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Targeting Endothelial Cells with Heme Oxygenase-1 Gene Using VE-Cadherin Promoter Attenuates Hyperglycemia-Mediated Cell Injury and Apoptosis

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ABSTRACT

Risk factors for cardiovascular diseases include hyperglycemia, TNF, and reactive oxygen species (ROS), which collectively contribute to vascular endothelial cell dysfunction and apoptosis. We examined, in vascular endothelial cells, whether the selective expression of heme oxygenase-1 (HO-1) offers cytoprotection against glucose- and TNF-mediated cell death. An adenoviral vector expressing human HO-1 was constructed using a VE-cadherin (VECAD) promotor fragment, and cell-specific expression of the recombinant adenovirus was examined using endothelial and vascular smooth muscle cells. The effects of HO-1 transduction (Ad-VECAD-HO-1 gene) on HO-1 expression, HO activity, and the response to TNF and hyperglycemia were studied. Human HO-1 gene was selectively expressed in endothelial cells after infection with the Ad-VECAD-HO-1 vector. Selective expression of HO-1 prevented TNF- and hyperglycemia-mediated superoxide (O_2^-) formation, DNA degeneration, and upregulation of caspase, but increased the expression of pAkt and Bcl-xL, proteins responsible for endothelial dysfunction in diabetes. These results demonstrate that endothelial cell survival after oxidative stress injury may be enhanced by targeting HO-1 expression, thus blocking inflammation, apoptosis, and thereby attenuating cardiovascular risk factors. *Antioxid. Redox Signal*. 9, 2065–2074.

INTRODUCTION

Cardiovascular risk factors, including TNF and hyperglycemia, are major contributors to endothelial cell dysfunction and apoptosis (1). Exposure of endothelial cells to elevated glucose levels causes glucose oxidation, resulting in the generation of excess reactive oxygen species (ROS) in endothelial cells. Superoxide (O_2^-) is a vasoconstrictor, which acts by removing vasodilators and stimulating vasoconstrictors [*e.g.*, O_2^- can convert nitric oxide (NO) to peroxynitrite, thereby consuming the endogenous vasodilator in the vasculature (14, 21)]. Moreover, O_2^- can induce assorted vasoconstrictors, such as endothelin, PDGF, TxA_2, and isoprostanes (14, 21). Reports indicate that oxidative stress, as a result of hyperglycemia, play an important role in the development and progression of diabetic vascular complications (24). Hyperglycemic rats have demonstrated increased urinary 8-epi-isoprostane, PGF_2, and O_2^- formation, as well as an increase in the number of circulating endothelial cells and fragments (3). High glucose–mediated local formation of ROS is considered to be the major contributing factor to endothelial dysfunction, including abnormalities in cell cycling (5, 31, 59).

The overexpression of human heme oxygenase-1 (HO-1) in endothelial cells has the potential to provide protection against a variety of agents that cause oxidative stress (2, 3). Recent studies imply that the products of HO-1–mediated heme degra-

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dation [*i.e.*, carbon monoxide (CO) and bilirubin] have antiapoptotic effects in diabetes models (52, 53). The role of endogenously produced CO and bilirubin in the prevention of endothelial cell apoptosis has been examined by determining the effect of conditions affecting HO activity and expression in diabetes (6, 43). The products of the HO catabolism of heme serve as a countervailing influence on oxidative damage in endothelial cells and in the vasculature by actions that include decreasing endothelial cell sloughing, inhibiting O_2^- and the expression of inflammatory molecules (*i.e.*, TNF, Ang II, ICAM), and a reduction in constrictor mechanisms (1). Our studies previously demonstrated that endothelial cells exposed to high glucose levels showed decreased HO-1 protein expression and HO activity *in vitro* (2, 3, 45) and *in vivo* (3, 25, 43).

The junctional complex plays a crucial role in the control of transendothelial permeability and migration of inflammatory cells in the blood vessels. Vascular endothelial cadherin (VECAD) is an adhesion molecule at the adherens junctions (a component of the junctional complex in endothelial cells). VECAD is a calcium-dependent adhesive molecule that is exclusively and constitutively expressed in the endothelial cells (49). It is paramount in the maintenance and control of vascular integrity and also is crucial for angiogenesis and the assembly of vascular tubes. The monoclonal antibody of VECAD inhibits formation of adherens junctions, angiogenesis, and tumor growth and metastasis (30).

Vascular endothelial cells serve as a barrier at the interface of vascular tissue. Alteration of this barrier has been correlated with diabetic complications; high levels of glucose alter endothelial cell function and disrupt the cell-to-cell connection (36). Endothelial dysfunction is recognized as the initial step in the atherosclerotic process and has been associated with pathogenesis in diabetic micro- and macroangiopathy (17, 18, 39, 46, 48, 58). Recent studies in patients with diabetes have shown increased numbers of circulatory endothelial cells, which are thought to be mature cells that have detached from the intimal monolayer in response to endothelial injury (33). HO-1 induction has been shown to reduce O_2^- production and decrease endothelial sloughing in hyperglycemic rats (3, 25). Upregulation of HO-1 decreases oxidant production, endothelial cell damage and shedding, and may attenuate vascular complications in diabetes (3, 25, 43). Caspase is a proapoptotic protein, and its activation represents one of the earliest biochemical indicators for apoptotic cell death. Measurement of caspase activity is a widely used and generally accepted method to determine apoptosis in *in vivo* and *in vitro* studies. CO, one of the byproducts of HO metabolism, has been shown to inhibit T-lymphocyte proliferation (47) and to inhibit apoptosis during ischemia/reperfusion lung injury by a caspase-dependent pathway (57). The role of caspase-dependent apoptosis has been implicated in diabetes complications (29, 55).

In this study, we assessed targeting endothelial cells with the HO-1 gene, using the endothelial cell–specific promoter VECAD (13). We examined the effect of the AD-VECAD-HO-1 gene on DNA damage and oxidative stress in the cells exposed to high glucose. The enhanced HO activity, brought about by Ad-VECAD-HO-1–mediated selective HO-1 gene transfer to endothelial cells, attenuated glucose-mediated abnormalities, including superoxide and caspase activity in endothelial cells.

METHODS

Cell cultures

Human dermal microvessel endothelial cells (HMEC-1 cells) were a kind gift of Michael Dillon (National Center for Infectious Diseases, Atlanta, GA) and grown in MCDB131 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% FBS, 10 ng/ml epidermal growth factor (EGF; Sigma, St Louis, MO), and 1 μg/ml hydrocortisone (Sigma). Human smooth muscle cells (CRL-1999) were obtained from ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere and maintained at subconfluency by passaging with trypsin–EDTA (Gibco-BRL).

Construction of adenoviral vector Ad-VECAD-HO-1

We constructed the VECAD-human HO-1 adenovirus vector as described in Fig. 1. In brief, VECAD-INT sequences were subcloned into the plasmid VQAd5K-NpA vector (ViraQuest, Inc, North Liberty, IA). The Xhol/sal site was eliminated. but the 3' Xhol site remained intact. The INT sequence was generated by PCR with Smal ends that eliminate the 5′ Xhol site in the fragment left intact the Xhol site at the 3' end of the INT. The resulting plasmid, VQ-VECAD-INT, has a single Xhol site at the 3' end of the INT sequence and was used as the substrate for the subsequent insertion of human HO-1 and later GFP. Originally, we generated the plasmid with lacZ, which was used for the current experiments, VQ-Ad-VECAD-LAZ plasmid. Once the shuttle was completed, it was linearized with PacI and transfected with the RAPAd.1 backbone. pVQ.VECAD-INT-HO-1 was digested with ScaI and co-transfected with the RAPAd.1 system (4), and the mixture was subsequently transfected into HEK 29 using the standard Ca phosphate method. Viral foci were noted commencing on day 6. The cells were harvested 8 days after transfection, pelleted by low-speed centrifugation, and the virus liberated by three cycles of freezing and thawing. The cell lysate (1 ml in10 ml Tris buffer, pH 8.1) containing the recombinant virus was amplified and purified using cesium chloride. The resultant particles had a concentration of 4×10^{12} to 8×10^{12} particles/ml.

Restriction digest of clones selected from the legation of the VECAD-HO1 to the adenoviral plasmid using Sca1 and Bgl II was performed to verify positive clones of the shuttle vector. Sca1 identified positive clones expressing VECAD-HO-1 size was used for the current study.

Cell-cycle progression, cell-proliferation, and apoptosis analysis

Control endothelial cells and cells targeted with Ad-VECAD-HO-1 and Ad-VECAD-Lac-Z were harvested, permeabilized, and stained with DAPI, as previously described (2). To evaluate the effect of TNF and glucose on cell-cycle progression, DNA distribution was analyzed by flow cytometry of endothelial cells treated with glucose (33 m*M*) and TNF (200 ng/ml) for 48 h in the presence of 1% FBS. The percentage of cells in G_2/M was estimated for each time point, as previously described

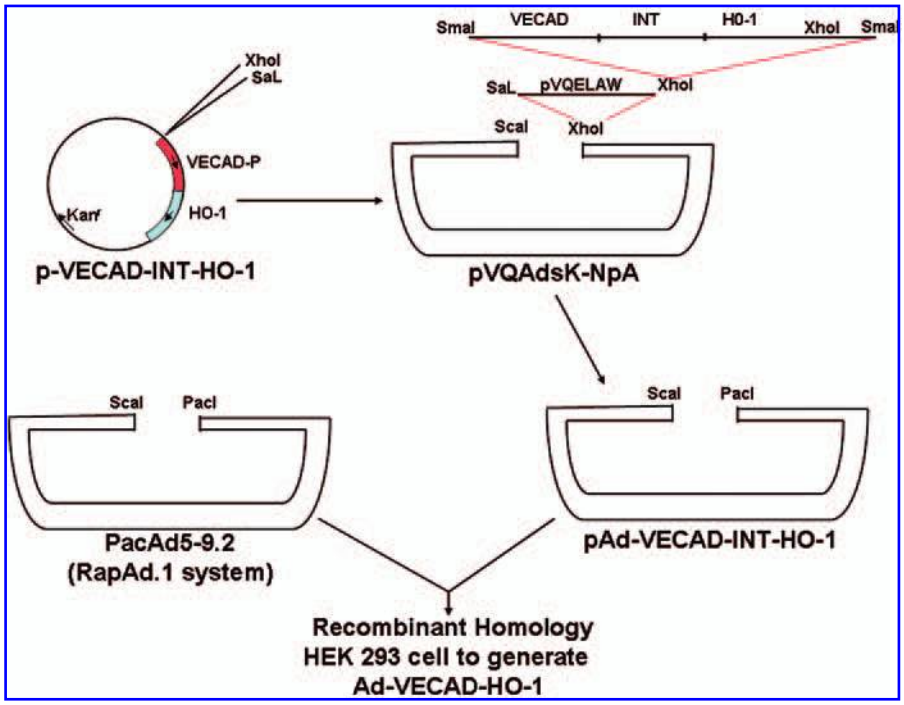

FIG. 1. Schematic presentation of the adenoviral vector, Ad-VECAD-HO-1, construction. This figure depicts the plasmid VQAd5K. NPA vector used to generate Ad5.VECAD-INT-HO-1. VQAd5.K.NPA was used to subclone INT sequences. The XhoI/sal site was eliminated, but the 3′ XhoI site remained intact. The INT sequence was generated by PCR with SmaI ends that eliminate the 5′ XhoI site in the fragment left intact the internal XhoI site at the 3′ end of the INT. The resulting plasmid, VQ.VECAD-INT, has a single XhoI site at the 3′ end of the INT sequence and was used as the substrate for the subsequent insertion of human HO-1 and later GFP. Originally, we generated the plasmid with lacZ, which was used for the current experiments, VQAd-VECAD-LAZ plasmid. Once the shuttle was completed, it was linearized with PacI and transfected with the RAPAd.1 backbone. PVQVECAD-INT-HO-1 was digested with ScaI and co-transfected with the RAPAd.1 system, and the mixture was subsequently transfected into HEK 293 using the standard Ca-phosphate method. (**A**) SMC, HMEC, MEL, and HEP-G2 were transfected with Ad-VECAD-HO-1 (20 pfu/cell), and Western blot shows an increase in expression of HO-1 in HMECs only. This signifies the specificity of the vector. (SMCs, human abdominal aorta smooth muscle cells; MEL, myeloid erythroleukemia cell; HEP-G_2, human hepatoma G_2 cells; HMECs, human dermal microvessel endothelial cells). (**B**) Western blot analysis to assess the functional expression of human HO-1 in endothelial cells after Ad-VECAD-HO-1 transduction. HMECs and VSM (T-75; 50% confluent) were transfected with a mixture containing 20 pfu/cell Ad-VECAD-HO-1 or Ad-VECAD-Lac-Z adenovirus. After 48 h, the cells were harvested using cell-lysis buffer. Ad-VECAD-HO-1 caused the pronounced expression of human HO-1 protein in endothelial cells, but not in VSMCs. (**C**) Cell lysate HO activity was assayed by measuring bilirubin using the difference in absorbency from λ460 to λ530 nm with an absorption coefficient of 40 m*M* and 40 per centimeter. HO activity (nmol/mg per hour bilirubin) is expressed as the mean ± SEM of three experiments. Statistical analyses were performed by *t* test; $*p < 0.0001$ *vs*. EC-Ad-VECAD-LAZ).

(2). The increase in frequency of G_2/M cells was then plotted as a function of time to estimate the kinetics of cell entry into mitosis. Cell proliferation was determined using a cell-counting kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) using 5×10^3 cells and a 96-well microtiter plate.

Western blot analysis for signaling molecules and HO activity

Endothelial cell homogenates were centrifuged at 27,000 *g* for 10 min at 4°C. The supernatant was used for the measurement of protein levels (Bradford method) and for the measurement of HO-1, HO-2, AKT, and pAKT (25). Protein levels were visualized by immunoblotting with antibodies against rat HO-1 and HO-2 (Stressgen Biotechnologies Corp., Victoria, BC, Canada) and pAKT, AKT, and BcL-xL (Santa Cruz Biotechnology, Santa Cruz, CA). HO activity and O_2^- levels were assayed in tissue homogenates with standard methods (25).

Caspase-activity assay

Caspase activity was determined using colorimetric assays (ApoTarget Kit) following the manufacturer's protocol (BioSource International, Camarillo, CA). In brief, cell lysates were prepared in lysis buffer (Tris-buffered saline containing detergent), and protein concentrations in samples were estimated using the Bradford method. Then 200–300 μg of protein lysate per sample was mixed with 200 μ*M* substrate in 2× reaction buffer (DEVD-*p*NA for caspase-3 and LEHD-*p*NA for caspase-9) and incubated at 37°C overnight in the dark. Developed color was measured at 405 nm in a microplate reader (Bio-Rad, Hercules, CA). Blank readings were subtracted from each sample before calculation. Caspase activity was expressed in terms of absorbance units (OD 405 nm) per mg of protein.

Measurement of vascular O_2^- levels

Using previously described methods (25, 43), endothelial cells were placed in plastic scintillation vials containing 5 μm lucigenin, for the detection of O_2^- and other additions, in a final volume of 1 ml of air-equilibrated Krebs solution buffered with 10 m*M* HEPES-NaOH (pH 7.4) Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC, Beckman, Instruments, San Diego, CA) at 37°C, and data are reported as counts/minute/milligram protein after background subtraction.

DNA analysis by COMET assay

The presence of DNA fragmentation was examined by single-cell electrophoresis (Comet assay). In brief, 0.5–0.8 $\times$ 10^5 cells were mixed with 75 μl of 0.5% low-melting agarose and spotted on slides. The "minigels" were maintained in lysis solution (*N*-laurosil–sarcosine 1%; NaCl, 2.5 *M*; Na_2EDTA, 100 m*M*; DMSO, 10%; pH 10) for 1 h at 4°C, then denatured in a high-pH buffer (NaOH, 300 m*M*; Na_2EDTA, 1 m*M*) for 20 min, and finally electrophoresed in the same buffer at 25 V for 50–60 min. At the end of the run, the minigels were neutralized in Tris-HCl, 0.4 *M*, pH 7.5, stained with 100 μl of ethidium bromide (2 μg/ml) for 10 min, and scored using a Nikon fluorescence microscope (Nikon Labophot) interfaced with a computer. Software Scion Image with a Comet 1.3 version macro (free download from the National Institutes of Health website) allowed us to analyze and quantify DNA damage by measuring (a) tail length (TL) and (b) tail DNA percentage (TDNA). These parameters are used by the software to determine the level of DNA damage as tail moment (TMOM), expressed as the product of TL and TDNA.

Statistical analyses

The data are presented as mean ± SEM for the number of experiments. Statistical significance ($p < 0.05$) between the experimental groups was determined by the Fisher method of analysis of multiple comparisons. For comparisons between treatment groups, the Null hypothesis was tested by a single-factor analysis of variance (ANOVA) for multiple groups or unpaired *t* test for two groups.

RESULTS

Functional expression of human HO-1 in endothelial cells transduced with Ad-VECAD-HO-1

To test the specificity of Ad-VECAD-HO-1, we examined the effect of Ad-VECAD-HO-1 or Ad-VECAD-LacZ transfection and expression of HO-1. Various cells (SMC, HMEC, MEL, and HEP-G2) were infected with Ad-VECAD-HO-1 (10 μl), to test the specificity of Ad-VECAD-HO-1. Western blot showed an increase in expression of HO-1 only in HMECs (Fig. 2A). Further, we examined the ability of the Ad-lacZ and Ad-VECAD-HO-1 to infect endothelial cells using 5–100 pfu/cell, and the ideal infection dose was 20 pfu/cell, which was used in subsequent infection, to express HO-1 protein in the endothelial cells. After 48 h of transfection with Ad-VECAD-HO-1 or the Ad-lacZ, the cells subjected to Western blot analysis and HO activity assay were used to assess the functional expression of human HO-1 in endothelial cells. Ad-VECAD-HO-1 caused the pronounced expression of human HO-1 protein in endothelial cells, but not in VSMCs. No significant HO-1 signal was detected in endothelial

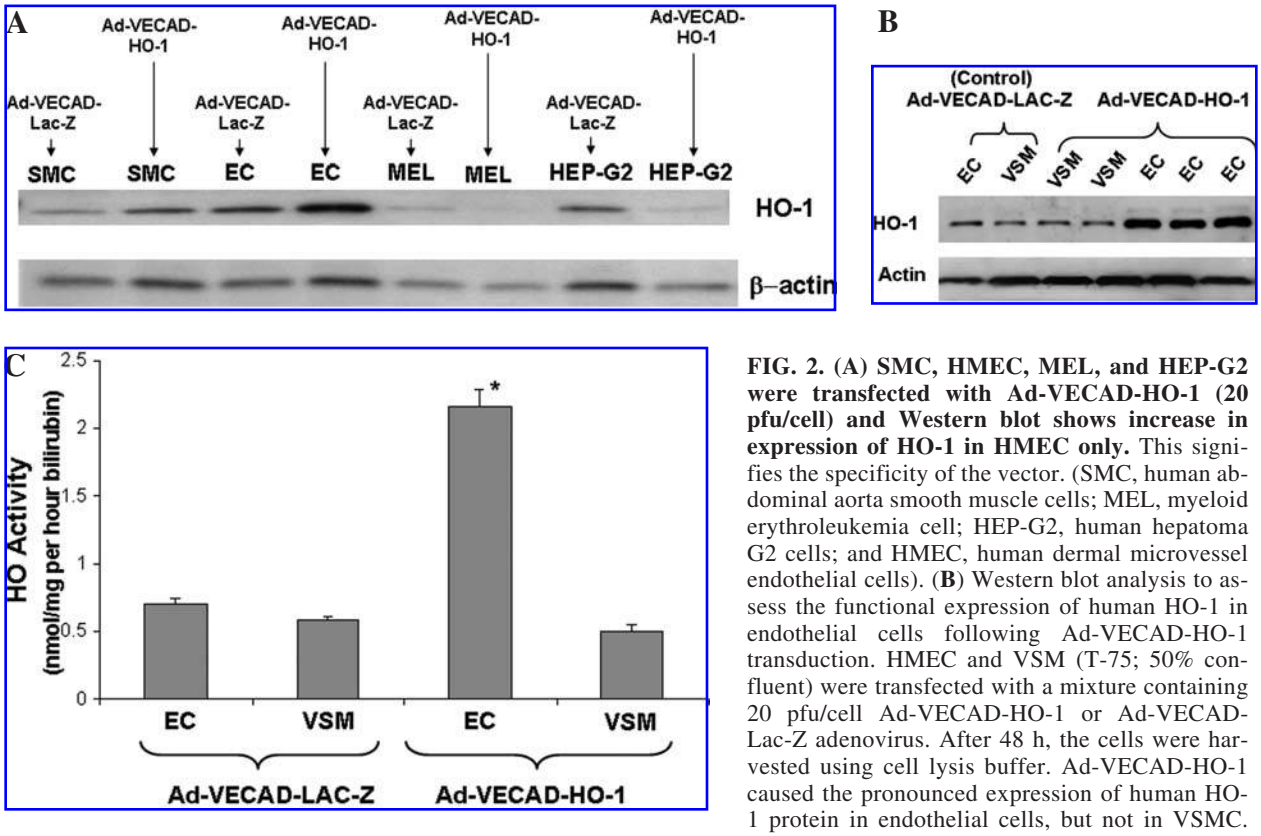

FIG. 2. (A) SMC, HMEC, MEL, and HEP-G2 were transfected with Ad-VECAD-HO-1 (20 pfu/cell) and Western blot shows increase in expression of HO-1 in HMEC only. This signifies the specificity of the vector. (SMC, human abdominal aorta smooth muscle cells; MEL, myeloid erythroleukemia cell; HEP-G2, human hepatoma G2 cells; and HMEC, human dermal microvessel endothelial cells). (**B**) Western blot analysis to assess the functional expression of human HO-1 in endothelial cells following Ad-VECAD-HO-1 transduction. HMEC and VSM (T-75; 50% confluent) were transfected with a mixture containing 20 pfu/cell Ad-VECAD-HO-1 or Ad-VECAD-Lac-Z adenovirus. After 48 h, the cells were harvested using cell lysis buffer. Ad-VECAD-HO-1 caused the pronounced expression of human HO-1 protein in endothelial cells, but not in VSMC. (**C**) Cell lysate HO activity was assayed by measuring bilirubin using the difference in absorbency from λ460 to λ530 mm with an absorption coefficient of 40 mmol/L^{-1} and 40 cm^{-1}. HO activity (nmol/mg per hour bilirubin) is expressed as the mean ±SE of 3 experiments. Statistical analyses were performed by *t* test. (*p value < 0.0001 vs. EC-Ad-VECAD-LAZ).

cells after infection with Ad-lacZ (Fig. 2B). This further confirms the specificity of the Ad-VECAD vector for endothelial cells.

HO activity was assayed in both VSMCs and endothelial cells after exposure to either Ad-VECAD-HO-1 or the control adenovirus, Ad-lacZ, for 2 days. HO activity in endothelial cells infected with Ad-VECAD-HO-1 was significantly higher than that in endothelial cells infected with Ad-lacZ ($p < 0.05$). However, no significant difference in HO activity was found between VSMCs infected with Ad-VECAD-HO-1 and Ad-lacZ, indicating that the Ad-VECAD-HO-1 increases HO-1 expression specifically in endothelial cells, but not in VSMCs (Fig. 2C).

Effect of Ad-VECAD-HO-1 on TNF-treated endothelial cells

TNF is an important risk factor for apoptosis. We examined DNA distributions in control ECs or ECs transduced with HO-1 by Ad-VECAD-HO-1. In Ad-VECAD-HO-1–treated cells, an increased percentage of the cells in S phase is noted, compared with those in Ad-lacZ cultures (Fig. 3). To investigate the influence of TNF on cell-cycle progression in ECs transfected with Ad-VECAD-HO-1, these cells were treated with TNF in the presence or absence of SnMP. As shown in Fig. 3, in Ad-lacZ cultures treated with TNF, the levels of G_2/M and apoptosis were significantly increased compared with cells transfected with Ad-VECAD-HO-1. After the addition of SnMP to TNF in ECs transduced with Ad-VECAD-HO-1, the DNA distribution more closely resembled that of control cultures, including the small percentage of cells undergoing apoptosis (see Fig. 3). These findings suggest that selective HO-1 gene expression in ECs resulted in increased resistance to TNF-mediated cell death and attenuated the increased in abnormalities in the DNA distributions.

Effect of Ad-VECAD-HO-1 transduction on glucose-mediated DNA damage

Because glucose has been shown to cause apoptosis (38, 42, 44, 59), we reasoned that the cell-specific expression of HO-1 in endothelial cells may attenuate glucose-mediated DNA damage. We previously analyzed DNA integrity in serum-starved endothelial cells exposed to glucose for 48 h, by COMET assay (3). Glucose increased TMOM levels from 54 ± 9 to 143 ± 12 in control cells. In contrast, in cells with increased expression of HO-1, the glucose-mediated DNA damage was greatly diminished, suggesting that HO-1 shields the cells from the initial toxic effect produced by glucose.

We analyzed DNA-distribution changes by flow cytometry after HO-1 transduction. Endothelial cells were exposed for 48 h to glucose and then were transfected with Ad-VECAD-HO-1 or Ad-VECAD-LacZ. To evaluate the effect of induction of

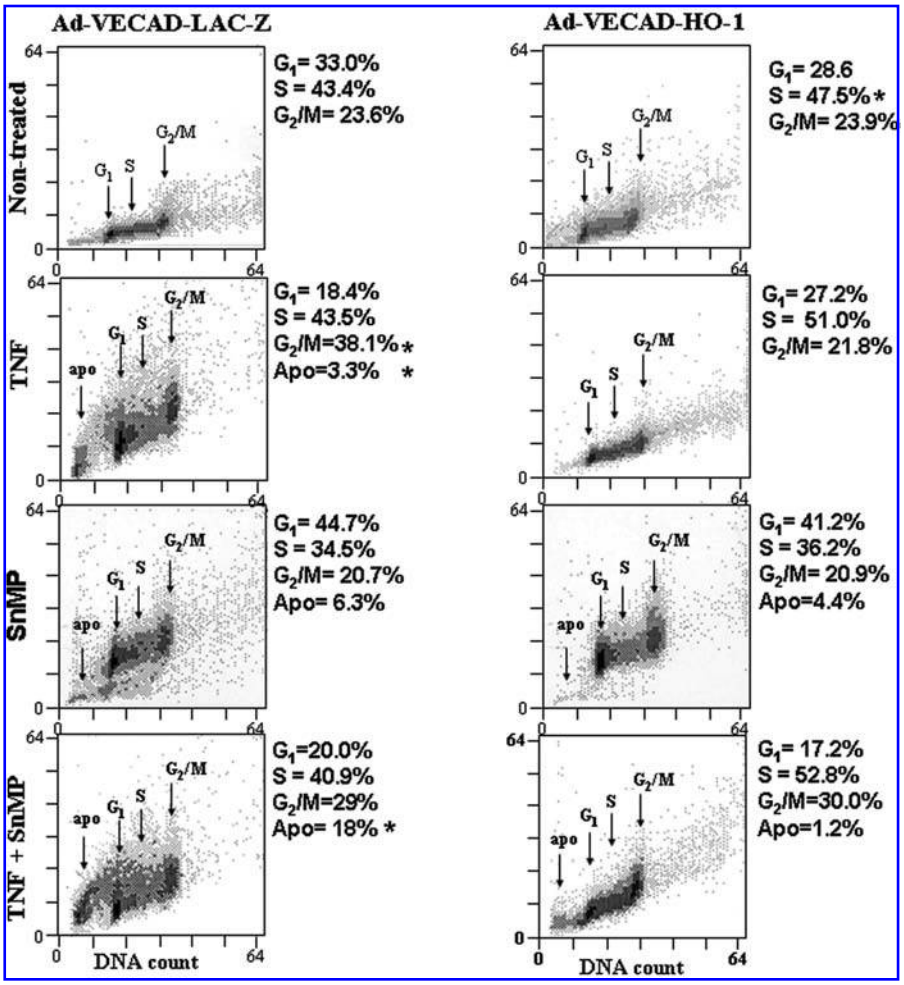

FIG. 3. DNA distribution in the control and HO-1–transduced cells treated with vehicle solution, and treated with TNF in the presence or absence of SnMP. Significant apoptosis was seen in cells treated with TNF compared with controls ($p < 0.05$), whereas a significant decrease in apoptosis appears in cells transduced with Ad-VECAD-HO-1, even with addition of a high concentration of TNF. (*$p < 0.05$).

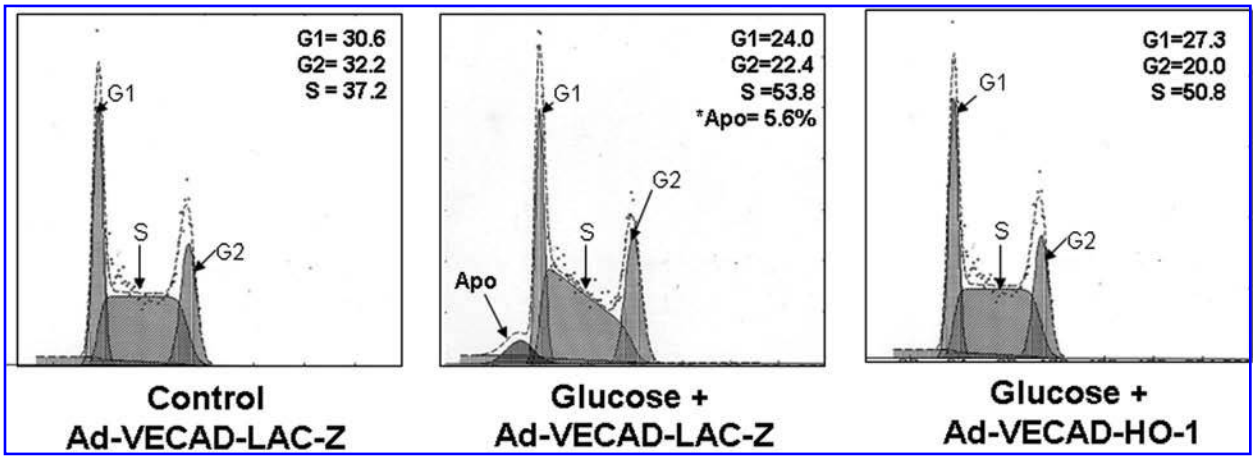

FIG. 4. Effect of high glucose on DNA distribution. Two types of endothelial cells, control cells and cells exposed to glucose, were transfected with Ad-VECAD-HO-1 or Ad-VECAD-LacZ. The cells were stained with DAPI and analyzed with flow cytometry. Representative DNA distributions are shown ($n = 4$ for each group).

the HO-1 gene using Adv-VECAD-HO-1 on glucose-induced DNA damage and cell-cycle abnormalities, DNA distribution was analyzed with flow cytometry in the two types of endothelial cells. As shown in Fig. 4 apoptosis was seen in endothelial cells transduced with Ad-VECAD-LacZ, but HO-1 induction by Ad-VECAD-HO-1 significantly reduced the apoptosis in endothelial cells exposed to high glucose levels ($p < 0.05$).

HO gene transfer downregulates caspase-3 activity in endothelial cells

We studied the effect of hyperglycemia on caspase expression and how the induction of HO in endothelial cells affects its expression. Hyperglycemia resulted in a significant increase in caspase-3 activity in endothelial cells exposed to glucose ($p < 0.05$) compared with control endothelial cells (Fig. 5). HO-1 gene transfer by Ad-VECAD-HO-1 strongly abrogated the increase in caspase-3 activity in transduced endothelial cells ($p < 0.05$). This demonstrates that selective increases in the HO-1 gene may attenuates diabetes-mediated endothelial cell death by the decrease in caspase activity.

Selective HO gene transfer upregulates BcL-xL and pAkt expression in endothelial cells

HO-1 induction has been shown to increase Bcl-xL expression through activation of the p38 MAPK and Akt pathways (51). We investigated whether selective increases in HO-1 by various agents (2, 3, 25) show a similar effect on pro- and an-

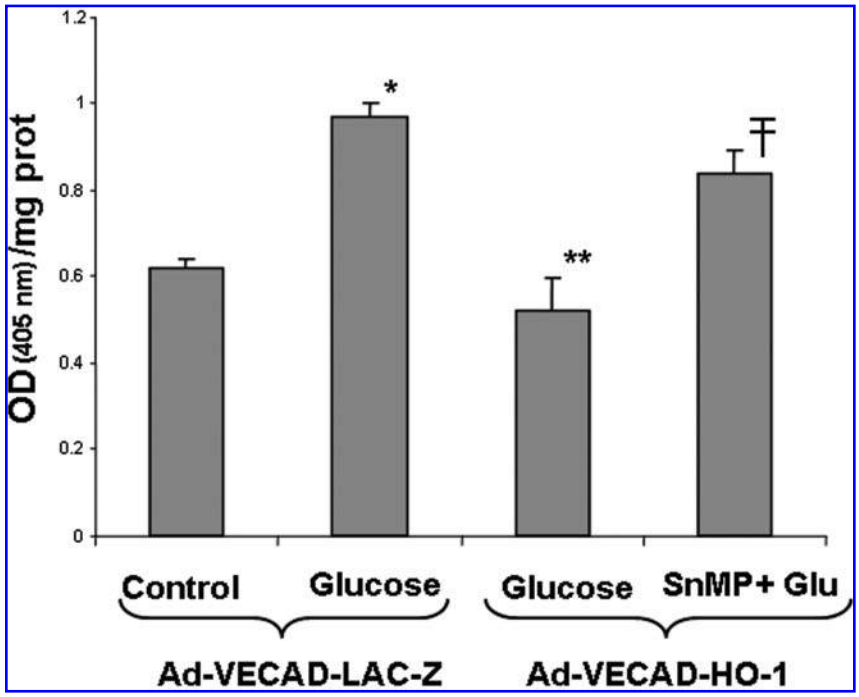

FIG. 5. Effect of HO-1 gene transfer on caspase-3 activity in endothelial cells. Caspase 3 activity in the Ad-VECAD-Lac-Z and Ad-VECAD-HO-1 and control were treated for 48 h, and caspase activity was measured as described. Results are expressed as mean ± SEM ($n = 4$ in each group). *$p = 0.001$, glucose-LAC-Z vs. control LAC-Z). **$p = 0.0006$, glucose-VECAD-HO-1 *vs.* glucose-LACZ. ‡$p = 0.009$, SnMP *vs.* glucose-VECAD-HO-1.

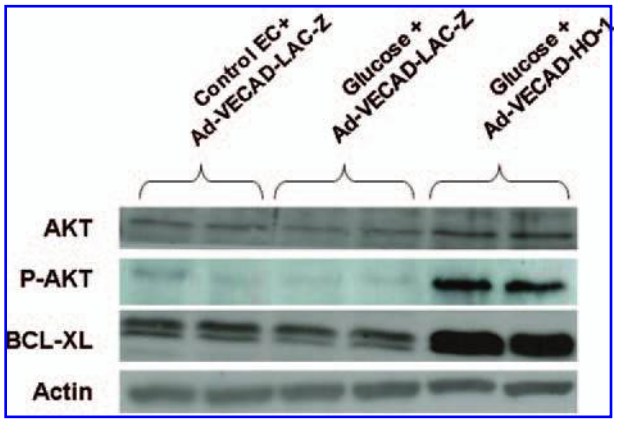

FIG. 6. Effect of HO-1 gene transfer on antiapoptotic proteins (Bcl-xL, Akt, and pAkt). Western blots of antiapoptotic proteins in endothelial cells (control endothelial cells and glucose-exposed endothelial cells) transfected with Ad-VECAD-HO-1 or Ad-VECAD-LacZ.

tiapoptotic protein expression by selective HO-1 gene transfer in endothelial cells exposed to high glucose levels. We determined the expression levels of BcL-xL and pAkt by Western blotting in cells infected with Ad-VECAD-HO-1. A significant increase in Bcl-xL expression was detected in HO-1–infected endothelial cells expressing HO-1 ($p < 0.05$). No such increase was detected in control and Ad-VECAD-HO-1–infected VSM cells. The ability of Ad-VECAD-HO-1 to upregulate Bcl-xL selectively in endothelial cells shows the selective affinity of the vector for endothelial cells (Fig. 6). Further examination of the status of Akt and phospho-Akt (SER 473), which are thought to be involved in the regulation of Bcl-xL expression, showed an association between Akt/pAkt and HO-1 induction in HO-1–expressing cells. Higher levels of Akt and pAkt were detected in Ad-VECAD-HO-1–transduced endothelial cells, whereas low levels of Akt/pAkt expression were found in control and VSM cells, confirming the selective upregulation of HO-1 in endothelial cells (see Fig. 6).

Effect of targeting HO-1 on glucose-mediated O_2^- formation

Targeting of the HO-1 gene to endothelial cells exposed to high levels of glucose was associated with a decrease in O_2^- compared with either untreated cells or cells exposed to Ad-VECAD-lacZ ($p < 0.05$) (Fig. 7). The level of O_2^- was reduced to normal levels in transduced endothelial cells compared with endothelial cells exposed to high glucose.

DISCUSSION

This study demonstrates, for the first time, that selective expression of endothelial cells with the HO-1 gene, using Ad-VECAD-HO-1 as a vector, exhibited a several-fold increase in HO-1 protein levels, which was accompanied by an increase in pAKT and decreases in caspase activity and O_2^- under hyperglycemic conditions. The Ad-VECAD-HO-1 vector did not express the HO-1 gene in control and VSM cells, showing its specificity for endothelial cells. This specific expression of HO-1 prevented glucose- and TNF-mediated cell injury. Four principal observations support this conclusion.

First, selective delivery of the human HO-1 gene attenuated the glucose-mediated oxidative stress. The increase in HO-1 activity prevented DNA degradation and oxidative damage in endothelial cells due to hyperglycemia. Several possible explanations exist as to the suppressive effect of glucose on HO activity. Zou *et al*. (59) demonstrated that high glucose *via* peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase in human aortic endothelial cells. Glucose results in deactivation of HO-1 *via* ROS generated during glucose oxidation (26). Kruger *et al*. (26) showed that the onset of diabetes coincided with an increase in HO-1 protein levels and a paradoxic decrease in HO activity in type II diabetes, which was restored by administration of Ebselen, ONOO- scavenger (26). An increase in HO-1 attenuates the increased O_2^- in diabetes, improves nitric oxide (NO) bioavailability, and prevents peroxynitrite (ONOO-) formation *via* an increase in extracellular superoxide dismutase (25, 53). The protective actions of HO-1 extend widely to such disease processes as the inflammation associated with atherosclerosis, ischemia/reperfusion injury, and renal disease (15, 16, 19, 25, 27, 28).

Peroxynitrite is a potent cytotoxic oxidant that has been shown to decrease the activity of several important enzymes (35, 59). In endothelial nitric oxide synthetase (eNOS), peroxynitrite has been shown to oxidize the cofactor tetrahydrobiopterin into inactive molecules, such as dihydrobiopterin (35). This results in a preferential increase in O_2^- production over NO production (35). Hyperglycemia-mediated oxidative stress

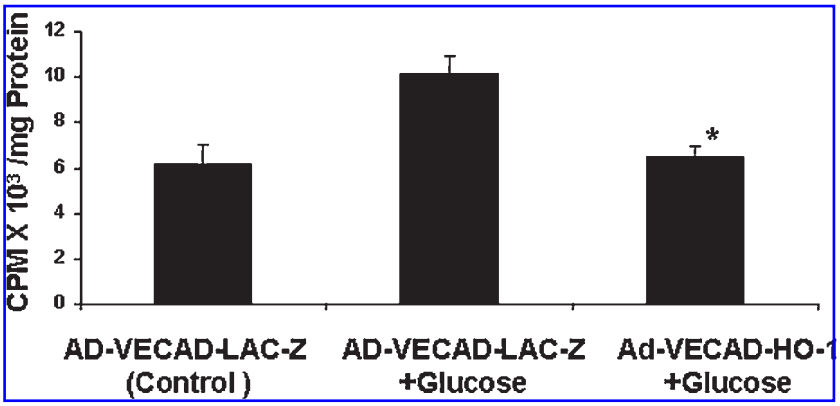

FIG. 7. Superoxide anion (O_2^-) production in the endothelial cells exposed to glucose (33 m*M*). Results are expressed as mean ± SEM, $n = 4$. *$p < 0.05$ Ad-VECAD-HO-1 *vs.* Ad-VECAD-LacZ in glucose-exposed cells.

has been associated with elevated levels of iNOS and reduced levels of eNOS in both rats and humans (37, 53). The increased generation of ROS causes endothelial injury, resulting in an accelerated rate of apoptosis and endothelial cell sloughing (10, 25). Kitamuro *et al*. (22) showed that Bach 1 functions as a hypoxia-inducible repressor for the HO-1 gene in human cells. Bach 1 is a basic leucine zipper protein. Upregulation of this factor by glucose may contribute to the decrease in HO-1 expression. We showed that high glucose decreases HO-1 promoter activity (40), which may also be regulated by hormonal levels and dexamethasone (7).

Second, the specific transduction of HO-1, using Ad-VECAD-HO-1 as vector, can decrease the apoptotic effects of TNF in endothelial cells. This protection is lost if HO-1 is inactivated by an inhibitor (SnMP). Abnormal levels of serum-soluble adhesion molecules have been detected in diabetic patients (54). High glucose and TNF increase IL-6 and adhesion molecules through the activation of NF-κB–controlled gene transcription (9, 20, 32, 41, 44). Elevation of these adhesion molecules and inflammatory cytokines (*e.g*., TNF, IL-6) is attributed to the pathogenesis of micro- and macroangiopathy in patients with diabetes (34). Wagener *et al*. (56) studied the effect of heme and TNF on HO and adhesion molecules, and suggested that upregulation of adhesion-molecule expression is associated with oxidative stress induced by hemoglobin/heme and that HO-1 may play a modulating role *via* its antioxidant properties. Thus, HO plays a crucial role in modulating adhesion molecules associated with endothelial cell activation in diabetes. The ability to induce HO in endothelial cells may be of great clinical relevance in patients with diabetes in preventing and treating vascular complications.

Third, a significant increase in Bcl-xL expression was detected in Ad-VECAD-HO-1–transduced endothelial cells expressing HO-1. The ability of Ad-VECAD-HO-1 to upregulate Bcl-xL selectively in endothelial cells further emphasizes the antiapoptotic action of HO-1 in endothelial cells. Moreover, higher levels of pAkt were detected in Ad-VECAD-HO-1–transfected endothelial cells expressing HO-1, compared with control. Increases in Bcl-xL have been reported to inhibit apoptosis *in vivo* in kidney ischemia/reperfusion injury and *in vitro* in cultured tubular cells (50). Additionally, it has been suggested that these two proteins, Bcl-xL and pAkt, can act by stabilizing the mitochondrial membranes and mitochondrial function in diabetes (8).

Phosphorylation of Akt has been demonstrated to increase ATP synthesis and the associated increase in the mitochondrial voltage-dependent anion channel, thereby blocking cytochrome *c* release and apoptosis (8). Thus, like Bcl-xL, activated Akt prevents a decline in oxidative phosphorylation, which precedes cytochrome c release and apoptosis. Fujio *et al*. (11, 12) showed that AKT signaling is essential and sufficient to protect against apoptosis, limiting infarct size induced by ischemia/reperfusion injury and promoting contractility and glucose uptake. An increase in pAKT may cause acceleration of glucose uptake *via* GLUT 4 translocation to plasma membranes (23), subsequently deceasing the rate of glucose oxidation and attenuating cardiac damage (11, 23).

Fourth, hyperglycemia results in a significant increase in caspase-3 activity in control endothelial cells, but selective HO-1 gene transfer *via* Ad-VECAD-HO-1 strongly abrogates the increase in caspase-3 activity in transfected endothelial cells expressing Ho-1; however, no such protective decrease was noted in the control. Thus, upregulation of HO-1 expression changed endothelial cells from a naïve to a defensive phenotype by producing a robust increase in pAKT and Bcl-xL and a decrease in caspase activity in endothelial cells.

In summary, the current study was intended as a "proof of principal" to determine whether targeting endothelial cells with Ad-VECAD-HO-1 may prevent hyperglycemia-mediated cell death. The enhanced HO activity, brought about by Ad-VECAD-HO-1–mediated selective HO-1 gene transfer in endothelial cells, attenuated glucose-mediated abnormalities, including DNA damage and oxidative injury to endothelial cells. Furthermore, an increase in HO-1 activity, elicited by gene transfer, may be beneficial in counteracting the endothelial dysfunction caused by hyperglycemia in diabetes. The findings of studies such as this may have important clinical and experimental relevance in the field of gene therapy and gene transfer in the pathogenesis of diabetic microangiopathy and macroangiopathy in patients.

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ABBREVIATIONS

Ang II, angiotensin II; CEC, circulating endothelial cell; EC, endothelial cell; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; HMEC, human microvessel endothelial cell; O_2^-, superoxide anion; PGF_2, prostaglandin F_2; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SnMP, tin mesoporphyrin IX dichloride; SMC, human smooth muscle cell; TNF, tumor necrosis factor; TXA_2, thromboxane A_2; TDNA, tail DNA percentage; TMOM, tail moment; VECAD, vascular endothelial cadherin.

REFERENCES

1. Abraham NG and Kappas A. Heme oxygenase and the cardiovascular-renal system. *Free Radic Biol Med* 39: 1–25, 2005.
2. Abraham NG, Kushida T, McClung J, Weiss M, Quan S, Lafaro R, Darzynkiewicz Z, and Wolin M. Heme oxygenase-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel endothelial cells. *Circ Res* 93: 507–514, 2003.
3. Abraham NG, Rezzani R, Rodella L, Kruger A, Taller D, Li VG, Goodman AI, and Kappas A. Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes. *Am J Physiol Heart Circ Physiol* 287: H2468–H2477, 2004.
4. Anderson RD, Haskell RE, Xia H, Roessler BJ, and Davidson BL. A simple method for the rapid generation of recombinant adenovirus vectors. *Gene Ther* 7: 1034–1038, 2000.
5. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J,

Gessl A, and Waldhausl W. High-glucose–triggered apoptosis in cultured endothelial cells. *Diabetes* 44: 1323–1327, 1995.
6. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, and Soares MP. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* 192: 1015–1026, 2000.
7. Deramaudt TB, daSilva JL, Remy P, Kappas A, and Abraham NG. Negative regulation of human heme oxygenase in microvessel endothelial cells by dexamethasone. *Proc Soc Exp Biol Med* 222: 185–193, 1999.
8. Di Noia MA, Van DS, Palmieri F, Yang LM, Quan S, Goodman AI, and Abraham NG. Heme oxygenase-1 enhances renal mitochondrial transport carriers and cytochrome C oxidase activity in experimental diabetes. *J Biol Chem* 281: 15687–15693, 2006.
9. Ehlermann P, Eggers K, Bierhaus A, Most P, Weichenhan D, Greten J, Nawroth PP, Katus HA, and Remppis A. Increased proinflammatory endothelial response to S100A8/A9 after preactivation through advanced glycation end products. *Cardiovasc Diabetol* 5: 6, 2006.
10. Endemann DH and Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol* 15: 1983–1992, 2004.
11. Fujio Y, Nguyen T, Wencker D, Kitsis RN, and Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 101: 660–667, 2000.
12. Fujioka M, Okuchi K, Sakaki T, Hiramatsu K, Miyamoto S, and Iwasaki S. Specific changes in human brain following reperfusion after cardiac arrest. *Stroke* 25: 2091–2095, 1994.
13. Gory S, Dalmon J, Prandini MH, Kortulewski T, de LY, and Huber P. Requirement of a GT box (Sp1 site) and two Ets binding sites for vascular endothelial cadherin gene transcription. *J Biol Chem* 273: 6750–6755, 1998.
14. Griendling KK, Sorescu D, and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494–501, 2000.
15. Haugen EN, Croatt AJ, and Nath KA. Angiotensin II induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro. *Kidney Int* 58: 144–152, 2000.
16. Hayashi S, Takamiya R, Yamaguchi T, Matsumoto K, Tojo SJ, Tamatani T, Kitajima M, Makino N, Ishimura Y, and Suematsu M. Induction of heme oxygenase-1 suppresses venular leukocyte adhesion elicited by oxidative stress: role of bilirubin generated by the enzyme. *Circ Res* 85: 663–671, 1999.
17. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A, Meinertz T, Griendling K, Harrison DG, Forstermann U, and Munzel T. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* 88: E14–E22, 2001.
18. Ii M, Takenaka H, Asai J, Ibusuki K, Mizukami Y, Maruyama K, Yoon YS, Wecker A, Luedemann C, Eaton E, Silver M, Thorne T, and Losordo DW. Endothelial progenitor thrombospondin-1 mediates diabetes-induced delay in reendothelialization following arterial injury. *Circ Res* 98: 697–704, 2006.
19. Ishikawa K, Navab M, Leitinger N, Fogelman AM, and Lusis AJ. Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL. *J Clin Invest* 100: 1209–1216, 1997.
20. Kalogeris TJ, Kevil CG, Laroux FS, Coe LL, Phifer TJ, and Alexander JS. Differential monocyte adhesion and adhesion molecule expression in venous and arterial endothelial cells. *Am J Physiol* 276: L9–L19, 1999.
21. Katusic ZS. Superoxide anion and endothelial regulation of arterial tone. *Free Radic Biol Med* 20: 443–448, 1996.
22. Kitamuro T, Takahashi K, Ogawa K, Udono RF, Takeda K, Furuyama K, Nakayama M, Sun J, Fujita H, Hida W, Hattori T, Shirato K, Igarashi K, and Shibahara S. Bach 1 functions as a hypoxia-inducible repressor for the heme oxygenase-1 gene in human cells. *J Biol Chem* 14: 9125–9133, 2003.
23. Kovacic S, Soltys CL, Barr AJ, Shiojima I, Walsh K, and Dyck JR. Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. *J Biol Chem* 278: 39422–39427, 2003.
24. Koya D, Hayashi K, Kitada M, Kashiwagi A, Kikkawa R, and Haneda M. Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats. *J Am Soc Nephrol* 14: S250–S253, 2003.
25. Kruger AL, Peterson S, Turkseven S, Kaminski PM, Zhang FF, Quan S, Wolin MS, and Abraham N.G. D-4F induces heme oxygenase-1 and extracellular superoxide dismutase, decreases endothelial cell sloughing and improves vascular reactivity in rat model of diabetes. *Circulation* 23: 3126–3134, 2005.
26. Kruger AL, Peterson SJ, Schwartzman ML, Fusco H, McClung JA, Weiss M, Shenouda S, Goodman AI, Goligorsky MS, Kappas A, and Abraham NG. Upregulation of heme oxygenase provides vascular protection in an animal model of diabetes through its antioxidant and anti-apoptotic effects. *J Pharmacol Exp Ther* 319: 1144–1152, 2006.
27. Laniado-Schwartzman M, Conners MS, Dunn MW, Levere RD, Kappas A, and Abraham NG. Heme oxygenase induction with attenuation of experimentally induced corneal inflammation. *Biochem Pharmacol* 53: 1069–1075, 1997.
28. Li P, Jiang H, Yang L, Quan S, Dinocca S, Rodriguez F, Abraham NG, and Nasjletti A. Angiotensin II induces carbon monoxide production in the perfused kidney: relation to protein kinase C activation. *Am J Physiol* Renal Physiol 287: F914–F920, 2004.
29. Liadis N, Murakami K, Eweida M, Elford AR, Sheu L, Gaisano HY, Hakem R, Ohashi PS, and Woo M. Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus. *Mol Cell Biol* 25: 3620–3629, 2005.
30. Liao F, Li Y, O'Connor W, Zanetta L, Bassi R, Santiago A, Overholser J, Hooper A, Mignatti P, Dejana E, Hicklin DJ, and Bohlen P. Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis. *Cancer Res* 60: 6805–6810, 2000.
31. Lorenzi M, Nordberg JA, and Toledo S. High glucose prolongs cell-cycle traversal of cultured human endothelial cells. *Diabetes* 36: 1261–1267, 1987.
32. Luo Y, Kwon HJ, Montano S, Georgiadis M, Goebl MG, and Harrington MA. Phosphorylation of Simpl modulates Rela associated NF-κB dependent transcription. *Am J Physiol Cell Physiol* 292: C1013–C1023, 2006.
33. McClung JA, Naseer N, Saleem M, Rossi GP, Weiss MB, Abraham NG, and Kappas A. Circulating endothelial cells are elevated in patients with type 2 diabetes mellitus independently of HbA(1)c. *Diabetologia* 48: 345–350, 2005.
34. Meleth AD, Agron E, Chan CC, Reed GF, Arora K, Byrnes G, Csaky KG, Ferris FL, III, and Chew EY. Serum inflammatory markers in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 46: 4295–4301, 2005.
35. Milstien S and Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* 263: 681–684, 1999.
36. Nobe K, Miyatake M, Sone T, and Honda K. High-glucose-altered endothelial cell function involves both disruption of cell-to-cell connection and enhancement of force development. *J Pharmacol Exp Ther* 318: 530–539, 2006.
37. Okon EB, Chung AW, Rauniyar P, Padilla E, Tejerina T, McManus BM, Luo H, and van Breemen C. Compromised arterial function in human type 2 diabetic patients. *Diabetes* 54: 2415–2423, 2005.
38. Piconi L, Quagliaro L, Assaloni R, Da RR, Maier A, Zuodar G, and Ceriello A. Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction. *Diabetes Metab Res Rev* 22: 198–203, 2006.
39. Pieper GM, Meier DA, and Hager SR. Endothelial dysfunction in a model of hyperglycemia and hyperinsulinemia. *Am J Physiol* 269: H845–H850, 1995.
40. Quan S, Kaminski PM, Yang L, Morita T, Inaba M, Ikehara S, Goodman AI, Wolin MS, and Abraham NG. Heme oxygenase-1 prevents superoxide anion associated endothelial cell sloughing in diabetic rats. *Biochem Biophys Res Commun* 315: 509–516, 2004.
41. Ramana KV, Friedrich B, Srivastava S, Bhatnagar A, and Srivastava SK. Activation of nuclear factor-kappaB by hyperglycemia in vascular smooth muscle cells is regulated by aldose reductase. *Diabetes* 53: 2910–2920, 2004.
42. Recchioni R, Marcheselli F, Moroni F, and Pieri C. Apoptosis in

human aortic endothelial cells induced by hyperglycemic condition involves mitochondrial depolarization and is prevented by N-acetyl-L-cysteine. *Metabolism* 51: 1384–1388, 2002.
43. Rodella L, Lamon B, Rezzani R, Colombrita C, Sangras B, Scapagnini G, Falck JR, and Abraham NG. Carbon monoxide and biliverdin prevent oxidant production and endothelial cell sloughing in rats with type I diabetes. *Free Radic Biol Med* 40: 2198–2205, 2006.
44. Ruan H, Hacohen N, Golub TR, Van PL, and Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* 51: 1319–1336, 2002.
45. Sacerdoti D, Olszanecki R, Li VG, Colombrita C, Scapagnini G, and Abraham NG. Heme oxygenase overexpression attenuates glucose-mediated oxidative stress in quiescent cell phase: linking heme to hyperglycemia complications. *Curr Neurovasc Res* 2: 103–111, 2005.
46. Shahab A. Why does diabetes mellitus increase the risk of cardiovascular disease? *Acta Med Indones* 38: 33–41, 2006.
47. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE, and Choi AM. Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J Immunol* 172: 1220–1226, 2004.
48. Stehouwer CD, Lambert J, Donker AJ, and van Hinsbergh VW. Endothelial dysfunction and pathogenesis of diabetic angiopathy. *Cardiovasc Res* 34: 55–68, 1997.
49. Stocker R and Keaney JF Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381–1478, 2004.
50. Tracz MJ, Alam J, and Nath KA. Physiology and pathophysiology of heme: implications for kidney disease. *J Am Soc Nephrol* 18: 414–420, 2007.
51. Tsui TY, Siu YT, Schlitt HJ, and Fan ST. Heme oxygenase-1-derived carbon monoxide stimulates adenosine triphosphate generation in human hepatocyte. *Biochem Biophys Res Commun* 336: 898–902, 2005.
52. Turkseven S, Drummond G, Rezzani R, Rodella L, Quan S, Ikehara S, and Abraham N.G. Impact of silencing HO-2 on EC-SOD and the mitochondrial signaling pathway. *J Cell Biochem* 100: 815–823, 2007.
53. Turkseven S, Kruger A, Mingone CJ, Kaminski P, Inaba M, Rodella L, Ikehara S, Wolin MS, and Abraham NG. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. *Am J Physiol Heart Circ Physiol* 289: H701–H707, 2005.
54. Utsumi K, Kawabe M, Hirama A, Ueda K, Kamada Y, Arii K, Komaba Y, Katsura K, Iino Y, and Katayama Y. Effects of selective LDL apheresis on plasma concentrations of ICAM-1, VCAM-1 and P-selectin in diabetic patients with arteriosclerosis obliterans and receiving maintenance hemodialysis. *Clin Chim Acta* 377: 198–200, 2007.
55. Vendrame F, Santangelo C, Misasi R, Dionisi S, Gizzi C, Realacci M, Grassetti D, Di MU, and Dotta F. Defective lymphocyte caspase-3 expression in type 1 diabetes mellitus. *Eur J Endocrinol* 152: 119–125, 2005.
56. Wagener FA, da Silva JL, Farley T, de Witte T, Kappas A, and Abraham NG. Differential effects of heme oxygenase isoforms on heme mediation of endothelial intracellular adhesion molecule 1 expression. *J Pharmacol Exp Ther* 291: 416–423, 1999.
57. Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, and Lee PJ. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. *J Biol Chem* 278: 1248–1258, 2003.
58. Zou MH, Cohen R, and Ullrich V. Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. *Endothelium* 11: 89–97, 2004.
59. Zou MH, Shi C, and Cohen RA. High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H2 receptor-mediated apoptosis and adhesion molecular expression in cultured human aortic endothelial cells. *Diabetes* 51: 198–203, 2002.

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2. Areum Daseul Kim, Rui Zhang, Kyoung Ah Kang, Ho Jin You, Kyung Goo Kang, Jin Won Hyun. 2012. Jeju Ground Water Containing Vanadium Enhances Antioxidant Systems in Human Liver Cells. *Biological Trace Element Research* **147**:1-3, 16-24. [CrossRef]
3. Xian-pei Heng, Ke-ji Chen, Zhen-feng Hong, Wei-dong He, Ke-dan Chu, Jiu-mao Lin, Hai-xia Zheng, Liu-qing Yang, Su-ping Huang, Yuan-long Lan, Ling Chen, Fang Guo. 2012. Toxicity features of high glucose on endothelial cell cycle and protection by Dan Gua-Fang (###) in ECV-304 in high glucose medium. *Chinese Journal of Integrative Medicine* . [CrossRef]
4. Jian Cao , Komal Sodhi , Kazuyoshi Inoue , John Quilley , Rita Rezzani , Luigi Rodella , Luca Vanella , Lucrezia Germinario , David E. Stec , Nader G. Abraham , Attallah Kappas . 2011. Lentiviral-Human Heme Oxygenase Targeting Endothelium Improved Vascular Function in Angiotensin II Animal Model of Hypertension. *Human Gene Therapy* **22**:3, 271-282. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
5. Xinghua Cheng , Richard C.M. Siow , Giovanni E. Mann . 2011. Impaired Redox Signaling and Antioxidant Gene Expression in Endothelial Cells in Diabetes: A Role for Mitochondria and the Nuclear Factor-E2-Related Factor 2-Kelch-Like ECH-Associated Protein 1 Defense Pathway. *Antioxidants & Redox Signaling* **14**:3, 469-487. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
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7. F. A. D. T. G. Wagener, D. Dekker, J. H. Berden, A. Scharstuhl, J. Vlag. 2009. The role of reactive oxygen species in apoptosis of the diabetic kidney. *Apoptosis* **14**:12, 1451-1458. [CrossRef]
8. Savita Khanna , Han-A Park , Chandan K. Sen , Trimurtulu Golakoti , Krishanu Sengupta , Somepalli Venkateswarlu , Sashwati Roy . 2009. Neuroprotective and Antiinflammatory Properties of a Novel Demethylated Curcuminoid. *Antioxidants & Redox Signaling* **11**:3, 449-468. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
9. Naglaa K. Idriss, Andrew D. Blann, Gregory Y.H. Lip. 2008. Hemoxygenase-1 in Cardiovascular Disease. *Journal of the American College of Cardiology* **52**:12, 971-978. [CrossRef]
10. Jozef Dulak . 2007. Changing Faces of Heme Oxygenases. *Antioxidants & Redox Signaling* **9**:12, 2043-2048. [Citation] [Full Text PDF] [Full Text PDF with Links]