO-1 induction preserving vascular integrity and protecting cardiac function involving an increase in anti-apoptotic proteins.

Introduction

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 (O_2^-), H_2O_2 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 (Abraham, et al., 2004;Zou, et al., 2002). Type 2 diabetic patients demonstrate impaired arterial function (Okon, et al., 2005), which may be due to circulating endothelial cells (CEC) (Kruger, et al., 2005) and the reduced bioavailability of NO (Endemann and Schiffrin, 2004). Increased CEC are seen in other cardiovascular diseases as well, and is 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 anti-apoptotic vasodilator, with the release of its iron moiety (Abraham, et al., 2004). Oxidant stress strongly induces heme oxygenase-1 (the inducible form of HO), which guards against oxidative insult, (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 pro-oxidant and pro-apoptotic properties that can be diminished by upregulation of HO-1 (Gonzalez-Michaca, et al., 2004). Upregulation 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 nitric oxide (NO) bioavailability, and prevents peroxynitrite ($ONOO^-$) formation via an increase in extra-cellular superoxide dismutase (Turkseven, et al., 2005;Kruger, 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 (Hayashi, et al., 1999;Laniado-Schwartzman, et al., 1997;Ishikawa, et al., 1997;Kruger, et al., 2005;Haugen, et al., 2000;Li, et al., 2004).

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 synthetase (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 (Turkseven, et al., 2005;Okon, 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 anti-apoptotic 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 anti-apoptotic effects (Zhang, et al., 2003;Reiter

and Demple, 2005). It has been demonstrated that the anti-apoptotic mechanism of CO involves increases in anti-apoptotic proteins including Bcl-2 (Zhang, et al., 2003) and p38-MAPK (Reiter and Demple, 2005). Upregulation of the HO gene has also been shown to increase Bcl-xl, an important part of the anti-apoptotic pathway (Ke, et al., 2002). Additionally, gene profiling analysis has shown that upregulation of HO-1 prevents growth arrest by increasing positive cell cycle regulators and decreasing caspase 2, caspase 8 and p27 (Sacerdoti, et al., 2005;Abraham, et al., 2003b).

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 employed two known HO-1 inducers, cobalt protoporphyrin (CoPP) Turkseven et. al, 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 utilized Ebselen, a non-specific $ONOO^-$ 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 MARK, anti-apoptotic signaling molecules.

Methods

Animal protocols

Male Zucker rats (Charles River Laboratory, Wilmington, MA) were maintained on standard rat diet and tap water ad libitum. In the first protocol, we utilized 8-week-old (pre-diabetic) and 22-week-old (diabetic) ZDF rats, and age-matched lean (ZL) controls (6 animals per group). Additional ZDF and ZL (n=6 per group) rats were also treated with Ebselen (5 mg/kg body 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 pre-conditioning, using twice weekly injections of cobalt protoporphyrin (CoPP) (0.5 mg/100 g bw s.c.) or $SnCl_2$ (5 mg/100 g bw 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 per group). Control rats were injected with an equal volume of vehicle (sodium citrate buffer). Animals were sacrificed at age 22 weeks, two 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 vs. 100.7±4.4 mg/dl) and increased body weight (764.3±18.7 vs. 429.7±14.9 grams) compared with ZL ($P<0.01$ and $P<0.00001$ 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 grams, $P<0.05$) compared with untreated ZDF. Stannous chloride ($SnCl_2$) 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, California) 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 hours at 4°C on a rotator. 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) to confirm endothelial origin. Quantification was undertaken using a hemacytometer under both bright-field and fluorescence microscopy (Olympus IX81 F).

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, 1mM EDTA, 0.1 mM PMSF and 0.1% tergitol, pH 7.5). Homogenates were centrifuged at 27,000-x g for 10 minutes at 4ºC. The supernatant was isolated, and

protein levels were assayed (Bradford method). The supernatant was used for measurement of HO-1, HO-2, EC-SOD and Cu/Zn-SOD (Stressgen Biotechnologies Corp., Victoria, BC), 3-NT (Upstate cell signaling solutions, Chicago, IL) and eNOS and iNOS (Santa Cruz Biotechnology, Santa Cruz, CA). Cellular mediators of anti and pro-apoptosis signaling proteins P-P38-MAPK, Bad, Bcl-2 , Bcl-xl (Cell Signaling, Danvers, MA). Protein levels 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, Chicago, IL), iNOS/eNOS 1:5000 (Santa Cruz Biotechnology, Inc., Santa Cruz, California) and 1:1000 for the cellular apoptotic mediators (Cell Signaling Technologies, Inc., Beverly, MA). Briefly, 20 µg of lysate supernatant was separated by 12% SDS/PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala Sweden) with a semi-dry transfer apparatus (Bio-Rad, Hercules, California). The membranes were incubated with 10% milk in 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.05% Tween 20 (TBST) buffer at 4°C overnight. After they were washed with TBST, the membranes were incubated with anti-HO-1, anti-HO-2, anti-3-NT, anti-iNOS or anti-eNOS antibodies for 1 hour 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). Chemiluminescence detection was performed with the Amersham ECL detection kit, according to the manufacturer's instructions.

Aortic HO activity was assayed as previously described (Abraham, et al., 2003a) using a technique in which bilirubin, the end product of heme degradation, was

extracted with chloroform and its concentration determined spectrophotometrically (Perkin-Elmer, Norwalk, CT, Dual UV/VIS Beam Spectrophotometer Lambda 25) using the difference in absorbance at a wavelength from λ460 to λ530 nm with an absorption coefficient of 40 mM^{-1} and 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 H_2O_2, kidneys were prepared using the same technique described above for preparation of the blood vessels. Supernatant was incubated with either peroxynitrite or H_2O_2 (250 μM) for 1 minute at 37°C. The reaction was stopped using 10 ml PBS (pH 7.4) and HO activity was assayed as above. Determination of heme content was done using the pyridine hemochromogen method as previously published (Abraham, et al., 2004). The absorbance difference between λ 557 and λ 530 nm was used to calculate heme content with an extinction coefficient of 20.7mM^{-1} and cm^{-1}.

NADPH oxidase assay

Superoxide production rates were determined as previously as described (Bonnevier, et al., 2004) with minor modifications. Briefly, fresh aortic tissue from 22 week old ZL and ZDF homogenates (3000 rpm supernatant) were used at a concentration of 100 ug/ml and incubated in glass tubes containing PBS, $CaCL_2$ (1.mM), $MgCl_2$ (1.mM) and 10 ul SOD, final concentrations 250 U/ml, were added to one tube and the other tube had 10 μl water and was incubated for 2 minutes before the addition of 50ul cytochrome C (30 mg/ml). The mixture was then incubated at 37^{O} C in a shaking water bath for 15 min and the reaction was stopped by placing the tubes on ice. The reduced cytochrome C was measured in a dual-beam PerkinElmer Lambda 25 UV/Vis

spectrometer and scanned between 570 and 530 (maximum difference is at 550), using SOD-inhabitable samples as the reference). The amount of O^- 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 minutes 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, Belmont, 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±SE 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 ANOVA for multiple groups or unpaired *t* test for two groups.

Results

Effect of onset of diabetes on the heme oxygenase system:

The Zucker diabetic fat (ZDF) rat 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 rat is associated with the development and progression of hyperglycemia as of 8 weeks, nephropathy, insulin resistance, and hyperlipidemia by 22 weeks old (Etgen and Oldham, 2000;Tanaka, et al., 1999). 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 to ZL (1.01±0.06). As seen in Figure 1A, at 22 weeks of age ZDF exhibited a markedly elevated HO-1/actin ratio (3.99±0.69) compared to 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 ($P<0.05$) while 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 (Figure 1A).

Since HO-catabolized 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 (Figure 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 to 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 vs. 0.52±0.05) the level of iNOS/actin compared to ZL ($P<0.05$, Figure 1C). In contrast, the level of eNOS in ZDF was dramatically reduced compared to ZL (0.48±0.15 vs. 5.86±2.88, $P<0.05$, Figure 1D). In fact, at 8 weeks of age ZDF already demonstrated a significant decrease in the level of eNOS compared to ZL (1.13±0.22 vs. 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) while 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 H_2O_2 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 peroxynitrite (250 μM) or H_2O_2 (250 μM) as potential candidates for HO inactivation. We performed dose response studies of HO activity for both $ONOO^-$ and H_2O_2 and the 250 μM dose gave the maximum effect for each (data not shown). As seen in Figure 2A, the basal levels of microsomal HO activity were diminished from 0.27±0.009 nmol

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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 H_2O_2, 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 $ONOO^-$. While 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 (Figure 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 ten-fold ($P<0.02$), while the level in ZL was unchanged (Figure 3A). Despite the diminished level of HO-1 protein in ZDF, there was a restoration of HO activity to normal levels seen in ZL rats but modestly increases compared to ZDF untreated from 0.13±0.01 (ZDF untreated) to 0.18±0.01 nmol bilirubin formed/mg protein (ZDF+ Ebselen) ($P<0.005$, Figure 3B). This restoration of HO activity to normal levels in ZDF by ebslen 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 (Figure 3C). Densitometry analysis was performed and the ratio of 3-NT to actin is expressed as mean±SEM. 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

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 $SnCl_2$ for 2 weeks. As seen in Figure 4A, increased HO activity was seen in ZDF following treatment with both CoPP (0.33±0.05 nmol bilirubin formed/mg protein, P<0.005) and $SnCl_2$ (0.45±0.05 nmol bilirubin formed/mg protein, P<0.0005) compared to control (0.13±0.01). HO induction also increased activity in ZL from 0.16±0.01 nmol bilirubin formed/mg protein 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 $SnCl_2$, respectively. The increased HO activity in ZDF treated with either CoPP (P<0.005) or $SnCl_2$ (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 $SnCl_2$. Induction of HO resulted in a robust decrease in 3-NT/actin ratio using either CoPP (0.27±0.10, P<0.05) or $SnCl_2$ (0.35±0.01, P<0.001) compared to untreated ZDF (0.65±0.01, Figure 4B). The increase in HO activity and decrease in 3-NT levels in response to CoPP or $SnCl_2$ were equivalent in ZDF.

Effect of HO-1 on vascular cellular heme and O_2^- levels in diabetes:

In order to demonstrate the anti-oxidant effects of HO induction, we measured the levels of cellular heme and O_2^- 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 $SnCl_2$ yielded similar changes in HO activity and 3-NT levels, further experiments were repeated using only $SnCl_2$ to more selectively demonstrate the effects of HO activity. Levels of both heme (Figure 5A) and O_2^- (Figure 5B) in aortic tissue from 22-week-old ZDF were significantly elevated compared to 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 $SnCl_2$ reduced cellular heme in ZDF 0.196±0.004 nmol/mg ($P<0.00001$ vs. untreated ZDF) and in ZL (0.191±0.021 nmol/mg, $P<0.01$ vs. untreated ZL). Treatment with Ebselen did not significantly affect heme levels (not shown) in ZL (0.267±0.029 nmol/mg) or in ZDF (0.396±0.009 nmol/mg). O_2^- 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 O_2^- in ZDF to 1.96±0.27 µmol/mg ($P<0.05$) while an observed decrease in ZL (2.676±0.31 µmol/mg) was not statistically significant. ZDF treated with $SnCl_2$ demonstrated O_2^- levels equivalent to 8-week old control ZL while heme levels were reduced to levels significantly below 8-week-old ZL ($P<0.05$) and ZDF ($P<0.005$) controls (Data for 8 week old ZL and ZDF not shown).

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 $SnCl_2$. Systolic blood pressure was significantly lower in control ZL (148.4±4.5 mmHg) than in ZDF (171.2±4.4 mmHg), demonstrating the link between T2DM and hypertension ($P<0.001$, Figure 6). Following HO induction with $SnCl_2$, SBP was decreased to 136.1±3.3 mm Hg

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in ZL ($P<0.05$) and to134.0±44.2 mm Hg in ZDF ($P<0.000001$). In fact, $SnCL_2$ 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 (Kruger, et al., 2005;Quan, et al., 2004). Endothelial cells typically have a round to oval shape and are 20- to 50- μm in size (Figure 7A). The number of CEC was assessed using 22-week-old ZL and ZDF (Figure 7B). The number of sloughed cells in untreated ZDF (21.1±4.5 cells/ml) was significantly increased compared to ZL (5.5±1.7 cells/ml, $P<0.005$) while induction with $SnCl_2$ 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 pro-apoptotic 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 figure 8, ZDF demonstrated a significant reduction in the amounts of the anti-apoptotic proteins Bcl-xl and Bcl-2 and decreased phosphorylation of p38-MAPK, a signaling mediator in the anti-apoptotic pathway. In contrast the level of the pro-apoptotic protein, Bad was not significantly increased in ZDF compared to ZL. These changes demonstrate a pro-apoptotic environment associated with diabetes, suggesting the rats of pro-apoptotic signaling may be a contributing to the increase in endothelial cells sloughing and death. Upregulation of HO activity by $SnCl_2$ resulted in a significant increase in the anti-apoptotic proteins and an increase in p38-MAPK phosphorylation to levels greater than

those seen in ZL. In contrast, the pro-apoptotic Bad seemed unaffected by HO induction (Figure 8). Densitometry analysis (Figure 8B-D) demonstrated the following reductions in protein levels relative to control (n=3): BCL-XL 45.9±8.3% ($P<0.05$), BCL-2 52.1±5.1% ($P<0.05$), and the phosphorylation of p38-MAPK (P-p38 MAPK) 34.3±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 a significant increases in BCL-XL 186.4±6.0% ($P<0.005$, Figure 8 b) but not significant increases in BCL-2 73.5±19% (Figure 8 C). In contrast to BCL2 , P-p38 MAPK increased by 142.2±11.6% ($P<0.005$, Figure 8 D). Induction of HO-1 in ZDF rats did not change Bad proteins levels

Discussion

This study demonstrates, for the first time, that upregulation 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 Ebselen, an $ONOO^-$ scavenger, 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 anti-oxidant in humans and its increase prevents cardiovascular disease as seen in Gilbert's disease (Vitek, et al., 2002). Therefore, upregulation of HO-1 has a dual effect by increasing the levels of CO but 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 PI3K/AKT phosphorylates HO-1 and increase 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 SnMP and activated AKT

was restored by upregulation of HO-1 using pharmacological inducers (Di Noia, et al., 2006). Our findings strongly suggest, 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, COX-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., 2004;Turkseven, et al., 2005;Abraham, et al., 2003a). Heme is normally protein-bound and present in only moderate quantities but 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;Kruger, et al., 2005;Agarwal, et al., 1995). 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;Abraham, et al., 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 upregulation of HO-1 is efficacious in preventing superoxide formation which can preserve renal function and attenuate hypertension.

Our finding of increased CEC in T2DM may suggest that loss of EC 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 EC 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 (McClung, et al., 2005;Kruger, et al., 2005;Abraham, et al., 2004). 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 which 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 anti-apoptotic Bcl-xl and phosphorylated p38-MAPK pathways, without affecting Bcl-2 and the pro-apoptotic Bad pathway (Figure 8). The increase in endothelial cell fragmentation in blood obtained from 22 week old ZDF was not seen in a similar group of rats treated with $SnCl_2$ (Figure 7B), suggesting that HO-1

derived CO and bilirubin enhance the levels of anti-apoptotic proteins. The Bcl-2 pathway appears of particular importance in diabetes as an increase in its level has been associated with pancreatic beta cell survival (Johnson, et al., 2003). Others have shown that HO-1 derived CO increases Bcl-xl and p38 (Zhang, et al., 2005). While this study was not designed to fully elucidate the mechanism of the anti-apoptotic actions of HO, our data provides important insight and suggest that HO "reprograms" vascular endothelial cells with a resultant increase in anti-apoptotic signaling molecules, Bcl-xl, Bcl-2 and p38 MAPK, and enhances EC integrity.

In summary, $SnCl_2$, a potent inducer of HO-1, decreases endothelial cell sloughing and fragmentation and normalizes blood pressure to control levels and presumably by a mechanism involving increased eNOS, Bcl-xl and p38 MAPK in an animal model of diabetes. In an era where polypharmacy has become the accepted practice in the treatment of many cardiovascular diseases, including hypertension and diabetes, pharmacological modulators of HO activity may have an unique role in the pharmacologic arsenal.

Acknowledgments

Adam L. Kruger and Stephen J. Peterson contributed equally to this work.

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Footnotes

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Legends For Figures

Figure1: Western blot analysis of HO-1, HO-2, eNOS and iNOS as well as HO activity in aorta from 8 and 22 week old ZL and ZDF rats are shown. Quantitative densitometry analysis of protein levels was determined and are expressed as a ratio to α-actin (mean ±SE, n=3). **1A:** Representative Western blots for HO-1, HO-2 and actin in 8 and 22 week old ZL and ZDF are shown along with densitometry analysis for HO-1/actin. HO-2 levels remained unchanged throughout all experiments. *****P<0.05 vs. 8 week old ZDF and P<0.001 vs. 22 week old ZL. **B:** HO activity was measured and is expressed as nmol bilirubin/mg protein (mean±SE, n=4); *****P<0.05 vs. 22 week old ZL. **C:** Western blot and densitometry for iNOS, *****P<0.05 vs. 22 week old ZL **D:** Western blot and densitometry for eNOS, *P<0.05 vs. ZL (8 weeks); **P<0.05 vs. ZL (22 weeks).

Figure 2A: In vitro assessment of HO activity in aortic tissue (expressed as mean±SE, n=4). The effects of peroxynitrite (250 μM) and H_2O_2 (250 μM) on HO activity were compared with control. *P<0.001 and **P<0.01 compared to control. **B:** Western blot analysis of HO-1 immunoprecipitate after $ONOO^-$ treatment. Anti-HO-1 and anti-nitrotyrosine antibodies were compared.

Figure 3: Aortic tissue from untreated and Ebselen treated ZL and ZDF was used to assess HO-1 level, HO activity and the levels of 3-NT. Representative Western blot for HO-1 and densitometry analysis normalized to actin (n=3) are shown. *P<0.02 vs. untreated ZL; **P<0.02 vs. untreated ZDF. **B:** HO activity in 22 week old Ebselen

treated ZL and ZDF was compared. *P<0.05 vs. ZL control; **P<0.005 vs. ZDF control. **C:** The effect of changes in HO activity on nitrosative stress was measured by examining 3-nitrotyrosine levels in 8 and 22 week old control and 22 week old Ebselen treated ZL and ZDF. Representative Western blots for 3-NT and actin are shown along with densitometry analysis (n=3). *P<0.05 vs. 22 week old ZL, **P<0.05 vs. untreated ZDF (22 weeks).

Figure 4A: Aortic HO activity, *P<0.05 vs. ZL control; **P<0.00005 vs. ZL control; ***P<0.005 vs. ZL and ZDF controls and ZL+CoPP; †P<0.00005 vs. ZL control; ‡P<0.005 vs. ZL+$SnCl_2$ and P<0.0005 vs. ZL and ZDF controls. **4B:** Western blot and densitometry analysis of 3-NT (mean density normalized to actin ±SE), *P<0.05 ZDF+CoPP vs. ZDF, **P<0.0005 ZDF+$SnCl_2$ vs. ZDF.

Figure 5A: Cellular heme content in aortic tissue from 22 week old ZL and ZDF (mean ±SE; n=4), *P<0.0005 vs. ZL control, **P<0.01 vs. ZL control; ***P<0.00001 vs. ZDF control. **B:** Superoxide levels in aortic tissue from 22 week old ZL and ZDF (mean ±SE; n=4), *P<0.005 vs. ZL control; **P<0.05 vs. ZDF control.

Figure 6 Systolic Blood pressure measurements from 22 week old ZL and ZDF, *P<0.001 vs. ZL control, **P<0.05 vs. ZL control; ***P<0.000001 vs. ZDF control and P<0.05 vs. ZL control.

Figure 7A: Morphology of endothelial cells isolated from zucker rats, using RECA-1 coated Dynabeads, is demonstrated under light (left) and fluorescent (right) microscopy. **B:** Endothelial cell sloughing *P<0.005 ZDF vs. ZL vehicle controls; **P<0.05 ZDF+$SnCl_2$ vs. ZDF vehicle control.

Figure 8: Aorta lysate from 22 week old untreated (ZL and ZDF) rats and $SnCl_2$ pre-treated (from age 12 to 22 weeks) ZDF was examined. Representative Western blots are shown for the anti-apoptotic mediators Bcl-xl and Bcl-2, the phosphorylation of p38-MAPK (P-p38 MAPK) and the pro-apoptotic 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 ±SE (n=3). **8 B)** BCL-XL decreased with ZDF but was restored with $SnCl_2$, *P<0.05 vs. control, †P<0.001 vs. ZDF untreated. **8 C)** BCL-2 decreased in ZDF rats but $SnCl_2$ only partially restored its level, *P<0.05 vs. control. **8 D)** the phosphorylation of p38-MAPK (P-p38 MAPK) decreased in ZDF rats and was restored with treatment with $SnCl_2$, *P<0.005 vs. control, †P<0.0001 vs. ZDF untreated.

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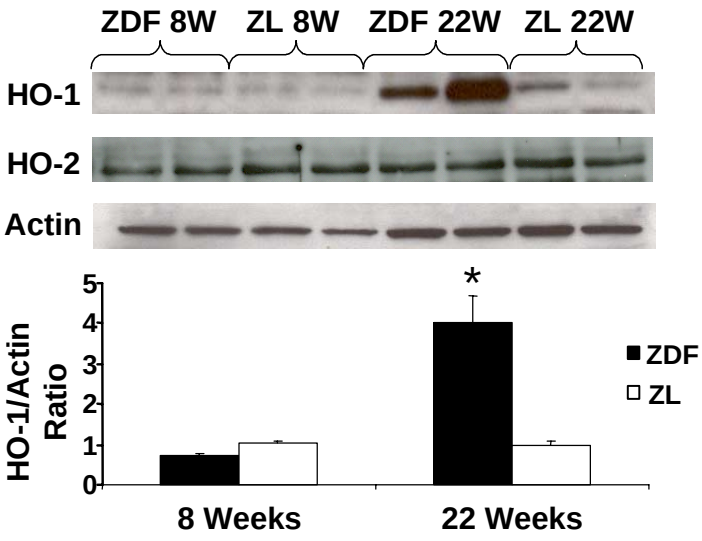
Figure 1A



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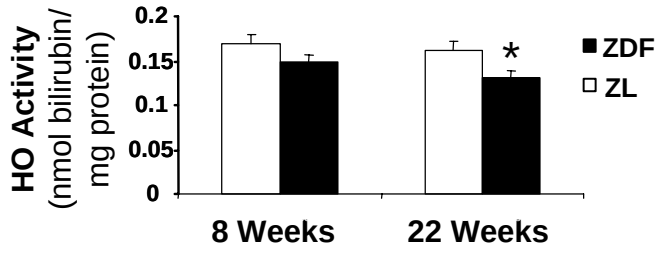

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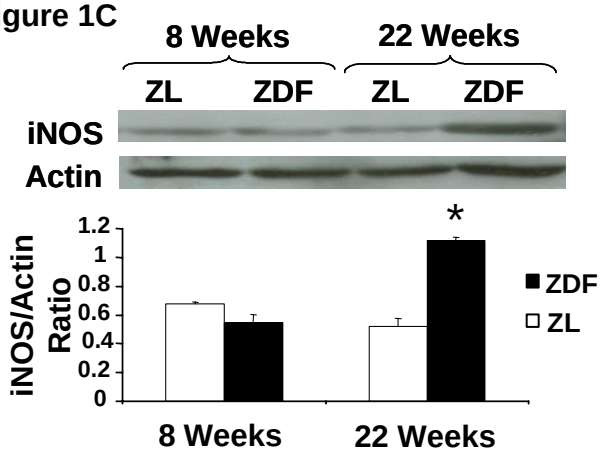

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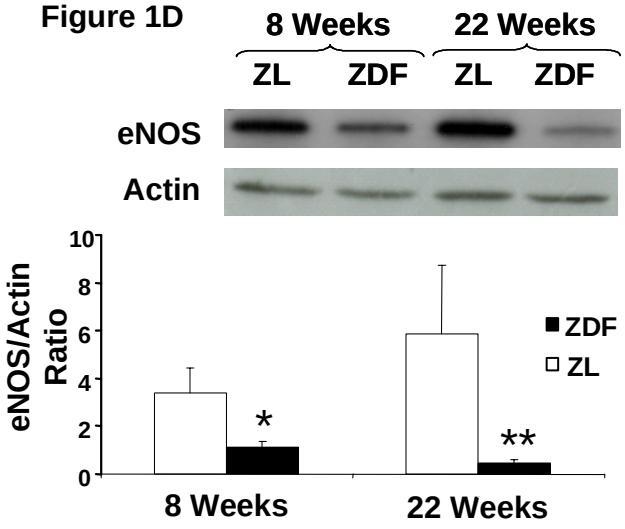

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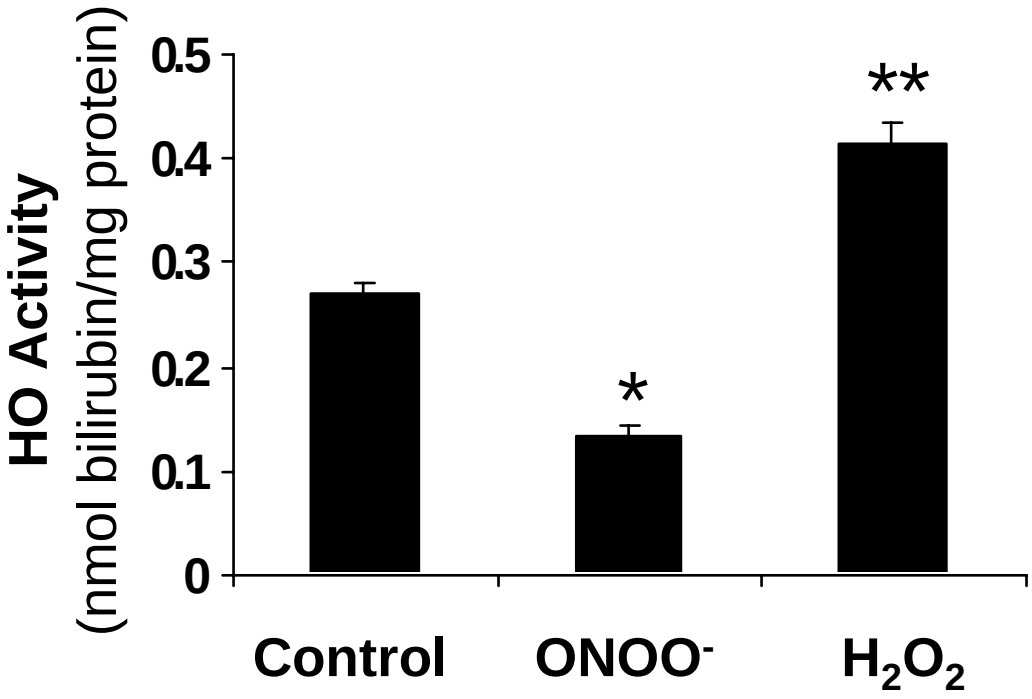

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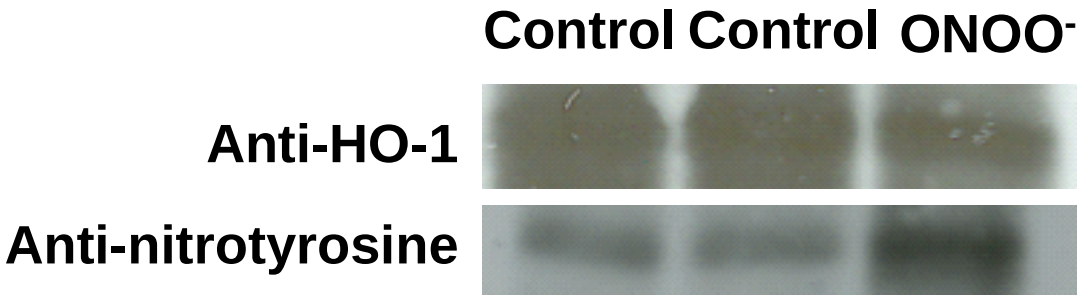

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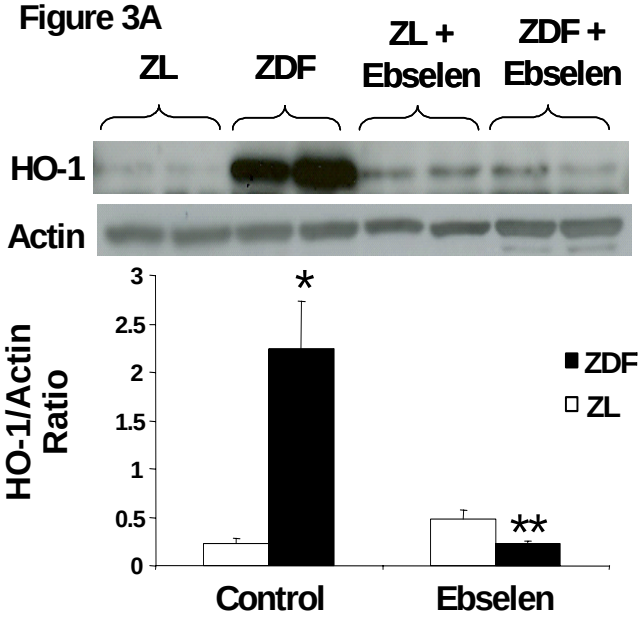
Figure 3A



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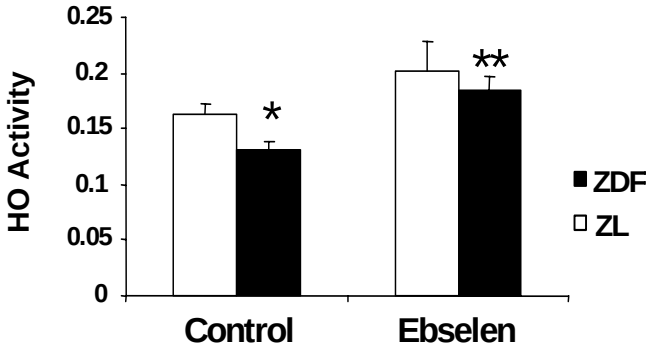

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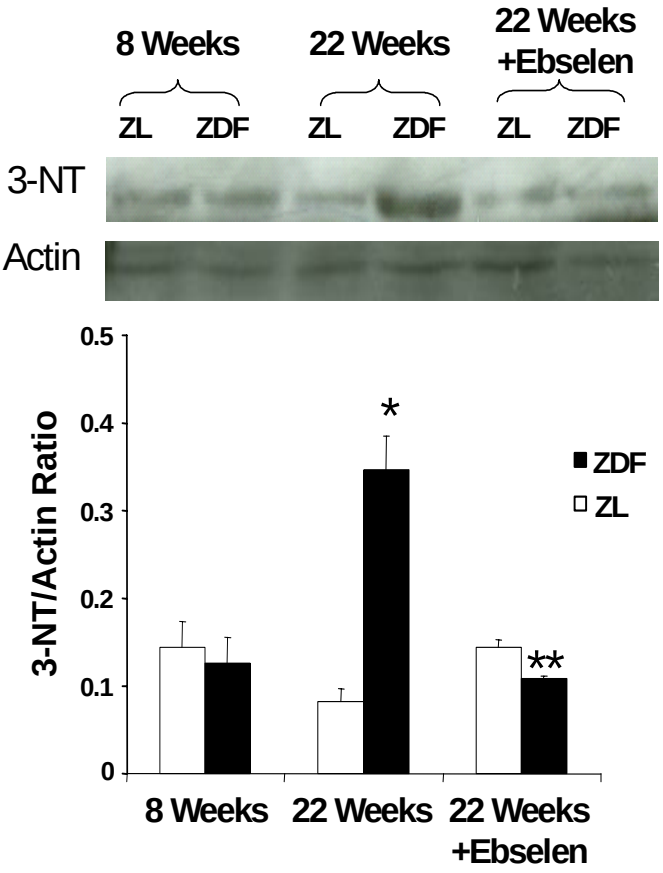

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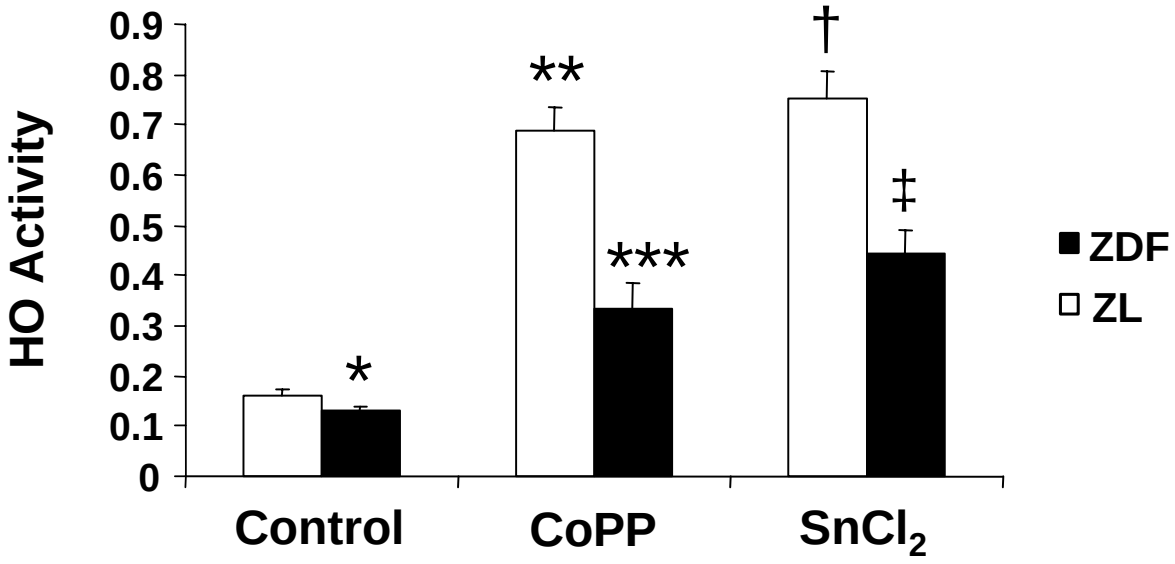
Figure 4A



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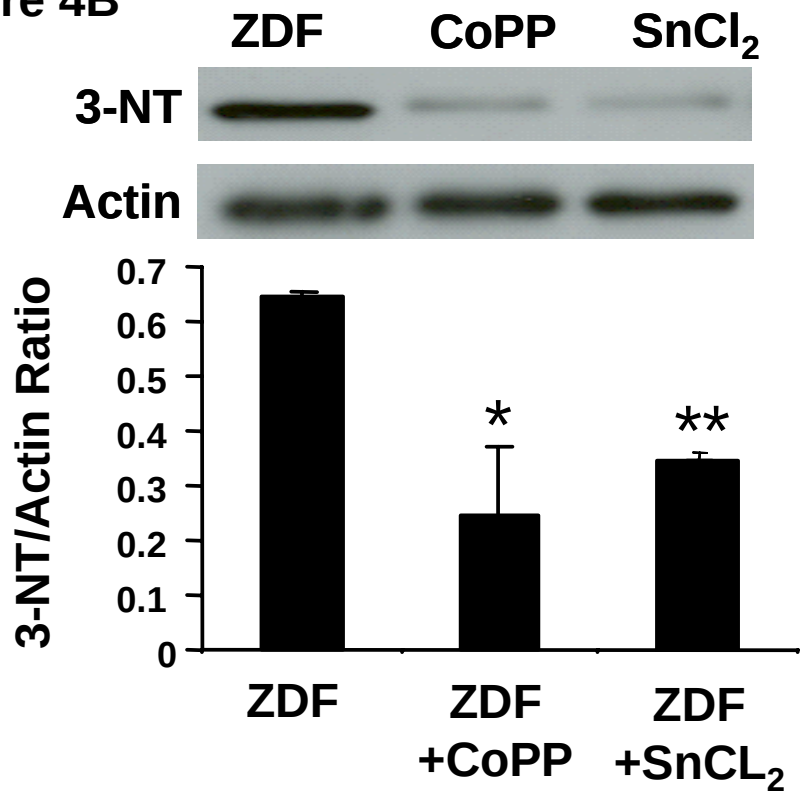

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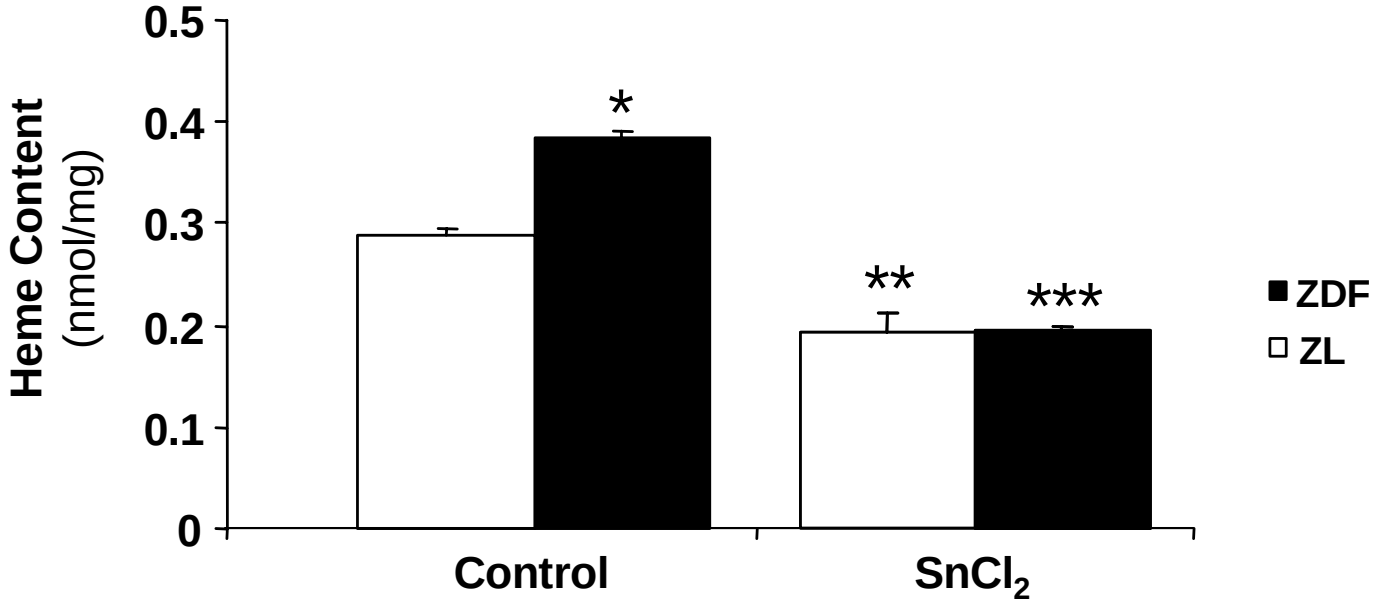
Figure 5A



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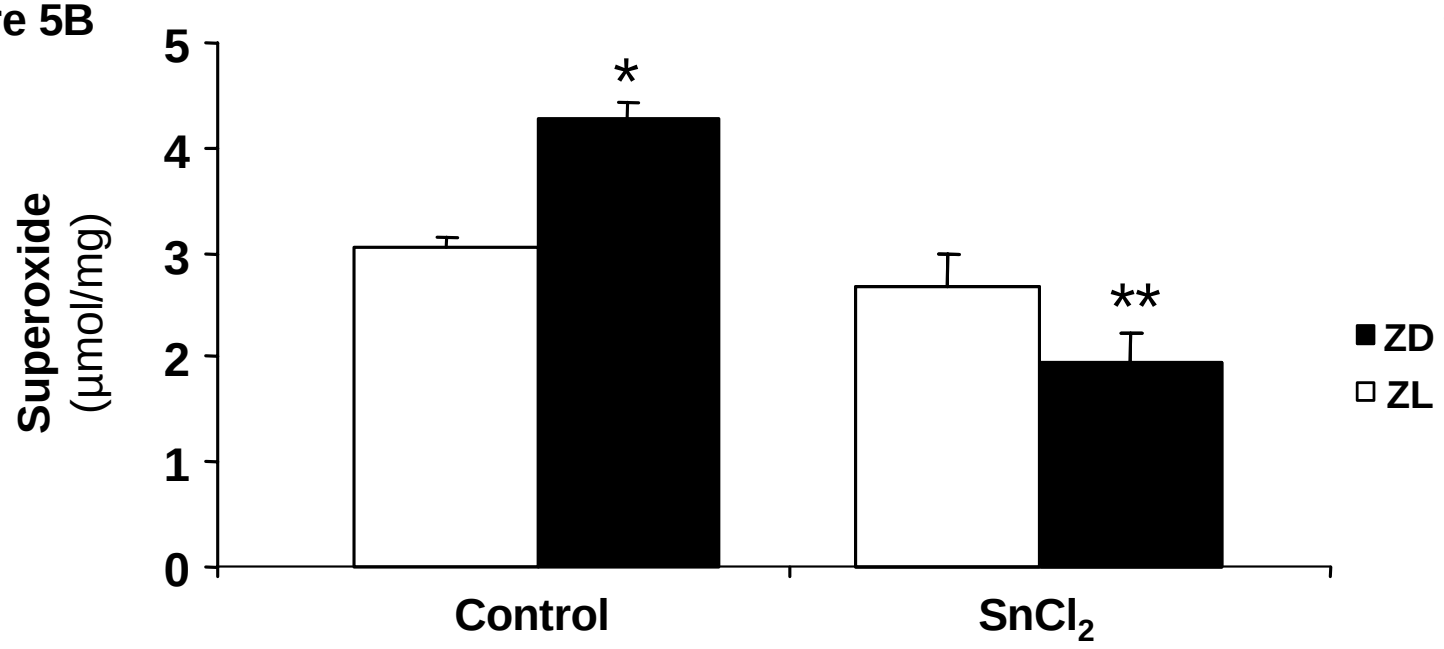

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Figure 6

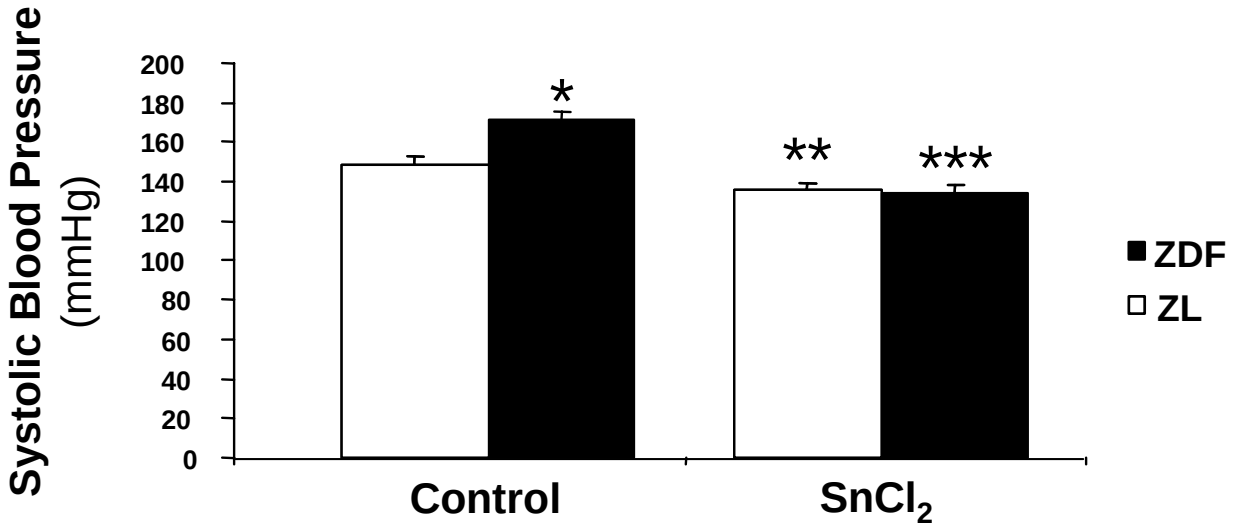

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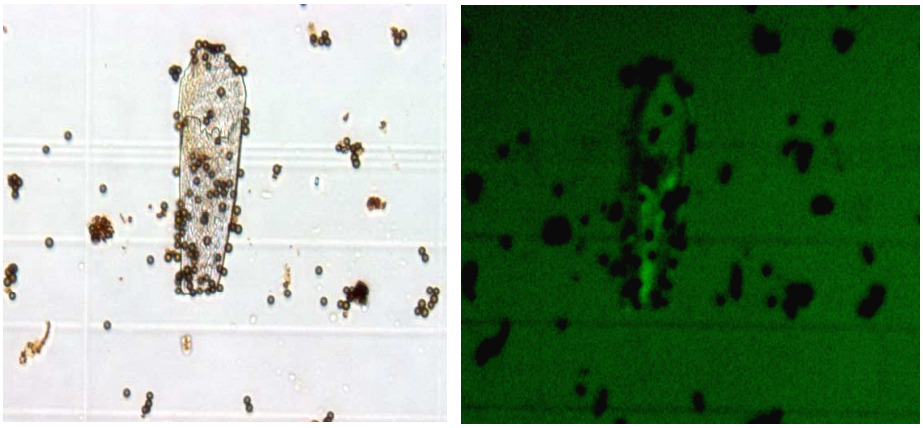
Figure 7A



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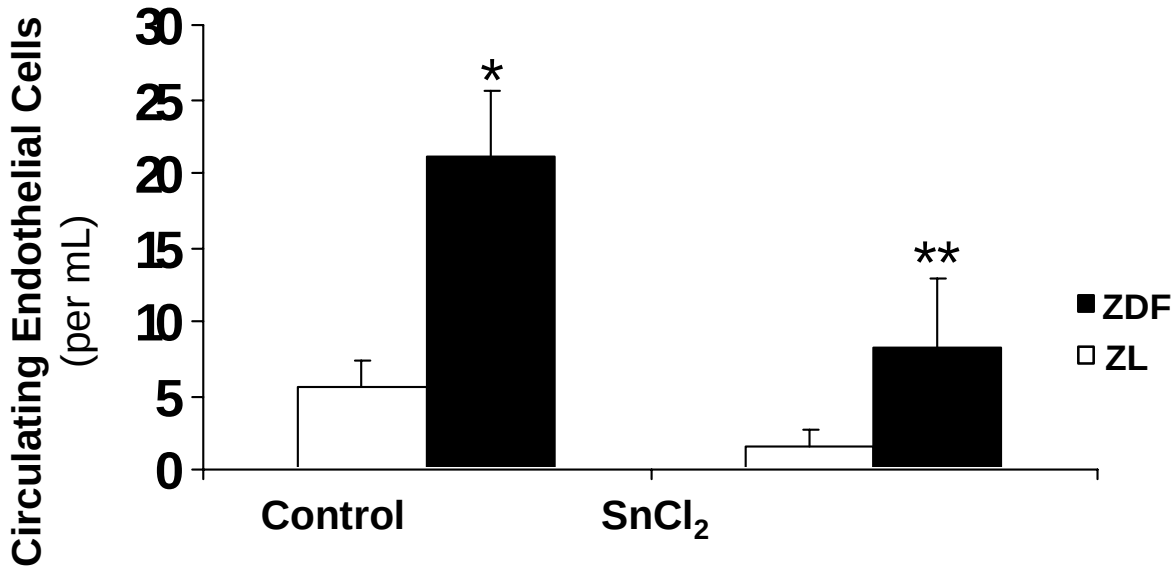

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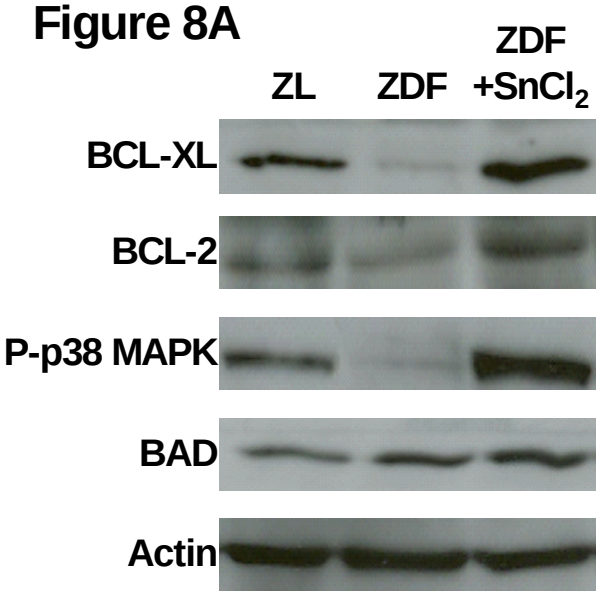
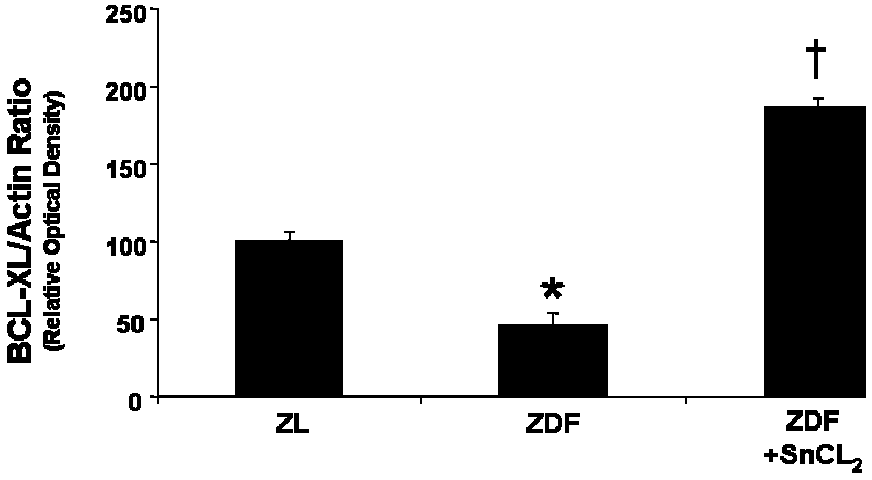
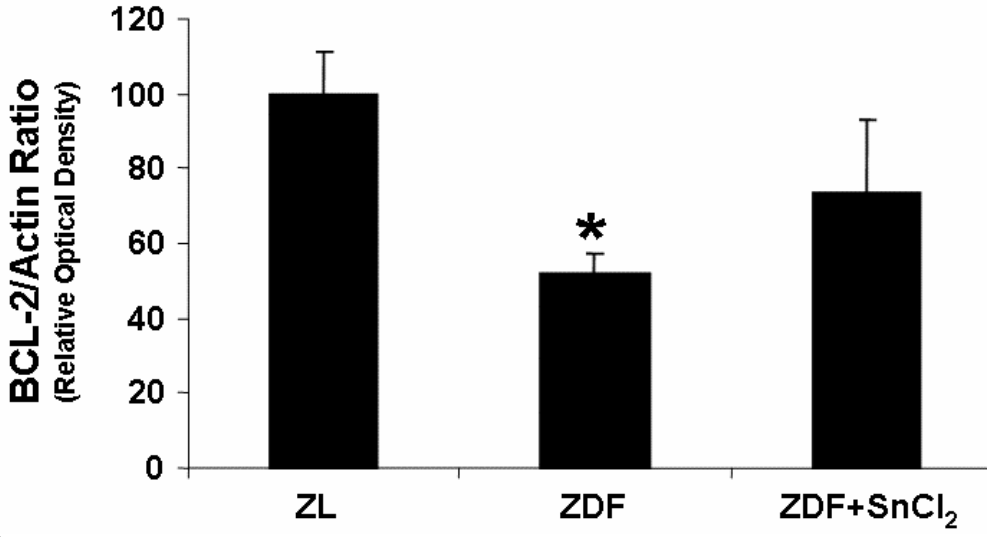
Figure 8A



B



C



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