Unconjugated bilirubin mediates heme oxygenase-1-induced vascular benefits in diabetic mice

Jian Liu¹, Li Wang¹, Xiaoyu Tian¹, Limei Liu², Wing-Tak Wong³, Yang Zhang¹, Quanbin Han⁴, Hing-Man Ho⁴, Nanping Wang⁵, Siu-Ling Wong¹, Zhenyu Chen⁶, Jun Yu⁷, Chi-Fai Ng⁸, Xiaoqiang Yao¹, Yu Huang¹,*

¹Institute of Vascular Medicine and Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong; ²Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing, China; ³Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, Texas; ⁴School of Chinese Medicine, Hong Kong Baptist University; ⁵Institute of Cardiovascular Science, Peking University, Beijing, China; ⁶School of Life Sciences, ⁷Department of Medicine and Therapeutics and ⁸Department of Surgery, Chinese University of Hong Kong, China;

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*Addressed to: Yu Huang, School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong SAR, China. Tel: (852)39436787; e-mail: yu-huang@cuhk.edu.hk

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Abstract
Heme oxygenase-1 (HO-1) exerts vaso-protective effects. Such benefit in diabetic vasculopathy however remains unclear. We hypothesize that bilirubin mediates HO-1-induced vascular benefits in diabetes. Diabetic \( db/db \) mice were treated with hemin (HO-1 inducer) for two weeks, and aortas were isolated for functional and molecular assays. Nitric oxide (NO) production was measured in cultured endothelial cells. Hemin treatment augmented endothelium-dependent relaxations (EDRs) and elevated Akt and eNOS phosphorylation in \( db/db \) mouse aortas, which were reversed by HO-1 inhibitor SnMP or HO-1 silencing virus. Hemin treatment increased serum bilirubin and \textit{ex vivo} bilirubin treatment improved relaxations in diabetic mouse aortas, which was reversed by Akt inhibitor. Biliverdin reductase silencing virus attenuated the effect of hemin. Chronic bilirubin treatment improved EDRs in \( db/db \) mouse aortas. High glucose-induced reductions in Akt and eNOS phosphorylation, and NO production were reversed by hemin and bilirubin. The effect of hemin but not bilirubin was inhibited by biliverdin reductase shRNA. Furthermore, bilirubin augmented EDRs in renal arteries from diabetic patients. In summary, HO-1-induced restoration of endothelial function in diabetic mice is most likely mediated by bilirubin which preserves NO bioavailability through the Akt/eNOS/NO cascade, suggesting bilirubin as a potential therapeutic target for clinical intervention of diabetic vasculopathy.
Cardiovascular disease (CVD) is the leading cause of death and disability for patients with diabetes (1). Hyperglycemia and insulin resistance reduce NO bioavailability and diminish the anti-atherogenic capacity of the endothelium, resulting in platelet aggregation, increased vascular contractility, and accelerated atherosclerosis. Thus, strategies aim at preserving endothelial function may help alleviate diabetic vasculopathy (2).

Heme oxygenase (HO) catalyzes heme to form carbon monoxide (CO), Fe$^{2+}$ and biliverdin; the latter is converted into unconjugated bilirubin by biliverdin reductase (BVR) (3). Two distinct isoforms of HO have been identified: heme oxygenase 1 (HO-1) and heme oxygenase 2 (HO-2). While HO-2 is constitutively expressed, the expression of HO-1 is normally low but inducible. HO-1 can be up-regulated by stimuli such as lipopolysaccharide and H$_2$O$_2$ in various cells or organs (4; 5). Limited studies show an altered HO-1 expression under metabolic conditions. HO-1 expression and activity is increased in human umbilical vein endothelial cells (HUVECs) when exposed to 10 mM glucose for 48 hours compared to 5.5 mM glucose, but remains unchanged when exposed to 20 mM glucose for 48 hours (6). HO-1 mRNA is down-regulated in skeletal muscle of diabetic patients, but it is increased in liver and visceral fat of obese patients (7; 8), suggesting that HO-1 expression can be differently regulated in major metabolic organs under different disease states. Growing evidence suggests HO-1 as a potential target for intervention of diabetes. Induction of HO-1 in obese mice increases plasma adiponectin and improves insulin sensitivity; while it decreases visceral and abdominal adiposity and plasma pro-inflammatory cytokines (9). HO-1 over-expression reduces lymphocytic infiltration in Langerhans islets and retards the progression of
type 1 diabetes (10). HO-1 also protects against high glucose-induced impairment of endothelial-dependent relaxations (EDRs) in rat aortas (11) and high glucose-induced endothelial cell apoptosis (6; 12). These studies indicate that HO-1 induction may serve as a potential therapeutic strategy to treat diabetic vascular complications. However, the precise intracellular mechanisms mediating the vaso-protective benefits of HO-1 remain largely unexplored.

Bilirubin is converted from biliverdin by BVR. Clinical studies show an inverse association between serum bilirubin concentration with incidences of myocardial infarction, peripheral artery disease, and stroke (13-16) and serum bilirubin level is lower in diabetic patients (17). Of note, Gilbert syndrome patients, that with higher serum unconjugated bilirubin, are less prone to major adverse cardiovascular events (18) and individuals with higher serum bilirubin are better protected from developing metabolic disorders in a 4-year retrospective longitudinal study (19). Bilirubin is a potent antioxidant and efforts have been directed to determine whether bilirubin can be used to treat diseases associated with oxidative stress. Indeed, atazanavir, a drug that raises unconjugated bilirubin levels, enhances the plasma antioxidant capacity and improves EDRs in diabetic patients (20).

This study examined the hypothesis that unconjugated bilirubin mediates vascular benefits of HO-1 induction to restore the impaired endothelial function in diabetic db/db mice and investigated the possible mechanisms involved. The present new findings support the clinical observation that bilirubin is vaso-protective in diabetes.
RESEARCH DESIGN AND METHODS

Animals and reagents

Animal care and experimental protocol were approved by Animal Research Ethics Committee, Chinese University of Hong Kong (CUHK) in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication no.85–23, revised 1996). Twelve-week-old male diabetic \( db/db \) mice on a C57BL/KsJ background and non-diabetic littermates \( db/m^+ \) mice were supplied by CUHK Animal Service Center, kept in a temperature-controlled room (\( \sim 23^\circ C \)) with a 12-h light/dark cycle, and fed a standard diet and water \textit{ad libitum}. \( db/db \) mice were treated with: vehicle, hemin [HO-1 inducer, 25 mg/kg body weight (BW), 3 times weekly, i.p.], hemin+SnMP (HO-1 inhibitor, 20 mg/kg BW, 3 times weekly, i.p.), hemin+scramble virus \( (10^9 \text{ pfu}) \), hemin+HO-1 shRNA virus \( (10^9 \text{ pfu}) \), bilirubin (5 mg/kg BW, 3 times weekly, i.p.). \( db/m^+ \) mice were treated with: vehicle and hemin (25 mg/kg BW, 3 times weekly, i.p.).

Diet-induced obese (DIO) mice were generated by feeding 6-week old C57BL/6J mice with high fat diet (Rodent diet with 45% kcal% fat, D12451, Research Diets Inc. New Brunswick, NJ, USA) for 10 weeks, and then treated with hemin or vehicle for two weeks (25 mg/kg BW, 3 times weekly, i.p.).

SnMP was purchased from Frontier Scientific (Utah, USA) and all other reagents were from Sigma (St Louis, MO, USA) unless otherwise stated.
Vessel preparation

After mice were sacrificed, thoracic aortas were removed, placed in ice-cold Krebs solution (in mmol/L: 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 D-glucose), and cut into ring segments. Changes in isometric tension in aortic rings were recorded by Multi Myograph System (Danish Myo Technology A/S, Denmark) (21). The baseline tension was set at 3 mN and all rings were equilibrated for 60 min was before the start of the experiments.

Functional study

Aortic rings were contracted by 1 µmol/L phenylephrine to induce a sustained tension before acetylcholine (ACh, 10⁻⁸ to 10⁻⁵ mol/L) was added cumulatively to trigger endothelium-dependent relaxations. Endothelium-independent relaxations induced by NO donor sodium nitroprusside (SNP, 10⁻⁸ to 10⁻⁵ mol/L) were compared in arteries from different groups.

Organ culture of aortic rings

Mouse aortas were cultured for 24-h at 37°C in Dulbeco’s Modified Eagle’s Medium (DMEM, Gibco, Gaithersberg, MD, USA) supplemented with 10% FBS (Gibco) and 100 IU/ml penicillin plus 100 µg/ml streptomycin as described (22). The db/db mouse aortas were treated with 5 µmol/L hemin or 1 µmol/L unconjugated bilirubin, in control and in the presence of 30 µmol/L SnMP or 5 µmol/L Akt inhibitor V.

For ex vivo viral transduction, mouse aortas were cultured with 10⁹ pfu BVR shRNA or GFP
shRNA adenovirus for 24-h and then treated with hemin or unconjugated bilirubin for another 24-h.

**Human artery specimen**

The use of human specimen was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Human renal arteries were obtained after informed consent from patients without cardio-metabolic complications and diabetic patients undergoing nephrectomy due to neoplasm at ages between 50 and 80 years. Diabetes is defined as having fasting plasma glucose level ≥7.0 mmol/L in patients.

**Oral glucose tolerance test and intra-peritoneal insulin tolerance test**

Mice were loaded with glucose (1.2 g/kg BW) for oral glucose tolerance test after 8-h fasting. For insulin tolerance test, mice were injected with insulin at 0.75 U/kg BW after 2-h fasting. Blood glucose was measured at 0, 15, 30, 60 and 120 min with glucometer (Ascensia ELITE®, Bayer, Mishawaka, IN).

**Plasma insulin level and lipid profile**

Plasma insulin levels were assayed by enzyme immunoassay (Mercodia, Sweden). Plasma levels of total cholesterol, triglyceride, high-density lipoprotein (HDL) and non-HDL were determined using enzymatic methods (Stanbio, Boerne, TX).
Liquid chromatography mass spectrometry measurement of unconjugated bilirubin

400 µl of 60% (v/v) acetonitrile in 0.01 M-phosphate buffer (pH 8.0) was added to 100 µl serum or culture medium, vortexed for 30 s, and centrifuged for 5 min at 1000 rpm (23). 100 µl of supernatant was applied on the Agilent 6460 Triple Quadrupole Mass Spectrometer with UHPLC (UHPLC–MS/MS, Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was performed on a ACQUITY UPLC® BEH C18 column (2.1mm×100mm, ID, 1.7µm particle size) (Waters, Milford, MA, USA) at 4 ºC with the mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) by a gradient elution method (55 to 100% of B from 0 to 12.5 min, 100% of B from 12.5 to 13 min, 55% of B from 13.1 to 16 min). The injection volume was 3 µl and the flow rate was 0.4 mL/min. The elution was directed to the mass spectrometer without splitting. The temperature of the auto-sampler was set at 4 ºC throughout the analysis. The mass spectrometer equipped with an ion source of ESI (Agilent Jet Stream) in negative ion mode was used for bilirubin detection. The source parameters were set as follows: gas temperature, 300 ºC; gas flow, 8 L/min; nebulizer: 30 psi; sheath gas temperature: 350 ºC; sheath gas flow: 10 L/min; capillary voltage: 3500 V, and nozzle voltage: 1000 V. The MS recordings were carried out in multiple reactions monitoring (MRM) mode. Nitrogen was used as the collision gas, the monitor ion and collision energy were m/z 583.2 → 285.2 and 20eV, respectively.
Measurement of nitrite in mouse aortas

Mouse aortas were treated with hemin (5 µmol/L) or bilirubin (1 µmol/L) for 24 h, followed by addition of 10 µmol/L acetylcholine (10 min) to stimulate NO generation with the presence of nitrate reductase to reduce nitrate to nitrite. Aortas were then homogenized and nitrite level in the supernatants was measured by a Griess reagent kit (Molecular Probes, Eugene, OR, USA) and normalized by the protein content.

Construction of HO-1 shRNA adeno-associated virus

Short hairpin (sh)RNA sequence targeting mouse HO-1 was obtained from sigma (TRCN0000234077). The 5’-GATCCACA GTGGCAGTGGAATTTATCTCGAGATAAATTCC CACTGCCACTGGTTTTTA-3’ and 5’-AGCTTAAAAAACAGTGGCAGTGGGAATTTATCTCG AGATAAATTCACCACACTGCTG-3’ were annealed and cloned into the pAAV-ZsGreen-shRNA (YRGene) shuttle vector to construct pAAV-ZsGreen-HO-1 shRNA plasmid, and then it was co-transfected into HEK-293T with RGDLRV5-AAV9 cap plasmid (a gift of Dr. OJ Müller, University Hospital Heidelberg, Germany) (24) and pHelper (Stratagene). AAV viral particles were harvested as reported (25). Mice were injected with 10⁹ pfu HO-1 shRNA or scramble (empty vector) virus via tail vein. The knockdown efficiencies were confirmed by Western blotting.

Knockdown of biliverdin reductase by adenoviral shRNA transduction

The U6 promoter and 1.9kb stuffer sequence were excised from pLKO.1 (addgene) with NotI/Hol
and cloned into pShuttle. shRNA targeting mouse BLVRA (sigma, TRCN0000042128) was generated similarly to the pLKO.1 system (26). Briefly, the forward (CCGGGCAAATGTAGGA GTCAATAACTCGAGTTATTGACTCCTACATTTGGCTTTTG) and reverse (TCGAGAAAAA GCAAAATGTAGGAGTCAATAACTCGAGTTATTGACTCCTACATTTGGC) oligos were annealed and ligated to pShuttle-U6 predigested with AgeI and XhoI. The pAd-U6-shBLVRA plasmid was produced as reported previously (27) and linearized by PacI and transfected to Hek-293 cell to produce the viral particle.

**Overexpression of HO-1 by adenoviral transduction**

db/db mice were injected with HO-1 overexpressing adenovirus (10⁹ pfu, a gift from Jun Yu, Prince of Wales Hospital in Hong Kong) through tail vein and kept for 4 days before sacrificed.

**Knockdown of Akt in HUVECs**

HUVECs were purchased from Lonza (San Diego, CA) and cultured in Endothelial Cell Growth Medium (EGM, Lonza) supplemented with 10 % FBS plus 1 % penicillin/streptomycin. HUVECs were transfected with a dominant negative Akt construct (DN-Akt) by electroporation using Nucleofector II machine (Amaxa/Lonza, Walkersville, MD, USA) according to the manufacturer’s instruction.
Measurement of NO and ROS generation in HUVECs

NO production in HUVECs was measured by Olympus Fluoview FV1000 laser scanning confocal system using 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-DA, Invitrogen) as indicator. The amount of NO produced in response to A23187 (100 nmol/L) was evaluated by measuring fluorescence intensity at excitation 488 nm and emission 515 nm. Changes in [NO]i were displayed as a ratio of fluorescence intensity after and before the addition of A23187 (F1/F0). For ROS measurement, HUVECs were incubated for 30 min in 5 µmol/L CM-H2DCFDA (Invitrogen) and measured using Fluoview FV1000 confocal system at excitation 488 nm and emission 520 nm.

To detect NO mediated antioxidant capacity of bilirubin, NO scavenger oxidized-hemoglobin (20 µmol/L) was used together with bilirubin in HG-treated HUVECs. HUVECs were incubated with 5 µmol/L dihydroethidium (DHE, Invitrogen) for 30 min and fluorescence intensity was measured using Fluoview FV1000 confocal system at excitation 515 nm and emission 585 nm.

Western blotting

Protein lysates from mouse aortas or HUVECs were separated by electrophoresis and then transferred onto an immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA, USA). The blots were then blocked with 1% BSA in 0.05% Tween-20 PBS for 1-h and incubated with primary antibodies overnight at 4°C, including: polyclonal anti-eNOS (1:500, Abcam, Cambridge, UK), anti-phospho-eNOS Ser1177 (1:1000, Abcam), polyclonal anti-HO-1 (1:1000, Assay Designs, Michigan, USA), monoclonal anti-phospho-Akt Thr308 (1:1000, Cell
Signaling, Danvers, MA, USA), monoclonal anti-Akt1 (1:1000, Cell Signaling), and polyclonal anti-IRS1 (1:1000, Cell Signaling). After washing, blots were incubated with horseradish peroxidase (HRP) -conjugated secondary antibody (DakoCytomation, Carpinteria, CA, USA). Protein expression was normalized by monoclonal anti-GAPDH (1:10000, Ambion, Texas, USA). Protein expression was determined by a densitometer (Flurochem, Alpha Innotech Corp., San Leandro, CA, USA).

**Immunohistochemical staining of biliverdin reductase**

Human renal arteries were frozen in OCT compound (Sakura Finetek, CA, U.S.A), cut into 10 µm-sections on a microtome (Leica Microsystems, Germany) and fixed with 4% paraformaldehyde. Sections were washed in PBS and blocked in 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA, USA), and incubated with anti-biliverdin reductase antibody (1:200, Enzo life sciences, USA) or PBS overnight at 4 ºC. Biotin-conjugated goat anti-rabbit secondary antibodies (1:500, Jackson Immunoresearch), streptavidin-HRP conjugate (1:500, Zymed laboratory, San Francisco, CA, USA), and DAB substrate (Vector laboratory, Burlingame, CA, USA) were used to visualize positive staining. Pictures were taken under Nicon TI-S microscope.

**Statistical Analysis**

Results are means±SEM of n experiments. The relaxation was expressed as percentage reduction in phenylephrine-induced contraction. Concentration-response curves were constructed using
GraphPad Prism software (Version 4.0, San Diego, CA, USA). Statistical significance was determined by two-tailed Student’s t-test or one-way ANOVA followed by Bonferroni post-hoc tests when more than two treatments were compared. p<0.05 indicates statistical significance.

RESULTS

HO-1 induction restored EDRs in diabetic mice

Acetylcholine (ACh)-induced EDRs were impaired in aortas of diabetic db/db mice compared with those from db/m+ mice. Two-week hemin treatment restored EDRs in db/db mouse aortas, which were reversed by co-treatment with HO-1 inhibitor SnMP (Fig. 1A). By contrast, SNP-induced endothelium-independent relaxations were similar in all groups (Supplemental Fig. 1). Hemin treatment induced ~3 fold increase of HO-1 expression in aortas from both db/db and db/m+ mice, which was unaffected by SnMP (Fig. 1B). Ex vivo treatment with hemin (5 µmol/L, 24-h) or tail vein injection of HO-1-overexpressing adenovirus (4 days) also improved EDRs in db/db mouse aortas (Fig. 1C&D) and elevated HO-1 expression (Supplemental Fig. 2A&B); the effect of hemin was again reversed by co-treatment with 30 µmol/L SnMP (Fig. 1C). To confirm that HO-1 mediates vascular benefits of hemin in db/db mice, HO-1 shRNA adeno-associated virus (AAV) was constructed and injected into db/db mice via tail vein and these mice were treated with hemin for 2 weeks. Compared with scramble virus, HO-1 shRNA AAV reversed the effect of hemin on EDRs and HO-1 expression (Fig. 1E&F). In addition, we also used DIO mice to confirm the findings in db/db mice. Hemin administration for two weeks augmented EDRs and HO-1
expression in DIO mouse aortas (Supplemental Fig. 3A&B), suggesting HO-1 is most likely to mediate hemin-induced improvement of EDRs in diabetic mice.

**PI3K/Akt/eNOS contributed to vascular benefits of HO-1 induction in diabetic mice**

The phosphorylation levels of Akt (Thr308) and eNOS (Ser1177) in aortas were lower in db/db mice than db/m+ mice. *In vivo* hemin treatment restored the diminished phosphorylation of Akt and eNOS and such effects were reversed by co-treatment with SnMP (Fig. 2A&B). Chronic hemin treatment also increased phosphorylation of Akt and eNOS in DIO mouse aortas (Supplemental Fig. 3C&D). HO-1 shRNA AAV transduction inhibited hemin-stimulated phosphorylation of Akt and eNOS (Fig. 2C-E). The hemin-improved EDRs were reversed by PI3K inhibitor wortmannin (100 nmol/L) or Akt inhibitor V (5 µmol/L) (Fig. 2F). However, neither inhibitor affected ACh-induced relaxations of db/m+ mouse aortas (data not shown).

**Bilirubin improved EDRs in db/db mouse aortas**

The concentration of serum unconjugated bilirubin was 77.5 ± 7.8 nmol/L in db/db and 350.0 ± 3.9 nmol/L in db/m+ mice. Hemin treatment increased bilirubin to 201.4 ± 13.6 nmol/L, which was inhibited by SnMP (Fig. 3A). *Ex vivo* bilirubin treatment (1 µmol/L, 24-h) improved EDRs in db/db mouse aortas, and such effect was inhibited by co-incubation with Akt inhibitor V (5 µmol/L) but not by SnMP (30 µmol/L) (Fig. 3B). To investigate whether bilirubin mediated the beneficial effect of HO-1, a biliverdin reductase (BVR) shRNA adenovirus was constructed. The vascular benefit of
hemin (Fig. 3C) but not that of bilirubin (Fig. 3D) was reversed by BVR shRNA. Successful inhibition of BVR expression by shRNA adenovirus was shown by Western blotting (Fig. 3E) and immunohistochemical staining (Fig. 3F). To confirm the role of bilirubin to mediate the vascular benefit of HO-1 in vivo, we treated db/db mice with bilirubin for 2 weeks. Chronic bilirubin administration improved EDRs in db/db mouse aortas (Fig. 4A) accompanied with increased phosphorylation of Akt (Thr308) and eNOS (Ser1177) (Fig. 4B-D). These results further indicate that bilirubin is most likely to mediate the effect of HO-1 induction to improve endothelial function.

**Hemin and unconjugated bilirubin restored NO production in HUVECs**

High glucose (HG, 36-h) reduced Ca^{2+} ionophore A23187 (100 nmol/L)-stimulated NO elevation detected by DAF-DA in HUVECs, compared with normal glucose (Fig. 5A&B). Hemin or bilirubin restored the HG-impaired NO production (Fig. 5A, B&C). Ex vivo treatment with hemin and bilirubin also raised nitrite level (which reflects tissue NO level) in db/db mouse aortas (Supplemental Fig. 4). Akt inhibitor V (5 µmol/L) abolished the effect of hemin and bilirubin while SnMP only inhibited the effect of hemin on NO production (Fig. 5B&C). Inhibition of Akt activity by Ad-DN-Akt, the plasmid expressing dominant negative Akt abolished the effect of both hemin and bilirubin (Fig. 5D). In addition, treatment with hemin or bilirubin increased phosphorylation of Akt and eNOS in HG-treated HUVECs. Again, the effect of hemin was abolished by SnMP while the effect of hemin and bilirubin was reversed by Akt inhibitor V (Fig. 5E-H).
Down-regulation of biliverdin reductase diminished the effect of hemin in HUVECs

GFP shRNA adenovirus served as the control virus. Hemin increased bilirubin level in medium culturing HUVECs, which was diminished by BVR shRNA adenovirus (Supplemental Fig. 5A). The stimulatory effect of hemin on Akt and eNOS phosphorylation in high glucose-treated HUVECs was reversed by BVR shRNA adenovirus (Fig. 6A-C). Likewise, hemin-stimulated NO production in HUVECs was inhibited by BVR shRNA adenovirus (Supplemental Fig. 5B&C). By contrast, the effect of bilirubin remained unchanged by BVR shRNA (Fig. 6D-F; Supplemental Fig. 5B&C).

Bilirubin improved EDRs in renal arteries from diabetic patients.

The HbA1c concentrations in diabetic patients are presented in Supplemental Table 1. Compared to renal arteries form patients without cardio-metabolic complications (control), EDRs in renal arteries from diabetic patients (DM) were impaired, which were augmented after 24-h exposure to bilirubin (1 µmol/L) (Fig. 7A&B). BVR expression was detected in the endothelium in human renal arteries by immunohistochemistry (Fig. 7C).
DISCUSSION

Although HO-1 is known to benefit vascular function, whether such beneficial effects were directly or indirectly induced by HO-1 induction remains poorly understood. HO-1 degrades heme to form biliverdin, CO, and Fe$^{2+}$; the former is further converted into unconjugated bilirubin. Bilirubin is increasingly recognized to be vaso-protective in cardio-metabolic diseases (18; 20) and total plasma bilirubin concentration is lower in diabetic patients (17). The present study also shows a reduced serum level of unconjugated bilirubin in db/db mice, which is increased following two-week hemin treatment. Importantly, ex vivo treatment with unconjugated bilirubin (at 1 µM, a concentration comparable to that detected in the serum of non-diabetic mice) and two-week administration of bilirubin (i.p.) to db/db mice resulted in similar vascular benefits as hemin to restore EDRs. More definitely, silencing BVR using shRNA adenovirus reverses ex vivo hemin-induced improvement of EDRs in db/db mouse aortas and hemin-stimulated NO production in HUVECs. By contrast, the beneficial effects of bilirubin were unaffected by BVR shRNA. Taken together, bilirubin is the most likely mediator of the vaso-protective action of HO-1 induction. Likewise, bilirubin is also reported to mediate the anti-atherogenic action of HO-1 through inhibiting vascular inflammation (28).

The present study provides the first piece of evidence showing that ex vivo treatment with bilirubin improves EDRs in renal arteries from diabetic patients, further suggesting that bilirubin is vaso-protective in human. It should be noted that the diabetic patients were with renal carcinoma and had different duration of diabetes and HbA1c levels (Supplemental Table 1); this may influence renal vascular responses to bilirubin. And also, in the future it would be important to investigate
whether bilirubin produces similar vascular benefit in other arteries such as coronary arteries, carotid arteries and femoral arteries, which are more clinically relevant.

The PI3K/Akt signaling is important to preserve endothelial function in diabetic mice by increasing eNOS phosphorylation at Ser\textsuperscript{1177} (22) and endothelial progenitor cell regenerative capacity (29; 30). The present study reveals a critical role of Akt signaling in mediating vascular benefits of both hemin and bilirubin, as inhibition of Akt activity abolishes the beneficial effects of hemin and bilirubin to improve EDRs in db/db mice and restores the impaired NO production in high glucose-treated HUVECs.

The biological action of bilirubin has long been considered due to its anti-oxidant property (31; 32). Bilirubin scavenges peroxyl radicals and reduces lipid peroxidation \textit{in vitro} (33); it down-regulates NAD(P)H oxidase activity and protects against diabetic nephropathy in rodents \textit{in vivo} (34). Bilirubin was also shown to mediate the effect of HO-1 to reduce reactive oxygen and nitrogen species in lipopolysaccharide-treated HUVECs (35). Reduced ROS contribute to the improved EDRs. Therefore, the antioxidant activity of bilirubin may partially accounts for its vascular benefits. The present study shows that bilirubin also increases NO production. NO can interact with superoxide anions to lower ROS levels. Our results show that bilirubin inhibits high glucose-stimulated ROS generation in HUVECs, which was attenuated by Akt inhibitor V and NO scavenger oxidized-hemoglobin (Supplemental Fig. 6), suggesting that increased NO is likely to mediate a significant part of the effect of bilirubin to reduce ROS in endothelial cells. Thus, the
increased NO bioavailability may directly contribute to bilirubin-induced improvement in EDRs and also indirectly by reducing ROS.

CO, one of the metabolic products of heme degradation, is reported to affect vascular reactivity, causing either endothelium-independent relaxations (36) or contractions (37). Most studies show that CO inhibits NO-mediated vasodilatation or NO production in endothelial cells (38; 39). Unlike hemin or bilirubin, *ex vivo* treatment with CO-releasing molecule tricarbonyl-dichloro-ruthenium (II) dimmer does not improve EDRs in *db/db* mouse aortas (Supplemental Fig. 7). Taken together with results from experiments using BVR shRNA virus and chronic bilirubin treatment, the present results suggest a minimal involvement of CO in hemin-induced endothelial protection in diabetic mice.

It is worthwhile to note that HO-1 induction *in vivo* moderately reduces fasting glucose and insulin levels in *db/db* mice and also improved insulin sensitivity (Supplemental Fig. 8). Previous studies showed that HO-1 induction improves insulin sensitivity and reduces adiposity, which is associated with increased adiponectin release (9; 40). Thus, the metabolic benefit is likely to contribute albeit to a lesser degree to the restoration of endothelial function in hemin-treated diabetic mice through HO-1 up-regulation in adipose tissues (40; 41). Although the role of vascular HO-1 induction in improved insulin sensitivity cannot be ruled out, increased expression of insulin receptor substrate 1 (IRS1) and Akt phosphorylation resulting from HO-1 over-expression in liver, adipose tissue, and skeletal muscle might play a greater role in hemin-induced increase in systematic insulin sensitivity (Supplemental Fig. 9). It is therefore probable that improved insulin
sensitivity upon hemin treatment is more likely a systematic effect.

In summary, the present study provides novel experimental evidence that bilirubin mediates HO-1 induction-induced vascular benefits through activation of the PI3K/Akt/eNOS signaling cascade in diabetic mice. The present findings enhance the prospective of using HO-1 inducers or bilirubin to ameliorate diabetic vasculopathy.

Author contributions

JL, LW, LL, YZ and SLW conducted the experiments and analyzed the data. YH, LJ, XYT and WTW designed the experiments and prepared the manuscript. QH and HMH performed bioassay. NW, ZYC, JY and XY conducted biochemical assay, provided plasmids and assisted with discussion. YH is the guarantor of this study.

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No potential conflicts of interest relevant to this work.
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Figure legends

FIG. 1. Effects of hemin, SnMP, HO1 over-expression and HO1 silencing on EDRs in mouse aortas. A: The impaired EDRs of db/db mouse aortas were rescued by hemin (HO1 inducer, 25 mg/kg, 3 times/week, 2-week, ip), which was antagonized by co-treatment with SnMP (HO1 inhibitor, 20 mg/kg, 3 times/week, 2-week, ip). *p<0.05 vs db/m+; #p<0.05 vs db/db; †p<0.05 vs db/db+Hemin. B: Hemin treatment increased HO1 expression in mouse aortas. *p<0.05 vs db/m+; #p<0.05 vs db/db. C: Ex vivo hemin treatment (5 µmol/L, 24 h) improved EDRs in db/db mouse aortas, which was inhibited by SnMP (30 µmol/L). *p<0.05 vs Control; #p<0.05 vs Hemin. D: HO1 overexpressing adenovirus transduction augmented EDRs of db/db mouse aortas. GFP or HO1 adenovirus was injected via tail vein 4 days before mice were sacrificed. *p<0.05 vs GFP Adv. E: HO1 shRNA virus transduction inhibited hemin-induced improvement in EDRs in db/db mouse aortas. *p<0.05 vs db/db; #p<0.05 vs db/db+Hemin+scramble. F: HO1 shRNA virus transduction suppressed hemin-induced HO1 expression in db/db mouse aortas. *p<0.05 vs db/db; #p<0.05 vs db/db+Hemin+scramble. Data are means±SEM of 4-6 experiments.

FIG. 2. PI3K/Akt/eNOS mediates the beneficial effect of HO1 in db/db mouse aortas. A&B: Chronic hemin treatment increased phosphorylation of Akt (Ser308) and eNOS (Ser1177). *p<0.05 vs db/m+; #p<0.05 vs db/db; †p<0.05 vs db/db+Hemin. C, D&E: HO1 shRNA virus transduction suppressed Akt (Ser308) and eNOS (Ser1177) phosphorylation after hemin treatment. *p<0.05 vs db/m+; #p<0.05 vs db/db; †p<0.05 vs db/db+Hemin+scramble. F: Improved EDRs in aortas from hemin-treated db/db mice were attenuated by wortmannin (100 nmol/L, 24 h) or Akt inhibitor V (5 µmol/L, 24 h). *p<0.05 vs Control. Data are means±SEM of 4-6 experiments.

FIG. 3. The effect of unconjugated bilirubin on EDRs. A: Hemin administration increased the level of serum unconjugated bilirubin in db/db mice. *p<0.05 vs db/m+; #p<0.05 vs db/db; †p<0.05 vs db/db+Hemin. B: Ex vivo treatment with bilirubin (1 µmol/L, 24-h) improved EDRs in db/db mouse aortas, with or without Akt inhibitor V (5 µmol/L) or SnMP (30 µmol/L). *p<0.05 vs Control; #p<0.05 vs Bilirubin. C&D: Mouse aortas were exposed to GFP shRNA or BVR shRNA adenovirus for 24-h and then treated with hemin (5 µmol/L) or bilirubin (1 µmol/L) for another 24-h before functional assay. *p<0.05 vs Control; #p<0.05 vs BVR shRNA+Hemin. E&F: Successful suppression of biliverdin reductase expression by BVR shRNA was shown by Western blotting and immunohistochemistry. *p<0.05 vs GFP shRNA. Data are means±SEM of 4-6 experiments.

FIG4. Effect of chronic bilirubin treatment on EDRs in db/db mouse aortas. A: Oral administration of bilirubin to db/db mice (5 mg/kg, 3 times/week, 2-week, ip) augmented EDRs in aortas. *p<0.05 vs db/m+; #p<0.05 vs db/db. B, C&D: Chronic bilirubin treatment increased phosphorylation of Akt (Ser308) and eNOS (Ser1177) in db/db mouse aortas. *p<0.05 vs db/m+; #p<0.05 vs db/db. Data are means±SEM of 4-6 experiments.
FIG. 5. The effects of hemin, bilirubin, or Akt activity inhibition on NO production in HUVECs. **A**: Representative images of DAF-DA fluorescence signal in response to A23187 (100 nmol/L) in HUVECs. The fluorescence before (F₀) and after (F₁) the addition of A23187 was analyzed. **B&С**: Summarized results showing the levels of NO production in HUVECs treated with hemin (5 µmol/L, 36-h) or bilirubin (1 µmol/L, 36-h) in the presence or absence of SnMP (30 µmol/L, 36-h) or Akt inhibitor V (5 µmol/L, 36-h) after exposure to NG or HG. *p<0.05 vs NG; #p<0.05 vs HG; †p<0.05 vs HG+Hemin/Bilirubin. **D**: Effects of DN-Akt on NO production in HUVECs co-incubated with hemin (5 µmol/L) or bilirubin (1 µmol/L). *p<0.05 vs NG. **E&F**: Akt and eNOS phosphorylation in HUVECs treated with hemin (5 µmol/L), with or without SnMP (30 µmol/L) exposed to NG or HG (30 mmol/L, 36-h). *p<0.05 vs NG; #p<0.05 vs HG; †p<0.05 vs HG+Hemin. **G&H**: Bilirubin (1 µmol/L) increased Akt and eNOS phosphorylation in HUVECs exposed to HG (30 mmol/L, 36-h). The effect was abrogated by Akt inhibitor V. *p<0.05 vs NG; #p<0.05 vs HG; †p<0.05 vs HG+Bilirubin. Data are means±SEM of 4-6 experiments.

FIG.6. The effects of BVR shRNA on Akt and eNOS phosphorylation in HUVECs. **A-C**: BVR shRNA reversed the effect of hemin (5 µmol/L) on Akt and eNOS phosphorylation. *p<0.05 vs NG; #p<0.05 vs HG; †p<0.05 vs HG+Hemin. **D-E**: BVR shRNA did not influence the effect of bilirubin (1 µmol/L) on Akt and eNOS phosphorylation. #p<0.05 vs HG; †p<0.05 vs HG+Bilirubin. Data are means±SEM of 4-6 experiments.

FIG.7. The effect of bilirubin on EDRs in renal arteries from diabetic patients. **A&B**: Bilirubin treatment (1 µmol/L, 24 h) improved EDRs in renal arteries from diabetic patients (DM). **C**: Immunohistochemistry staining showing the expression of biliverdin reductase in the endothelium in human renal arteries. *p<0.05 vs Control, #p<0.05 vs DM. Data are means±SEM of 3 experiments.
FIG. 2
FIG. 3
FIG. 5
FIG. 6
FIG. 7

A. Human renal artery

B. Relaxation (% Phe tone)

C. Negative control

Biliverdin reductase
Data Supplement

Unconjugated bilirubin mediates HO-1-induced vascular benefits in diabetic mice

Jian Liu¹, Li Wang¹, Xiaoyu Tian¹, Limei Liu², Wing-Tak Wong³, Yang Zhang¹, Quanbin Han⁴, Hing-Man Ho⁴, Nanping Wang⁵, Siu-Ling Wong¹, Zhenyu Chen⁶, Jun Yu⁷, Chi-Fai Ng⁸, Xiaoqiang Yao¹, Yu Huang¹

¹Institute of Vascular Medicine and Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong; Department of Physiology and Pathophysiology, Peking University Health Science Center, China; ³Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, USA; ⁴School of Chinese Medicine, Hong Kong Baptist University; ⁵Institute of Cardiovascular Science, Peking University, China; ⁶School of Life Sciences, ⁷Department of Medicine and Therapeutics and ⁸Department of Surgery, Chinese University of Hong Kong, China

Additional Results

Sodium nitroprusside-induced relaxations of aortas were unaffected by in vivo hemin treatment in db/db mice. Endothelium-independent relaxations induced by sodium nitroprusside (SNP) were similar among all groups (Supplemental Fig. 1).

Supplemental Figure 1. The effect of hemin treatment on sodium nitroprusside-induced relaxations in mouse aortas. Data are means±SEM of 4-6 mice.

in vitro hemin treatment and in vivo transduction with HO-1 overexpressing adenovirus increased HO-1 expression in mouse aortas. Hemin treatment (5 µmol/L, 24-h) and tail injection of HO-1-overexpressing adenovirus increased HO-1 expression in mouse aortas (Supplemental Fig. 2A&B).
Supplemental Figure 2. (A) HO-1 expression after hemin treatment (5 µmol/L, 24-h). *p<0.05 vs Control. (B) HO-1 expression after transduction with control virus (GFP AdV) and HO-1 overexpressing adenovirus (HOI-1 AdV). *p<0.05 vs GFP AdV. Data are means±SEM of 4-6 mice.

In vivo hemin treatment improved endothelial function in diet-induced obese mice. Compared to lean mice, ACh-induced endothelium-dependent relaxations were impaired in diet-induced obese (DIO) mice (Supplemental Fig. 3A), which were reversed by two-week hemin treatment. Hemin treatment also increased HO-1 expression, phosphorylation of Akt (Thr308) and eNOS (Ser1177) in mouse aortas (Supplemental Fig. 3B, C&D).

Supplemental Figure 3. The effect of two-week hemin treatment on endothelial function in DIO mice. (A) ACh-induced relaxations in DIO mouse aortas. *p<0.05 vs Control; #p<0.05 vs DIO. (B-D) HO-1 expression, phosphorylation of Akt (Thr308) and eNOS (Ser1177) after hemin treatment. *p<0.05 vs DIO. Data are means±SEM of 4 mice.
Hemin and bilirubin treatment increased nitrite level in the aorta. Treatment with hemin (5 µmol/L, 24-h) and bilirubin (1 µmol/L, 24-h) increased nitrite levels in mouse aortas (Supplemental Fig. 4).

**Supplemental Figure 4.** The effect of hemin and bilirubin treatment on nitrite levels in mouse aortas. *p<0.05 vs db/m; #p<0.05 vs db/db. Data are means±SEM of 4-6 mice.

BVR shRNA adenovirus transduction inhibited the effect of hemin in HUVECs
Hemin treatment (5 µmol/L, 36-h) of HUVECs increased the bilirubin level in culture medium, which was reversed by transduction with BVR shRNA adenovirus (Supplemental Fig. 5A). In addition, hemin restored NO production that was impaired by high glucose (HG, 30 mmol/L, 36 h) in HUVECs. This effect of hemin was reversed after transduction of BVR shRNA adenovirus. However, the effect of bilirubin to restore NO production was not affected by BVR shRNA adenovirus transduction (Supplemental Fig. 5B&C).

**Supplemental Figure 5.** The effect of BVR shRNA transduction on bilirubin and NO production in HUVECs. (A) Bilirubin level in culture medium after hemin treatment (5 µmol/L, 36-h). *p<0.05 vs NG; #p<0.05 vs HG+Hemin. (B&C) NO production in HUVECs treated with hemin (5 µmol/L, 36-h) or bilirubin (1 µmol/L, 36-h) in the presence or absence of BVR shRNA adenovirus in HUVECs exposed to NG or HG. *p<0.05 vs NG; #p<0.05 vs HG; †p<0.05 vs HG+Hemin. Data are means±SEM of 4 experiments.
Bilirubin reduced reactive oxygen species (ROS) generation in HUVECs. Exposure to high glucose (HG, 30 mmol/L) for 36-h increased ROS production in HUVECs compared to normal glucose (NG, 5 mmol/L). Co-treatment with bilirubin (1 µmol/L) inhibited HG-stimulated ROS production, and Akt inhibitor V (5 µmol/L) and oxidized-hemoglobin inhibited the effect of bilirubin (Supplemental Fig. 6).

Supplemental Figure 6. The effect of bilirubin on high glucose-stimulated ROS production when used together with Akt inhibitor V or NO scavenger oxidized-hemoglobin. (A&B) The effect of Akt inhibitor (A&B) or oxidized-hemoglobin (C&D) on ROS production when used together with bilirubin as detected by CM-H2DCFDA or dihydroethidium (DHE). Data are means±SEM of 4 experiments. *p<0.05 vs NG; #p<0.05 vs HG; †p < 0.05 vs HG+Bilirubin.

Carbon monoxide-releasing molecule did not modulate EDRs. Ex vivo treatment with carbon monoxide-releasing molecule (CORM) tricarbonyldichlororuthenium (II) dimmer (10-100 nmol/L) for 24 hours did not change EDRs in db/db mouse aortas, with or without co-treatment of Akt inhibitor V (5 µmol/L) (Supplemental Fig. 7).

Supplemental Figure 7. Lack of the effect of carbon monoxide-releasing molecule (CORM) on EDRs in db/db mouse aortas. Data are means±SEM of 4 experiments.
Hemin slightly increased insulin sensitivity in \textit{db/db} mice. Chronic hemin treatment did not change body weight and lipid profile in \textit{db/db} mice (Supplemental Table 2). But fasting blood glucose and plasma insulin level was decreased. Oral glucose tolerance test and intraperitoneal insulin tolerance test showed an improved glucose tolerance and insulin sensitivity (Supplemental Fig. 8).

\begin{figure}[ht]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{The effect of chronic hemin treatment on fasting blood glucose (A), plasma insulin (B), oral glucose tolerance test (C) and intra-peritoneal insulin tolerance test (D) in \textit{db/db} mice. Data are means±SEM of 4-6 mice. *p<0.05 vs \textit{db/m}+; #p<0.05 vs \textit{db/db}; †p<0.05 vs \textit{db/db}+Hemin.}
\end{figure}

\textbf{Supplemental Figure 8.} The effect of chronic hemin treatment on fasting blood glucose (A), plasma insulin (B), oral glucose tolerance test (C) and intra-peritoneal insulin tolerance test (D) in \textit{db/db} mice. Data are means±SEM of 4-6 mice. *p<0.05 vs \textit{db/m}+; #p<0.05 vs \textit{db/db}; †p<0.05 vs \textit{db/db}+Hemin.

Hemin increased insulin receptor substrate 1 (IRS1) expression, Akt phosphorylation and HO-1 expression in liver, adipose tissue and skeletal muscle of \textit{db/db} mice. Chronic hemin treatment increased IRS1 expression and Akt phosphorylation, as well as HO-1 expression in the liver, epididymal fat and skeletal muscle of \textit{db/db} mice, which may account for the improved systematic insulin sensitivity (Supplemental Fig. 9).
Supplemental Figure 9. The effect of chronic hemin treatment on IRS1 expression, Akt phosphorylation and HO-1 expression in liver, epididymal fat and skeletal muscle of db/db mice. Data are means±SEM of 4-6 mice. *p<0.05 vs db/m; #p<0.05 vs db/db; †p<0.05 vs db/db+Hemin+Scramble.

Supplemental Table 1. HbA1c values and the duration of diabetes of patients

<table>
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<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
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<tbody>
<tr>
<td>HbA1c</td>
<td>9.1%/76 mmol/mol</td>
<td>7.2%/55 mmol/mol</td>
<td>6.7%/50 mmol/mol</td>
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<tr>
<td>Duration (years)</td>
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Supplemental Table 2. Basic parameters after chronic hemin treatment

<table>
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<tr>
<th></th>
<th>db/m⁺</th>
<th>db/m⁺ + Hemin</th>
<th>db/db</th>
<th>db/db + Hemin</th>
<th>db/db + SnMP + Hemin</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29.8 ± 0.7</td>
<td>29.3±0.5</td>
<td>55.0 ± 4.6*</td>
<td>53.8±1.3</td>
<td>51.5 ± 3.8</td>
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<tr>
<td>Plasma levels of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>77.2 ±3.3</td>
<td>79.9± 2.4</td>
<td>133.6 ± 10.5*</td>
<td>124.4 ± 4.4</td>
<td>146.7 ± 10.5</td>
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<tr>
<td>Triglyceride</td>
<td>63.4 ± 2.4</td>
<td>60.9±2.5</td>
<td>210.7 ± 26.3*</td>
<td>204.8 ± 16.2</td>
<td>210.7 ± 13.6</td>
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<tr>
<td>HDL (mg·mL⁻¹)</td>
<td>42.1±1.1</td>
<td>42.1±1.1</td>
<td>83.1 ± 4.7*</td>
<td>89.4 ± 5.3</td>
<td>80.3 ± 2.7</td>
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<tr>
<td>non-HDL (mg·mL⁻¹)</td>
<td>35.0± 2.4</td>
<td>37.7 ± 1.5</td>
<td>50.5 ± 5.2*</td>
<td>48.0 ± 4.5</td>
<td>46.4 ± 4.0</td>
</tr>
</tbody>
</table>

The levels of total cholesterol, triglyceride, HDL, and non-HDL in the plasma from mice were measured by enzymatic methods (Stanbio, Boerne, TX, USA). Data are means±SEM of 4-6 mice *p< 0.05 vs db/m⁺.

Additional Discussion

Akt is one of eNOS activators in endothelial cells. Reduced Akt phosphorylation is reported in arteries of diabetic animals or in high glucose-treated endothelial cells while Akt activation augments endothelium-dependent relaxations in aortas of diabetic mice (1). Previous studies showed that high glucose exposure suppresses Akt phosphorylation probably through increasing oxidative stress and protein kinase C activation (2-5). The present study shows a decreased Akt phosphorylation in db/db mouse aortas. Serum bilirubin concentration is reported to be lower in diabetic patients than in healthy subjects (6). We also detected less serum bilirubin level in diabetic mice, indicating that lower serum bilirubin may partly account for the reduced aortic Akt phosphorylation in diabetic mice. In view of the serum antioxidant capacity of bilirubin (7), oxidative stress in association with reduced bilirubin is probably another factor involved in decreased Akt phosphorylation under diabetic conditions (2-5). Our in vitro experiments show that high glucose exposure stimulates ROS generation and reduces Akt phosphorylation in human endothelial cells without changing the levels of HO-1 and bilirubin. In vitro and in vivo results show a different impact of hyperglycemia on bilirubin levels in cultured endothelial cells and in serum of diabetic mice probably due to far more complex mechanisms regulating circulating bilirubin concentration which is likely controlled by the balance between production and excretion of bilirubin in vivo. Although the present study does not provide direct data to support the possibility that decreased serum bilirubin is associated with decreased Akt phosphorylation in db/db mouse aortas, increased bilirubin production by hemin treatment and bilirubin administration to db/db mice can effectively increase Akt phosphorylation. The present study shows that bilirubin activates Akt/eNOS/NO cascade to lower oxidative stress as the major mechanism to restore the impaired endothelial function in hemin-treated diabetic mice.

References
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