Free Radical Research, Volume 38 Number 12 (December 2004), pp. 1301–1310

Taylor & Francis
healthsciences

Pro-oxidant Role of Heme Oxygenase in Mediating Glucose-induced Endothelial Cell Damage

SHALI CHEN[a], ZIA A. KHAN[a], YOUSEF BARBIN[a] and SUBRATA CHAKRABARTI[a,b,*]

[a]*Department of Pathology, University of Western Ontario, London, Ontario, Canada;* [b]*Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada*

Accepted by Professor E. Niki

(Received 3 May 2004; In revised form 9 October 2004)

Oxidative damage to the vascular endothelial cells may play a crucial role in mediating glucose-induced cellular dysfunction in chronic diabetic complications. The present study was aimed at elucidating the role of glucose-induced alteration of highly inducible heme oxygenase (HO) in mediating oxidative stress in the vascular endothelial cells. We have also investigated the interaction between HO and the nitric oxide (NO) system, and its possible role in alteration of other vasoactive factors.

Human umbilical vein endothelial cells (HUVECs) were exposed to low (5 mmol/l) and high (25 mmol/l) glucose levels. In order to determine the role of HO in endothelial dysfunction and to elucidate a possible interaction between the HO and NO systems, cells were exposed to HO inducer (hemin, 10 μmol/l), HO antagonist (SnPPIX, 10 μmol/l), and NO synthase blocker (L-NAME, 200 μmol/l) with or without NO donor (arginine, 1 mmol/l). mRNA expression of HO and NO isoforms was measured by real time RT-PCR. HO activity was measured by bilirubin production and cellular oxidative stress was assessed by 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine staining. We also determined the expression of vasoactive factors, endothelin-1 (ET-1) and vascular endothelial growth factor (VEGF).

In the endothelial cells, glucose caused upregulation of HO-1 expression and increased HO activity. A co-stimulatory relationship between HO and NO was observed. Increased HO activity also associated with oxidative DNA and protein damage in the endothelial cells. Furthermore, increased HO activity augmented mRNA expression of vasoactive factors, ET-1 and VEGF. These data suggest that HO by itself and via elaboration of other vasoactive factors may cause endothelial injury and functional alteration. These findings are of importance in the context of chronic diabetic complications.

Keywords: Endothelial cells; Heme oxygenase; Oxidative stress; Nitric oxide; Glucose

INTRODUCTION

Oxidative stress has been suggested to be a key pathogenetic mechanism in causing tissue damage in chronic diabetic complications.[1,2] Endothelial cell dysfunction may play a significant role in late complications of chronic diabetes. As glucose enters freely in vascular endothelial cells via insulin-independent glucose transporter type 1 (GLUT1),[3] high intracellular levels of glucose may lead to accelerated flux through glycolytic pathway, activate protein kinase C via diacylglycerol synthesis, and alter $NADP^+$/NADPH ratio via augmented polyol pathway.[4–6] Several of these pathways may be linked with increased oxidative stress and are also potentially activated by oxidative stress in endothelial cells.[4–6] As a defense mechanism against such oxidative stress, highly inducible heme oxygenase (HO) enzyme system may be activated.[7,8] HO system consists of HO-1, also known as heat shock protein 32, HO-2 and HO-3.[9,10] HO enzyme system physiologically degrades heme into biliverdin, free iron, and carbon monoxide.[9,10]

Several agents, such as hydrogen peroxide, UV irradiation, and endotoxin exposure may induce HO system suggesting a major role in cellular homeostasis.[9–12] Although it has been suggested that induction of stress-responsive HO may be a defense mechanism, it may also induce cellular damage in

*Corresponding author. Address: Department of Pathology, The University of Western Ontario, London, Ontario, N6A 5C1. Canada. Tel.: +1-519-685-8500. Ext. 36350. Fax: +1-519-663-2930. E-mail: schakrab@uwo.ca

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400017228

diabetes.[8] Bilirubin, produced from HO-catalyzed biliverdin, and CO have been suggested to be responsible antioxidant effect of HO.[13,14] However, iron production and accumulation may have cytotoxic effects by participating in fenton reaction.[15] We have previously demonstrated that induction of HO is of importance in the pathogenesis of diabetes-induced heart damage.[8] Recent evidence also suggests an interaction of the HO system with the nitric oxide (NO) pathway.[16] Increased HO activity may upregulate inducible and endothelial NO synthase (iNOS and eNOS) expression and NO activity.[8,16] Inhibitors of HO activity have also been shown to inhibit NOS activity.[17] Furthermore, increased NO levels have been shown to upregulate HO enzyme expression.[16,17] It is possible that in the context of diabetic complications, HO may interact with the NO pathway and lead to oxidative stress. Augmented HO activity in non-diabetic context has been demonstrated to increase vascular endothelial growth factor (VEGF) expression via interaction with the NO.[18]

The aim of the present study was to investigate whether glucose causes endothelial cell dysfunction via alteration of the HO system, and its interaction with the NO pathway. We have also investigated the effect of HO activation on some of the effector molecules in the context of chronic diabetic complications, such as endothelin-1 (ET-1) and VEGF.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from the Clonetics (Walkersvile, Maryland). Cells were plated at 2500 cells/cm^2 in Endothelial Cell Growth Medium (EGM) (Clonetics, Walkersvile, Maryland). EGM is a modified MCDB 131 formulation and supplied with 10 ng/ml human recombinant epidermal growth factor, 1.0 μg/ml hydrocortisone, 50 μg/ml Gentamicin, 50 ng/ml Amphotericin B, 12 μg/ml bovine brain extract, and 2% fetal bovine serum. Cells were grown in 25 cm^2 tissue culture flasks and incubated at 37° C in 5% CO_2 until 80% confluent as described by us previously.[19,20] Sub-confluent cells were incubated in 25 mmol/l D-glucose (high glucose; HG). L-Glucose was used as a control. In all experiments, HO agonist hemin and HO inhibitor tin protoporphyrin IX (SnPPIX; Porphyrin products, Inc., Logan, Utah) were used at 10 μmol/l. NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), was used at 200 μmol/l. L-Arginine was used at 1 mmol/l. All experiments were carried out after 24 h of incubation unless otherwise indicated. Three different batches of cells, each in duplicate, were investigated.

RNA Isolation and cDNA Synthesis

TRIzol™ reagent (Invitrogen, Burlington, ON, Canada) was used to isolate RNA as previously described.[19–21] RNA was extracted with chloroform followed by centrifugation to separate the sample into aqueous and organic phases. RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in diethylpyrocarbonate-treated water.

cDNA synthesis was performed using the Superscript-II system (Invitrogen) as previously described.[19–21] Total RNA (2 μg) was added to oligo(dT) primers (Invitrogen), denatured at 70°C, and quenched on ice for 10 min. Reverse transcription was carried out by the addition of moloney murine leukemia virus reverse transcriptase and dNTPs at 42°C for 50 min in a total reaction volume of 20 μl. The reaction was terminated by incubation at 70°C for 15 min. The resulting cDNA products were stored at −20°C.

Real Time RT-PCR

Real time RT-PCR for HO-1, HO-2, iNOS, eNOS, ET-1 and VEGF was carried out in the LightCycler™ (Roche Diagnostics Canada, Laval, PQ, Canada) using the SYBR Green I detection platform.[22,23] This system allows amplification and detection of products in a single reaction tube. PCR reactions were performed in microcapillary tubes (Roche Diagnostics Canada), with a final volume of 20 μl. The reaction mixture consisted of 10 μl SYBR® Green Taq ReadyMix (Sigma-Aldrich, Canada), 1.6 μl of 25 mM $MgCl_2$, 1 μl of each forward and reverse 10 μM primers (Table I),[22–24] 4.4 μl H_2O, and 2 μl cDNA template. The PCR reaction mixture for ET-1 consisted of 2.5 μl 10 × PCR Buffer (Invitrogen), 1.25 μl of 5 mM dNTP, 1.2 μl 50 mM $MgCl_2$, 1 μl primers, 9.8 μl H_2O, 2 μl cDNA, and 0.75 μl 15 mM Taqman probe (Table I).[22,23] The data was normalized to housekeeping gene (β-actin) to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures. As the PCR protocols for the genes were optimized, melting curve analysis (MCA) was used to determine melting temperature (T_m) of specific amplification products and primer-dimmers. For each gene, the specific T_m values were used to create a signal acquisition step (2–3°C below T_m), which was added onto each elongation period. This phase allows for signal acquisition from specific products without signal interference from primer-dimers and non-specific amplification products. All PCR reactions were subjected to MCA to determine specificity of amplification. In addition, PCR products were subjected to gel electrophoresis (2% agarose) to determine size of the amplified products.

TABLE I Oligonucleotide sequences for real time RT-PCR

Gene	Sequence 5′–3′	Temperature profile*	
HO	HO-1 (Ref. [24])	Denaturation	95°–0 s
		Annealing	50°–10 s
	TGATAGAAGAGGCCAAGA	Extension	72°–17 s
	TTTCCAGACAGAGGGACA	Signal	84°–1 s
	HO-2 (Ref. [24])	Denaturation	95°–0 s
		Annealing	50°–10 s
	TGGAGCGCAACAAGGACCAT	Extension	72°–17 s
	CCGGTAGAGCTGCTTGAACT	Signal	84°–1 s
NOS	iNOS	Denaturation	95°–0 s
		Annealing	55°–5 s
	TAACTTGCAGGTCCAAATCT	Extension	72°–8 s
	TCTTTGAAGGAGCCGTAATA	Signal	81°–1 s
	eNOS	Denaturation	95°–0 s
		Annealing	55°–5 s
	CCCAAATGTGCTGGTTAC	Extension	72°–8 s
	AGTGAAGGCGACAATCCT	Signal	81°–1 s
ET-1	AAGCCCTCCAGAGAGCGTTAT	Denaturation	95°–0 s
	CCGAAGGTCTGTCACCAATGT	Annealing	55°–5 s
	6FAM-TGACCCACAACCGAG-MGBNFQ	Extension	72°–4 s
		Signal acquisition following extension	
VEGF	GGCCTCCGAAACCATGAACTTTCTGCT	Denaturation	95°–0 s
	GCATGCCCTCCTGCCCGGCTCACCGC	Annealing	55°–10 s
		Extension	72°–20 s
		Signal	80°–1 s
β-actin	CCTCTATGCCAACACAGTGC	Denaturation	95°–0 s
	CATCGTACTCCTGCTTGCTG	Annealing	55°–5 s
		Extension	72°–8 s
		Signal	80°–1 s

*Initial denaturation was carried out at 95°C–1 min. Ramp rate for all PCR phases was 20°C/s.

Western Blotting

HUVECs were lysed in lysis buffer (HEPES 50 mmol/l, pH 7.6, NaCl 150 mmol/l, NaF 50 μmol/l, EDTA 2 mmol/l, sodium vanadate 1 mmol/l, 1% NP-40, and phenylmethylsulphonyl fluoride 2 mmol/l). The protein concentrations were determined by BCA protein assay (Pierce, IL). A 10 μg proteins for HO-1 and 30 μg for iNOS were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were analyzed by Western blotting using the rabbit anti-HO-1 and iNOS polyclonal antibodies (Stressgen Biotechnologies, Victoria, Canada). The signals from Western blots were obtained using horseradish peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology, CA) and visualized by the chemiluminescent substrate (Amersham Pharmacia Biotechnology, Amersham, UK). The blots were quantified by densitometry using Mocha software (SPSS, Chicago, IL).

HO Activity

Confluent endothelial cells in 150-mm culture dishes were incubated for 24 h in the presence of control media (5 mmol/l glucose), 25 mmol/l glucose, 10 μmol/l hemin, 10 μmol/l SnPPIX, or 200 μmol/l L-NAME with or without 1 mmol/l L-arginine. The method used for determination of HO activity via bilirubin formation follows the protocol published with some modifications.[25] Briefly, cells were washed twice with phosphate-buffered saline following treatment, gently scraped off the dish and centrifuged (1000*g*, 10 min, 4°C). The cell pellets were suspended in 800 μl solution A (0.25 mol/l sucrose, 20 mmol/l Tris–HCI, pH 7.4) containing 1 × protease inhibitor cocktail and sonicated on ice before centrifugation at 15,000*g* for 20 min at 4°C. The supernatant was centrifuged at 105,000*g* for 1 h at 4°C and microsomal pellet was resuspended in 200 μl solution A and sonicated. The suspension was recentrifuged at 15,000*g* at 4°C for 5 min. The supernatant containing the microsomal fraction was collected and stored at −70°C. Half mg microsomal fraction was added to a NADPH-generating system containing 1 mmol/l β-NADPH, 2 mmol/l glucose-6-phosphate, 1 U glucose-6-phosphate-1-dehydrogenase, 1 mg protein of rat liver cytosol prepared from the 105,000*g* supernatant fraction as a source of biliverdin reductase, 25 μmol/l hemin, 0.25 mmol/l sucrose, 20 mmol/l Tris–HCI (pH 7.4) in a final volume of 500 μl. The reaction was conducted for 1 h at 37°C in the dark and terminated by addition of 0.5 ml of chloroform.

The extracted bilirubin concentration was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette using an extinction coefficient of 40 mmol/l^{-1} cm^{-2}. HO activity was measured as picomoles of bilirubin formed per milligram of protein per hour. Basal HO activity was in a range between 200 and 600 pmol bilirubin/mg protein/hour.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) Staining

HUVECs, grown on 8 well chambered cell culture glass slides (Becton Dickinson, Franklin Lake, NJ), were incubated for 24 h in the presence of experiment agents and were stained for 8-OHdG as described previously.[26] Briefly, fixed slides were treated with RNase (100 μg/ml) for 1 h at 37°C and proteinase K (10 μg/ml) for 10 min at room temperature. DNA was denatured with 4 N HCl for 10 min at room temperature. Endogenous peroxidase was blocked by treating the slides with 3% H_2O_2 in methanol for 10 min at room temperature and 10% normal goat serum was used to block nonspecific antibody binding sites. The cells were then incubated with the goat anti-OHdG polyclonal antibody (1:150) (Chemicon Lab, Temecula, CA) at room temperature for 1 h followed by biotinylated rabbit anti-goat IgG for 30 min. Horseradish peroxidase conjugated streptavidin was added and the slides were incubated for 30 min at 37°C. Cells were treated with chromogen, diaminobenzidine. The negative controls were produced by replacing the primary antibody with non-immune goat serum. After three repetitions of the experiment, the slides were read by two investigators unaware of the particular treatment and were arbitrarily scored. Number of positive cells were quantified in five random fields were quantified. The data was expressed as percentage (%) of total cells.

Nitrotyrosine Staining

HUVECs were fixed with a mixture of cold acetone/methanol (1:1) for 5 min at −20°C. Non-specific binding was blocked by incubation for 1 h with 10% normal goat serum in phosphate-buffered saline plus 0.3% Triton X-100 at room temperature. The cells were then incubated with an affinity-purified antinitrotyrosine monoclonal antibody (1:200) (Cayman Chemical, Ann Arbor, MI). Cells were washed three times and incubated with rabbit anti-mouse IgG conjugated with the streptavidin–biotin reaction described in the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). ABC reagent, avidin conjugated to horseradish peroxidase, was added, and the slides were incubated for 30 min at 37°C. Cells were treated with chromogen, diaminobenzidine. The negative controls were produced by replacing the primary antibody with non-immune mouse serum. After three repetitions of the experiment, the slides were read by two investigators unaware of the particular treatment and were arbitrarily scored. Number of positive cells were quantified in five random fields were quantified. The data was expressed as percentage (%) of total cells.

Statistical Analysis

The data are expressed as means ± SE and were analyzed by ANOVA followed by Student's *t*-test. Differences were considered significant at values of $p < 0.05$.

RESULTS

Glucose Causes HO Upregulation and Increased Activity in the Endothelial Cells

We have used a real time RT-PCR, a sensitive and accurate method, for the assessment of HO isozyme mRNA expression in vascular endothelial cells (Figs. 1 and 2). Incubation of cells with 25 mmol/l glucose caused significant increase in the HO-1 mRNA expression. The mRNA expression peaked at 24 h. Hence in subsequent experiments we investigated after 24 h of exposure (Fig. 1, $p < 0.05$). Incubation of the cells with hemin also demonstrated upregulation of HO-1 mRNA. SnPPIX treatment, on the other hand, did not prevent glucose-induced increased HO-1 mRNA expression. No effect of SnPPIX treatment on HO-1 mRNA expression in low glucose was observed (Fig. 2B). The results with SnPPIX treatment are consistent with previous studies in other systems which demonstrate potent inhibition of HO activity but no inhibition of HO mRNA expression with SnPPIX.[27] Compared to the HO-1 mRNA, HO-2 mRNA

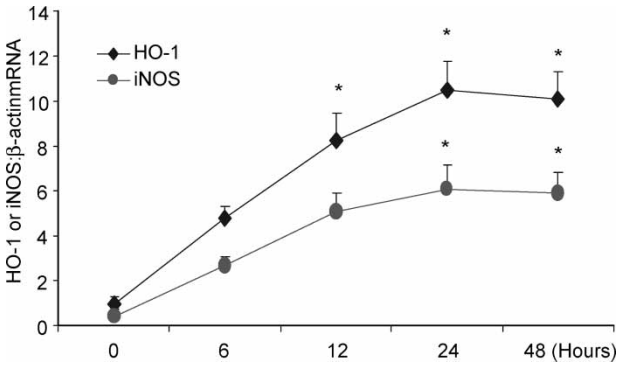

FIGURE 1 Quantification of HO-1 and iNOS mRNA expression as assessed by real time RT-PCR shows that incubation of HUVECs in D-glucose (25 mmol/l) causes time-dependent increase in HO-1 and iNOS mRNA. Transcripts were expressed as ratio of target mRNA to β-actin mRNA. *$p < 0.05$ compared to NG, $n = 5$/treatment.

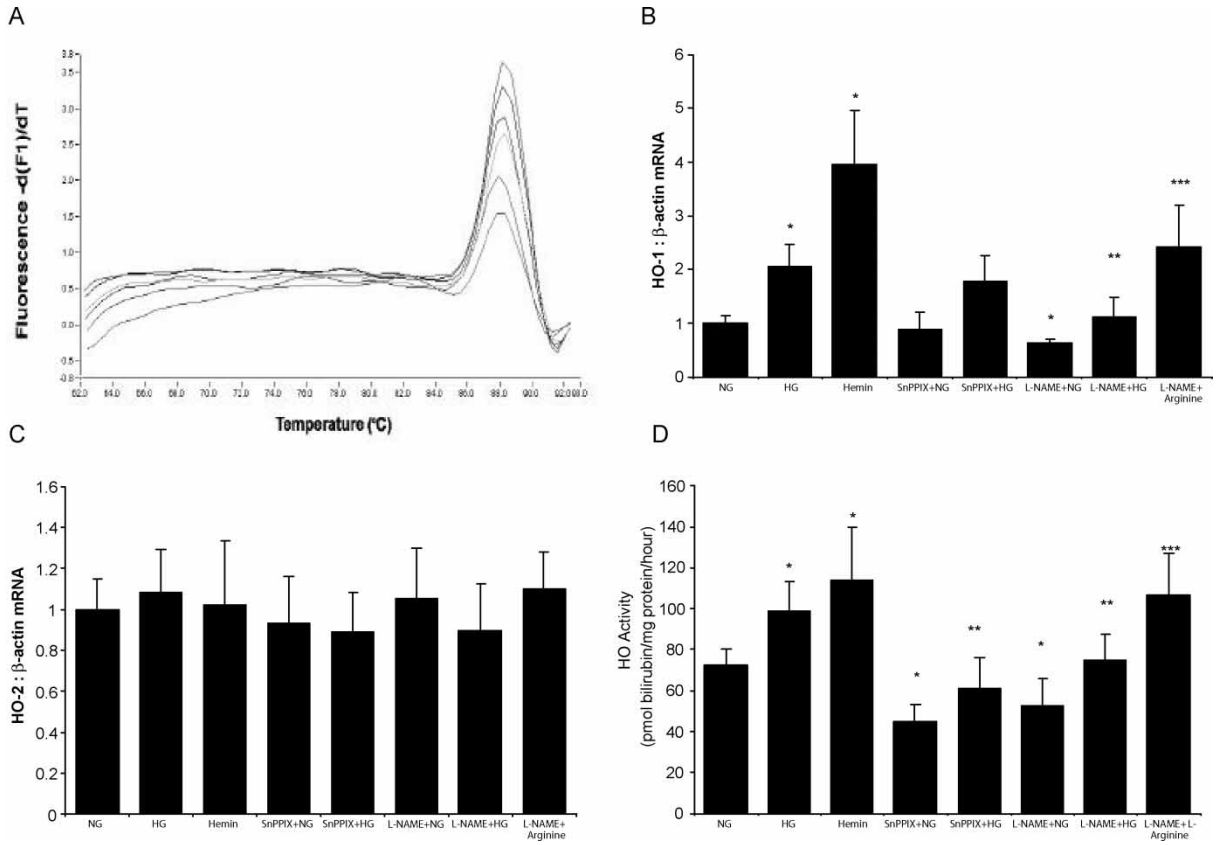

FIGURE 2 Quantification of HO mRNA and activity as assessed by real time RT-PCR and HO activity assay, showing (A) melting curve analysis of post-PCR HO-1 products, (B) quantification of HO-1 mRNA, (C) quantification of HO -2 mRNA, and (D) quantification of HO activity as concentration of bilirubin produced per hour per milligram of protein. [Each trace in (A) represents cDNA sample from endothelial cells; Melting curve analysis was used to determine specificity of PCR amplification; HO isozyme transcripts were expressed as ratio of target mRNA to β-actin mRNA (relative to control); NG = 5 mM glucose, HG = 25 mM glucose, Hemin = 10 μmol/l hemin, SnPPIX = 10 μmol/l SnPPIX, L-NAME = 200 μmol/l L-NAME, and L-NAME + arginine = 200 μmol/l L-NAME + 1 mmol/l arginine; *$p < 0.05$ compared to NG, **$p < 0.05$ compared to hemin; ***$p < 0.05$ compared to L-NAME; $n = 3$/treatment].

showed no significant glucose-induced alteration (Fig. 2C). Protein analysis data paralleled mRNA data (Fig. 3A and B).

In order to determine whether glucose-induced upregulation of HO-1 mRNA coincides with increased activity, we measured bilirubin production, a standard method of assessing HO activity. Compared to low glucose (5 mmol/l), incubation with high glucose levels caused increased HO activity (Fig. 2D; $p < 0.05$). Cells incubated with hemin, HO inducer, also demonstrated an increased HO activity. Our results further show that glucose-induced HO activity was blocked by incubation with SnPPIX. Basal HO activity was also slightly reduced.

A Co-stimulatory Relationship may exists between HO and NO in Endothelial Cells Following Glucose Exposure

An intricate relationship may exist between the HO and the NO systems. Hence, we examined such a relationship in the context of glucose-induced HO alteration. Incubation of the cells in 25 mmol/l glucose caused increased iNOS and eNOS mRNA expression as measured by real-time quantitative RT-PCR (Fig. 4; $p < 0.05$). A pronounced upregulation of NOS mRNA expression was also observed following incubation with HO-inducer, hemin. In parallel with these results, incubation with SnPPIX prevented basal- and glucose-induced upregulation of NOS expression.

In order to further characterize the interaction between HO and NO systems, we treated cells with NOS inhibitor and investigated alteration of HO enzymes. Our results show that basal- and glucose-induced increased HO-1 mRNA expression was prevented by incubation of the cells with NO blocker L-NAME. This phenomenon also could be reversed by arginine (Fig. 2; $p < 0.05$). These data indicate that an important interaction may exist between the HO and NO systems, by which, these pathways may upregulate expression of each other. Furthermore, protein analysis of iNOS paralleled the mRNA expression data (Fig. 3).

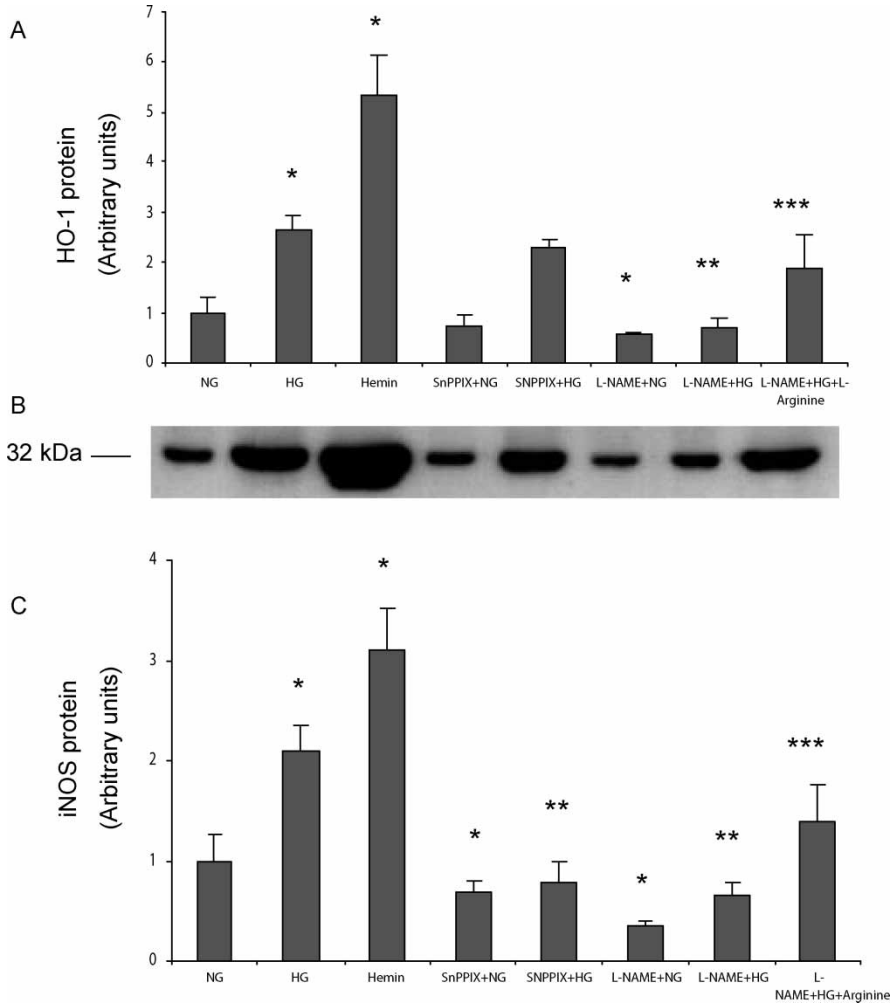

FIGURE 3 HO-1 and iNOS protein expression as assessed by western blotting, showing (A) quantification of HO-1 protein (B) representative HO-1 immunoblot and (C) quantification of iNOS protein from endothelial cells. NG = 5 mM glucose, HG = 25 mM glucose, Hemin = 10 μmol/l Hemin, SnPPIX = 10 μmol/l SnPPIX, L-NAME = 200 μmol/l L-NAME, and L-NAME + arginine = 200 μmol/l L-NAME + 1 mmol/l arginine; *$p < 0.05$ compared to NG, **$p < 0.05$ compared to HG; ***$p < 0.05$ compared to L-NAME; $n = 3$/treatment.

HO and NO Alteration in Mediating Cellular Damage

To investigate whether HO and NO alteration may mediate cellular damage, we measured oxidative stress using 8-OHdG and nitrotyrosine staining. 8-OhdG immunoreactivity measures DNA damage by reactive oxygen species. As expected, 25 mmol/l glucose caused significant increase in 8-OHdG positive cells (Fig. 5; $p < 0.05$). Hemin, the HO-inducer, and arginine also caused an increase in oxidative DNA damage. Both HO antagonist, SnPPIX, and NOS inhibitor, L-NAME, prevented glucose-induced DNA damage.

We further measured oxidative protein damage by nitrotyrosine staining. Incubation of the endothelial cells in 25 mmol/l glucose caused a significant increase in the nitrotyrosine positivity. Incubation with hemin, L-NAME, SnPPIX as well as arginine paralleled the changes seen by 8-OHdG staining (Fig. 5; $p < 0.05$). These data indicate that both HO and NO may cause cellular oxidative damage.

Glucose-induced HO Alteration may change other Vasoactive Molecules

As both carbon monoxide and NO have potent vasoactive functions, we studied the effect of HO upregulation in mediating alteration of other vasoactive factors which are upregulated by glucose. We targeted vasoconstrictor ET-1 and VEGF, the molecule of significant importance in glucose-induced increased vascular permeability and angiogenesis.[5,6] High glucose increased mRNA expression of both of these molecules (Fig. 6; $p < 0.05$). A similar result was obtained when cells

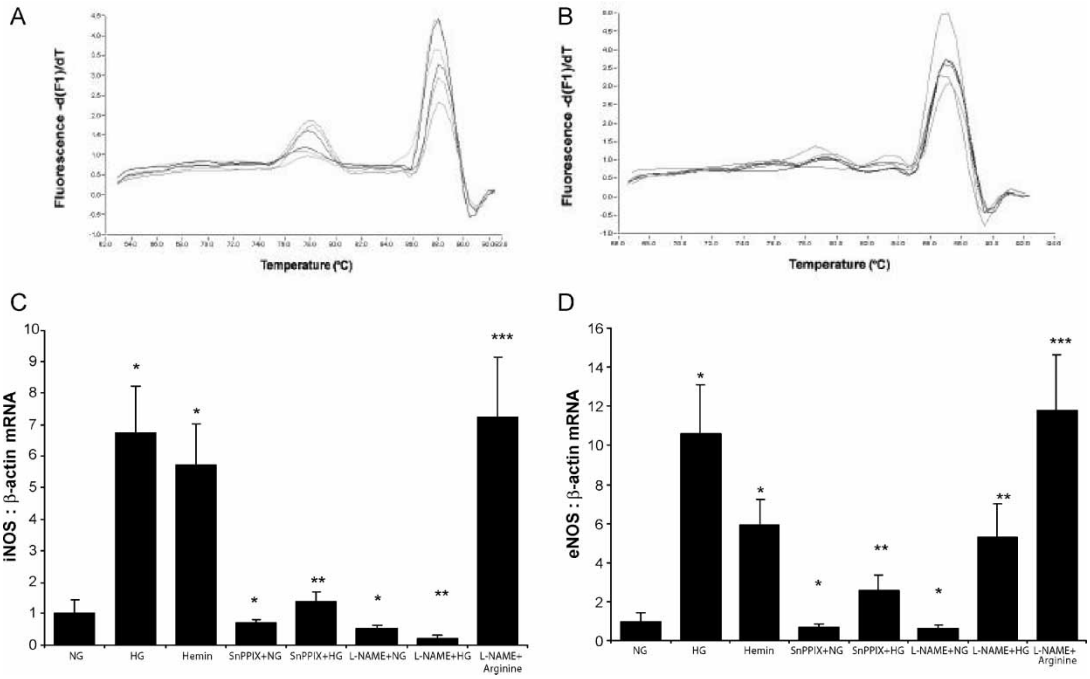

FIGURE 4 Quantification of NOS mRNA expression as assessed by real time RT-PCR, showing (A) melting curve analysis of iNOS RT-PCR, (B) melting curve analysis of eNOS RT-PCR, (C) quantification of iNOS mRNA, and (D) quantification of eNOS mRNA. [Each trace in (A and B) represents cDNA sample from endothelial cells; Melting curve analysis was used to determine specificity of PCR amplification; NOS isozyme transcripts were expressed as ratio of target mRNA to β-actin mRNA (relative to control); NG = 5 mM glucose, HG = 25 mM glucose, Hemin = 10 μmol/l hemin, SnPPIX = 10 μmol/l SnPPIX, L-NAME = 200 μmol/l L-NAME, and L-NAME+ arginine = 200 μmol/l L-NAME + 1 mmol/l arginine; *$p < 0.05$ compared to NG, **$p < 0.05$ compared to hemin; ***$p < 0.05$ compared to L-NAME; $n = 3$/treatment].

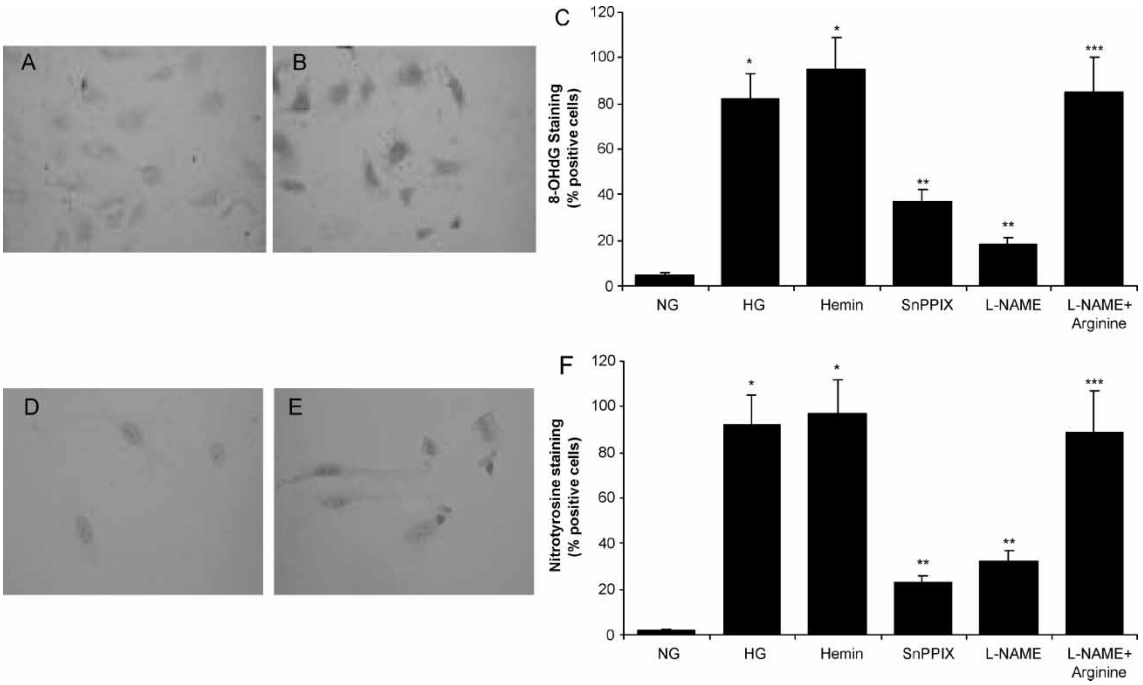

FIGURE 5 Oxidative DNA and protein damage in endothelial cells as assessed by 8-OHdG and nitrotyrosine immunoreactivity, showing (A) 8-OHdG positivity in NG, (B) 8-OHdG positivity in HG, and (C) semi-quantitative analysis of 8-OHdG, (D) nitrotyrosine positivity in NG, (E) nitrotyrosine positivity in HG, and (F) semiquantitative analysis of nitrotyrosine staining. [Original magnification 400X; NG = 5 mM glucose, HG = 25 mM glucose, Hemin = 10 μmol/l hemin, SnPPIX = 10 μmol/l SnPPIX, L-NAME = 200 μmol/l L-NAME, and L-NAME + arginine = 200 μmol/l L-NAME + 1 mmol/l arginine; *$p < 0.05$ compared to NG, **$p < 0.05$ compared to hemin; ***$p < 0.05$ compared to L-NAME; $n = 3$/treatment].

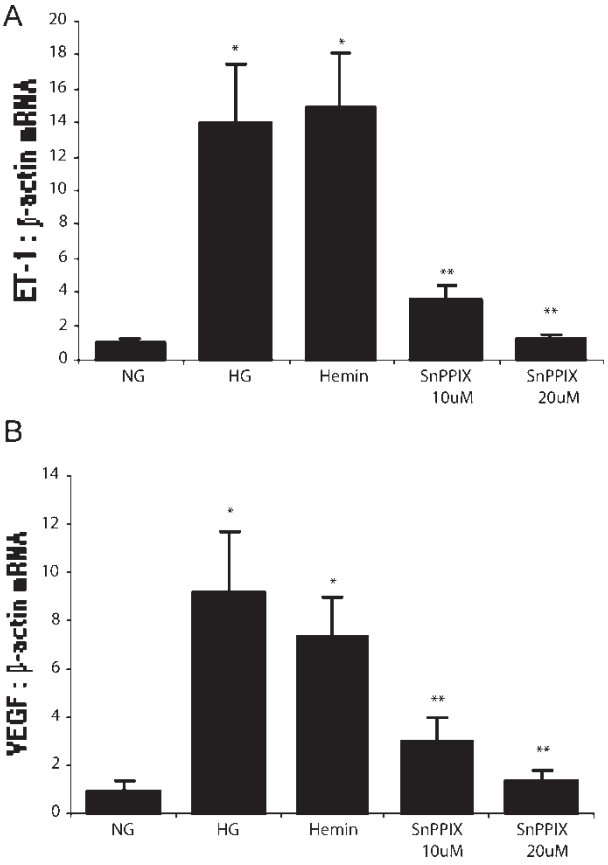

FIGURE 6 Quantification of ET-1 and VEGF mRNA expression as assessed by real time RT-PCR, showing (A) ET-1 transcript level, and (B) VEGF transcript levels. [Target transcripts were expressed as ratio of target mRNA to β-actin mRNA (relative to control); NG = 5 mM glucose, HG = 25 mM glucose, Hemin = 10 μmol/l hemin, SnPPIX = 10 μmol/l SnPPIX; *$p < 0.05$ compared to NG, **$p < 0.05$ compared to hemin; $n = 3$/treatment].

in 5 mmol/l glucose were incubated with HO inducer, hemin. Furthermore, glucose-induced upregulation of both ET-1 and VEGF was prevented by incubation of the cells with SnPPIX (Fig. 6; $p < 0.05$).

DISCUSSION

In this study, we have demonstrated that in the endothelial cells, glucose causes upegulation of HO - 1 expression and increases HO activity. Our results demonstrate that a co-stimulatory relationship between HO and NO pathway mediates oxidative damage in the endothelial cells and augmented expression of vasoactive factors such as ET-1 and VEGF.

HO system has generally been accepted to have a protective role in several disease processes.[28–30] The antioxidant effects are mediated by generation of carbon monoxide, biliverdin and its metabolite bilirubin, as well as sequesteration of redox active iron by ferritin.[31–33] However, HO exhibits both pro- and antioxidant activity depending on the redox potential and fate of iron.[34–36] We have previously demonstrated that diabetes-induced upregulation of HO in the heart may lead to increased oxidative stress, in part, via iron accumulation.[8] Previous studies have further shown increased expression of HO in glomerular cells of diabetic rat kidneys.[29] It has been demonstrated that administration of insulin and antioxidants in these animals normalizes HO upregulation. These findings provide evidence that diabetes-induced oxidative stress, which plays a pivotal role in late vascular complications, may be mediated via HO alteration. The present study confirms a possible pro-oxidant role of HO in the vascular endothelial cells. We have demonstrated that glucose causes increased expression and activity of the HO system and subsequent oxidative damage.

In the present study, we have also demonstrated an important interaction between the HO and the NO pathway which is of significance in chronic diabetic complications. We have shown that both glucose and HO inducer cause NOS upregulation which can be normalized by HO anatogonist. Furthermore, glucose-induced HO-1 upregulation was blocked by L-NAME. These data suggest that a co-stimulatory relationship exists between these two systems. As both CO and NO may act as vasodilators, an interaction between them is expected. In several other systems, such interactions have also been demonstrated.[16] We have also previously demonstrated that in the heart of diabetic rats, hyperglycemia-induced eNOS and iNOS upregulation can be prevented by SnPPIX.[8] It is, however, important to realize that gene upregulation does not necessarily reflect an increased enzyme activity. Inhibition of HO-1 upregulation by L-NAME also indicates regulation of HO-1 gene expression, at least in part, by NO in diabetes. These data are in accordance with other studies. However, a non-NO mediated regulation of HO-1 expression may also exist in diabetes.

Our studies have also, for the first time, shown that inhibiting the activity of HO prevents glucose-induced ET-1 and VEGF upregulation. Such regulatory interrelationship is demonstrated here in the context of diabetic complications. However in others systems, it has been demonstrated that HO inhibition may have an anti-angiogenic effect by reducing VEGF.[37,38] Carbon monoxide generated as a result of HO activity has been suggested to reduce vascular sensitivity and reactivity to ET-1.[39] However in our previous studies, we have shown that SnPPIX treatment ameliorates diabetes-induced retinal vasoconstriction via modulation of NOS without modifying retinal ET-1 mRNA expression. Hence, the present data suggest that interaction of HO system with other vasoactive factors may be tissue specific. Exact mechanism of HO-mediated alteration of other vasoactive factors

is not evident. HO system activation may lead to activation of redox sensitive transcription factor nuclear factor-κB (NFκB).[40] We have previously demonstrated NFκB activation by glucose in endothelial cells as well as in several target organs of diabetic complications.[20,23] Hence, HO-mediated NFκB activation and aleration of vasoactive factors such as ET-1 and NO is conceptually possible. However, other mechanisms may also exist, which require further investigation.

In summary, the present study has demonstrated that high glucose level may cause oxidative cellular damage via upregulation and increased activity of HO. Augmented HO-1 activity may have a co-stimulatory relationship with NO system and may, at least in part, regulate gene expression of other vasoactive factors, such as ET-1 and VEGF. Delineation of such intricate relationship is of importance to understand pathogenetic mechanisms of glucose-induced cellular damage.

Acknowledgements

Authors acknowledge grant supports from Canadian Institutes of Health Research (MOP 43841), and Lawson Health Research Institute.

References

[1] Giugliano, D., Ceriello, A. and Paolisso, G. (1995) "Diabetes mellitus, hypertension, and cardiovascular disease: which role for oxidative stress?", *Metabolism* **44**, 363–368.
[2] Mohamed, A.K., Bierhaus, A., Schiekofer, S., Tritschler, H., Ziegler, R. and Nawroth, P.P. (1999) "The role of oxidative stress and NF-kappaB activation in late diabetic complications", *Biofactors* **10**, 157–167.
[3] Pessin, J.E. and Bell, G.I. (1992) "Mammalian facilitative glucose transporter family: structure and molecular regulation", *Annu. Rev. Physiol.* **54**, 911–930.
[4] Brownlee, M. (2001) "Biochemistry and molecular cell biology of diabetic complications", *Nature* **414**, 813–820.
[5] Chakrabarti, S., Khan, Z.A., Cukiernik, M., Fukuda, G., Chen, S. and Mukherjee, S. (2002) "Alteration of endothelins: a common pathogenetic mechanism in chronic diabetic complications", *Int. J. Exp. Diabetes Res.* **3**, 217–231.
[6] Khan, Z.A. and Chakrabarti, S. (2003) "Endothelins in chronic diabetic complications", *Can. J. Physiol. Pharmacol.* **81**, 622–634.
[7] Cukiernik, M., Mukherjee, S., Downey, D. and Chakrabarti, S. (2003) "Heme oxygenase in the retina in diabetes", *Curr. Eye Res.* **27**, 301–308.
[8] Farhangkhoee, H., Khan, Z.A., Mukherjee, S., Cukiernik, M., Barbin, Y.P., Karmazyn, M. and Chakrabarti, S. (2003) "Heme oxygenase in diabetes-induced oxidative stress in the heart", *J. Mol. Cell Cardiol.* **35**, 1439–1448.
[9] Maines, M.D. (1988) "Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications", *FASEB J.* **2**, 2557–2568.
[10] McCoubrey, W.K., Huang, T.J. and Maines, M.D. (1997) "Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3", *Eur. J. Biochem.* **247**, 725–732.
[11] Buelow, R., Tullius, S.G. and Volk, H.D. (2001) "Protection of grafts by hemoxygenase-1 and its toxic product carbon monoxide", *Am. J. Transplant.* **1**, 313–315.
[12] Otterbein, L.E. and Choi, A.M. (2000) "Heme oxygenase: colors of defense against cellular stress", *Am. J. Physiol. Lung Cell Mol. Physiol.* **279**, L1029–L1037.
[13] Clark, J.E., Foresti, R., Sarathchandra, P., Kaur, H., Green, C.J. and Motterlini, R. (2000) "Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction", *Am. J. Physiol. Heart Circ. Physiol.* **278**, H643–H651.
[14] Fujita, T., Toda, K., Karimova, A., Yan, S.F., Naka, Y., Yet, S.F. and Pinsky, D.J. (2001) "Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis", *Nat. Med.* **7**, 598–604.
[15] Dennery, P.A., Spitz, D.R., Yang, G., Tatarov, A., Lee, C.S., Shegog, M.L. and Poss, K.D. (1998) "Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2", *J. Clin. Investig.* **101**, 1001–1011.
[16] Foresti, R. and Motterlini, R. (1999) "The heme oxygenase pathway and its interaction with nitric oxide in the control of cellular homeostasis", *Free Radic. Res.* **31**, 459–475.
[17] Takahashi, K., Hara, E., Suzuki, H., Sasano, H. and Shibahara, S. (1996) "Expression of heme oxygenase isozyme mRNAs in the human brain and induction of heme oxygenase-1 by nitric oxide donors", *J. Neurochem.* **67**, 482–489.
[18] Dulak, J., Jozkowicz, A., Foresti, R., Kasza, A., Frick, M., Huk, I., Green, C.J., Pachinger, O., Weidinger, F. and Motterlini, R. (2002) "Heme oxygenase activity modulates vascular endothelial growth factor synthesis in vascular smooth muscle cells", *Antioxid. Redox Signal.* **4**, 229–240.
[19] Chen, S., Apostolova, M.D., Cherian, M.G. and Chakrabarti, S. (2000) "Interaction of endothelin-1 with vasoactive factors in mediating glucose-induced increased permeability in endothelial cells", *Lab. Investig.* **80**, 1311–1321.
[20] Chen, S., Mukherjee, S., Chakraborty, C. and Chakrabarti, S. (2002) "High glucose-induced, endothelin-dependent fibronectin synthesis is mediated via NF-kappa B and AP-1", *Am. J. Physiol. Cell Physiol.* **284**, C263–C272.
[21] Evans, T., Deng, D.X., Chen, S. and Chakrabarti, S. (2000) "Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement membrane thickening in the retina of diabetic and galactose-fed rats", *Diabetes* **49**, 662–666.
[22] Khan, Z.A., Cukiernik, M., Gonder, J.R. and Chakrabarti, S. (2004) "Oncofetal fibronectin in diabetic retinopathy", *Investig. Ophthalmol. Vis. Sci.* **45**, 287–295.
[23] Chen, S., Khan, Z.A., Cukiernik, M. and Chakrabarti, S. (2003) "Differential activation of NF-kappa B and AP-1 in increased fibronectin synthesis in target organs of diabetic complications", *Am. J. Physiol. Endocrinol. Metab.* **284**, E1089–E1097.
[24] McLean, M., Bowman, M., Clifton, V., Smith, R. and Grossman, A.B. (2000) "Expression of the heme oxygenase-carbon monoxide signalling system in human placenta", *J. Clin. Endocrinol. Metab.* **85**, 2345–2349.
[25] Ryter, S.W., Kvam, E. and Tyrrell, R.M. (2000) "Heme oxygenase activity. Current methods and applications", *Methods Mol. Biol.* **99**, 369–391.
[26] Yarborough, A., Zhang, Y.J., Hsu, T.M. and Santella, R.M. (1996) "Immunoperoxidase detection of 8-hydroxydeoxyguanosine in aflatoxin B1-treated rat liver and human oral mucosal cells", *Cancer Res.* **56**, 683–688.
[27] Sardana, M.K. and Kappas, A. (1987) "Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver", *Proc. Natl Acad. Sci. USA* **84**, 2464–2468.
[28] Cosso, L., Maineri, E.P., Traverso, N., Rosatto, N., Pronzato, M.A., Cottalasso, D., Marinari, U.M. and Odetti, P. (2001) "Induction of heme oxygenase 1 in liver of spontaneously diabetic rats", *Free Radic. Res.* **34**, 189–191.
[29] Hayashi, K., Haneda, M., Koya, D., Maeda, S., Isshiki, K. and Kikkawa, R. (2001) "Enhancement of glomerular heme oxygenase-1 expression in diabetic rats", *Diabetes Res. Clin. Pract.* **52**, 85–96.
[30] Hammes, H.P., Bartmann, A., Engel, L. and Wulfroth, P. (1997) "Antioxidant treatment of experimental diabetic retinopathy in rats with nicanartine", *Diabetologia* **40**, 629–634.
[31] Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. and Ames, B.N. (1987) "Bilirubin is an antioxidant of possible physiological importance", *Science* **235**, 1043–1046.

[32] Stocker, R. and Ames, B.N. (1987) "Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in bile", *Proc. Natl Acad. Sci. USA* **84**, 8130–8134.
[33] Balla, G., Jacob, H.S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J.W. and Vercellotti, G.M. (1992) "Ferritin: a cytoprotective antioxidant strategem of endothelium", *J. Biol. Chem.* **267**, 18148–18153.
[34] Noguchi, M., Yoshida, T. and Kikuchi, G.A. (1983) "Stoichiometric study of heme degradation catalyzed by reconstituted heme oxygenase system with special consideration of the production of hydrogen peroxide during the reaction", *J. Biochem.* **93**, 1027–1036.
[35] Cadenas, E. (1989) "Biochemistry of oxygen toxicity", *Ann. Rev. Biochem.* **58**, 79–110.
[36] Aust, S.D., Morehouse, S.A. and Tomas, C.E. (1985) "Role of metals in oxygen radical reactions", *Free Radic. Biol. Med.* **1**, 3–25.
[37] Bussolati, B., Ahmed, A., Pemberton, H., Landis, R.C., Di Carlo, F., Haskard, D.O. and Mason, J.C. (2003) "Bifunctional role for VEGF-induced heme oxygenase-1 *in vivo*: induction of angiogenesis and inhibition of leukocytic infiltration", *Blood* **103**, 761–766.
[38] Jozkowicz, A., Huk, I., Nigisch, A., Weigel, G., Dietrich, W., Motterlini, R. and Dulak, J. (2003) "Heme oxygenase and angiogenic activity of endothelial cells: stimulation by carbon monoxide and inhibition by tin protoporphyrin-IX", *Antioxid. Redox Signal* **5**, 155–162.
[39] Zhang, F., Kaide, J.I., Yang, L., Jiang, H., Quan, S., Kemp, R., Gong, W., Balazy, M., Abraham, N.G. and Nasjletti, A. (2003) "CO modulates pulmonary vascular response to acute hypoxia: relation to endothelin", *Am. J. Physiol. Heart Circ. Physiol.* **286**, H137–H144.
[40] Nishio, Y., Kashiwagi, A., Taki, H., Shinozaki, K., Maeno, Y., Kojima, H., Maegawa, H., Haneda, M., Hidaka, H., Yasuda, H., Horrike, K. and Kikkawa, R. (1998) "Altered activities of transcription factors and their related gene expression in cardiac tissues of diabetic rats", *Diabetes* **47**, 1318–1325.