Heme Oxygenase-2 Deletion Causes Endothelial Cell Activation Marked by Oxidative Stress, Inflammation, and Angiogenesis

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ABSTRACT

In previous studies, we have shown that heme oxygenase (HO)-2 null [HO-2(–/–)] mice exhibit a faulty response to injury; chronic inflammation and massive neovascularization replaced resolution of inflammation and tissue repair. Endothelial cells play an active and essential role in the control of inflammation and the process of angiogenesis. We examined whether HO-2 deletion affects endothelial cell function. Under basal conditions, HO-2(–/–) aortic endothelial cells (mAEC) showed a 3-fold higher expression of vascular endothelial growth factor receptor 1 and a marked angiogenic response compared with wild-type (WT) cells. Compared with WT cells, HO-2(–/–) mAEC showed a 2-fold reduction in HO activity and marked

increases in levels of gp91^{phox}/NADPH oxidase isoform, superoxide, nuclear factor κ B activation, and expression of inflammatory cytokines, including interleukin (IL)-1 α and IL-6. HO-2 deletion transforms endothelial cells from a “normal” to an “activated” phenotype characterized by increases in inflammatory, oxidative, and angiogenic factors. This switch may be the result of reduced HO activity and the associated reduction in the cytoprotective HO products, carbon monoxide and biliverdin/bilirubin, because addition of biliverdin to HO-2(–/–) cells attenuated angiogenesis and reduced superoxide production. This transformation underscores the importance of HO-2 in the regulation of endothelial cell homeostasis.

The integrity of the vascular endothelium is critical for the maintenance of vascular homeostasis. This layer of cells actively participates in the regulation of vascular tone, blood fluidity, growth of vascular smooth muscle cells, and local inflammation by synthesizing and releasing paracrine factors in response to humoral, mechanical, and neural stimuli. Under normal conditions, the endothelium maintains a vasodilatory, antithrombotic, and anti-inflammatory state. One of the systems that contribute to the maintenance of this state is the heme-heme oxygenase (HO) system.

HO is the rate-limiting enzyme in heme catabolism. It

cleaves heme into iron, sequestered by ferritin, carbon monoxide (CO), and biliverdin, which is reduced to bilirubin by biliverdin reductase (Abraham and Kappas, 2008). Two isoforms, HO-1 and HO-2, are the major source of HO activity in most tissues. Both are alike in terms of mechanisms of heme oxidation, cofactor and substrate specificity, and susceptibility to inhibition by porphyrins (Maines, 1988; Abraham and Kappas, 2008). They differ in their postulated function; HO-2 functions as the constitutive HO activity contributing to cell homeostasis, whereas HO-1 expression is relatively low in most normal tissues. After injury, however, HO-1 expression is greatly enhanced to play a significant role in cytoprotection (Abraham and Kappas, 2008).

The catalytic activity of HO is considered the underlying principle of HO cytoprotective actions. Heme functions as a double-edged sword in that in moderate quantities and when bound to protein, heme forms an essential element for various biological processes, but when accumulated or unleashed

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ABBREVIATIONS: HO, heme oxygenase; CO, carbon monoxide; HO-2(–/–), HO-2 null; EGM-MV, microvascular endothelial cell growth medium; PCR, polymerase chain reaction; mAEC, mouse aortic endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EC-SOD, extracellular superoxide dismutase; eNOS, endothelial nitric oxide synthase; NF- κ B, nuclear factor κ B; NOX, NADPH oxidase isoform; VCAM, vascular cell adhesion molecule; DHE, dihydroethidium; WT, wild-type; ROS, reactive oxygen species; peNOS, phosphorylated endothelial nitric oxide synthase; IL, interleukin.

in large amounts, it is a pro-oxidant and enhances oxidative stress and inflammation (Wagener et al., 2003). Hence, the antioxidant effects arise, in part, from the capacity of HO to degrade the heme from destabilized hemoproteins (Nath et al., 2000) while reducing heme availability for oxidative reactions (Jeney et al., 2002). On the other hand, CO and bilirubin protect against tissue damage by exerting antioxidant (Stocker et al., 1987) and anti-inflammatory effects, including inhibition of adhesion molecules, leukocyte recruitment (Wagener et al., 2003), and suppression of cytokine/chemokine expression (Otterbein et al., 2000; Minamino et al., 2001; Sarady-Andrews et al., 2005).

Even though catalytic activity is the primary basis of HO cytoprotective properties, the contribution of HO-2, the main source of bilirubin and CO in uninjured tissues, to cytoprotection has been largely unexplored. In recent reports, we have showed that deletion of the *HO-2* gene markedly impairs the inflammatory and reparative response of the cornea to injury and leads to unresolved corneal inflammation and chronic inflammatory complications, including ulceration, perforation, and neovascularization (Seta et al., 2006; Bellner et al., 2008). In the current study, we explore possible mechanisms underlying the increased susceptibility of the HO-2 null [HO-2(–/–)] mice to neovascularization by examining whether HO-2 deletion affects the phenotype of endothelial cells, the major player in the neovascular response.

Materials and Methods

Cell Isolation. All the animal experiments followed an institutionally approved protocol in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). The HO-2(–/–) mice are direct descendents of the HO-2 mutants produced by Poss et al. (1995). These well characterized HO-2(–/–) mice have a C57BL/6 × 129/Sv genetic background (Rogers et al., 2003), which was used on age- and gender-matched controls. Mice were anesthetized with ketamine (50 mg/kg) and xylazine (20 mg/kg) intramuscularly; blood was withdrawn by heart puncture; and the abdominal aortas were dissected. Serum cytokines were measured by enzyme-linked immunosorbent assay (Search-Light array; Aushon Biosystems Inc., Billerica, MA). Isolated aortas free of connective tissue were dipped in 70% alcohol to inhibit fibroblast cell growth and placed in complete microvascular endothelial cell growth medium (EGM-MV; Lonza Walkersville, Inc., Walkersville, MD). Aortas were cut into rings, and opened sections were placed with the inside facing down onto Matrigel drops (Becton Dickinson, Franklin Lakes, NJ) and immersed in 0.5 ml of EGM-MV. Two weeks later, aortic segments were removed, and cells were harvested with trypsin/EDTA (Invitrogen, Carlsbad, CA). Cells were transferred to six-well plates and grown in EGM-MV to confluence. The endothelial phenotype was routinely verified by staining for von Willebrand factor and with *Ulex* lectin (Sigma-Aldrich, St. Louis, MO) and by uptake of acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA). Cells were also examined for HO-2 mRNA levels by real-time polymerase chain reaction (PCR) to ascertain their genotype. Cells of passages 3 through 12 were used for all the experiments.

In Vitro Angiogenesis Assay. Assessment of in vitro capillary tube-like formation used growth factor-reduced Matrigel as described previously (Mezentsev et al., 2002). In brief, Matrigel was thawed overnight at 4°C and mixed to homogeneity using cooled pipette tips. Aliquots of Matrigel (200 μ l) were placed in 24-well cell culture plates and left to polymerize at 37°C for 30 min. Quiescent mouse aortic endothelial cells (mAECs) were resuspended in endothelial basal medium containing 0.5% fetal bovine serum, plated

onto the Matrigel-coated surface (5×10^4 cells/well), and incubated for 1 h in a 37°C humidified incubator. The medium was aspirated, and fresh medium containing vehicle or vascular endothelial growth factor (VEGF) (50 ng/ml), biliverdin (10 μ M), SN50 (10 μ M), and vascular endothelial growth factor receptor (VEGFR)-1 inhibitory antibody (20 μ g/ml) was added. Tube-like structure formation was examined 24 h after treatment. Cultures were photographed; the length of the tube-like structures and the number of lumen were quantified using Image Pro-Express Software (MediaCybernetics, Inc., Bethesda, MD).

Genotyping and Real-Time PCR. DNA was isolated from the tail and genotyped using HO-2 and *neo*-specific primers as described previously (Rogers et al., 2003). Total RNA was isolated with RNeasy Protect Mini Kit (QIAGEN, Valencia, CA) and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription reaction of total RNA (400 ng) was performed using the Superscript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using QuantiTect SYBR Green PCR Kit (QIAGEN) and the Mx3000 real-time PCR system (Stratagene, La Jolla, CA) as described previously (Bellner et al., 2008). Specific primers were designed based on published sequences (GenBank). RNA expression of target genes was calculated based on the real-time PCR efficiency and the threshold crossing point and is expressed in comparison to the reference gene *18S*. The results were analyzed by the comparative threshold cycle method.

Western Blot Analysis. Cells were lysed in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN) and 1 mM phenylmethanesulfonyl fluoride. Unbroken cells or cell debris was removed by centrifugation at 10,000g for 15 min at 4°C, and protein concentration was determined by the Bradford (1976) method. Nuclear and cytosolic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Immunoblotting was performed using the following primary antibodies: anti-HO-1, anti-HO-2, and anti-extracellular superoxide dismutase (EC-SOD) (Assay Designs, Ann Arbor, MI); anti-phospho(S1177)-endothelial nitric oxide synthase (eNOS) (1:1000); anti-nuclear factor κ B (NF- κ B) p65, anti-phospho-NF- κ B p65, and gp91phox/NADPH oxidase isoform (NOX)-2 (Cell Signaling Technology Inc., Danvers, MA); anti-eNOS, anti-vascular cell adhesion molecule (VCAM)-1, and anti-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-histone h3 (Abcam Inc., Cambridge, MA); and β -actin antibody (Sigma-Aldrich). Secondary antibodies were either horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology, Inc.) or anti-mouse (Santa Cruz Biotechnology, Inc.; GE Healthcare, Piscataway, NJ).

HO Activity. The amount of HO-dependent CO was measured as described previously (Seta et al., 2006). In brief, cells ($0.5\text{--}1 \times 10^6$) were incubated in 1 ml of oxygenated Krebs' solution containing NADPH (1 mM) with and without chromium mesoporphyrin (10 μ M), an inhibitor of HO activity, for 30 min in sealed vials. Subsequently, internal standards made of isotopically labeled CO ($^{13}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{18}\text{O}$) were injected into the vials, and the CO content of the headspace gas was determined by gas chromatography/mass spectroscopy using an HP-5989A mass spectrometer interfaced to an HP-5890 gas chromatograph (Hewlett Packard, Palo Alto, CA). Aliquots (100 μ l) of the headspace gas were injected using a gas-tight syringe into the splitless injector having a temperature of 120°C. Abundance of ions at 28, 29, and 31 mass-to-charge ratio (*m/z*) corresponding to $^{12}\text{C}^{16}\text{O}$, $^{13}\text{C}^{16}\text{O}$, and $^{13}\text{C}^{18}\text{O}$, respectively, was acquired via selected ion monitoring. The amount of CO in samples was calculated from standard curves constructed with abundance of ions at 28, 29, or 31 *m/z*. Both standard curves were linear over the range of 0.01 to 5.0 μ M, and both yielded comparable results when used for determining the concentration of endogenous CO. The sensitivity of

the assay is 1 pmol of CO. The results were expressed as picomoles of CO per 10^6 cells.

Detection of Superoxide by Fluorescence Microscopy. Dihydroethidium (DHE; Invitrogen) is widely used as a superoxide probe because two-electron oxidation by superoxide of membrane-permeable DHE results in the impermeable fluorescent product ethidium (Peshavariya et al., 2007). Superoxide-specific DHE fluorescence was determined as the difference between fluorescence intensity in the absence and presence of Tiron, a superoxide-selective scavenger. Cells were cultured on four-well chamber slides at 37°C to ~70% confluence. Cells were washed and incubated in Hanks' balanced salt solution with and without Tiron (10 mM) and in the presence or absence of biliverdin (10 μ M) for 30 min before loading with DHE (5 μ M) for an additional 30 min. Cells were then washed with Hanks' balanced salt solution, and images (four to six per well) were acquired using an Olympus (Center Valley, PA) FluoView FV300 confocal laser-scanning microscope. Fluorescence intensity was measured using Axiovision 4.6 imaging software (Carl Zeiss Inc., Thornwood, NY). Parallel image acquisitions of control and treated cells were performed with fixed parameters. Background fluorescence was estimated by capturing an image in an area free of cells and subtracted from the fluorescence intensity of cells on the same slide. Each treatment group was performed in triplicate. To minimize any potential artifactual fluorescence due to visible light, the entire procedure was performed under dark conditions. Results are presented as the difference in fluorescence intensity between control and Tiron-treated cells normalized to the number of cells.

NF- κ B Immunofluorescence. Cells were grown on four-chamber glass slides (Lab Tek; Thermo Fisher Scientific) at a density of 10,000 cells/well. Cells were fixed, immunostained using rabbit anti-

NF- κ B subunit p65 antibody (1:50; Santa Cruz Biotechnology, Inc.) and Cy-3 conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and counterstained for nuclei with 4',6-diamino-2-phenylindole as described previously (Ishizuka et al., 2008). Immunofluorescence was visualized using an Axioplan-2 fluorescent microscope (Carl Zeiss Inc.). Cell images were captured and analyzed using the AxioVision 4.6 image processing software (Carl Zeiss Inc.). The number of NF- κ B-positive cells versus the total number of cells was measured in four randomly chosen fields for each experiment. The results were based on three independent analyses.

Statistical Analysis. Results are expressed as the mean $\pm$ S.E.M. Significance of difference in mean values was determined using either the *U* test or one-way analysis of variance followed by Newman-Keuls post hoc test for multiple comparisons; *p* < 0.05 was considered to be significant.

Results

HO Expression and Activity. Cells derived from HO-2(−/−) mice lacked expression of HO-2 mRNA and protein (Fig. 1A) while expressing significantly higher levels of HO-1 protein, compared with wild-type (WT) cells (Fig. 1B). However, assessment of HO activity indicated that despite the high levels of HO-1 protein in HO-2(−/−) cells, the HO activity (CO production) was more than 2-fold lower in HO-

Fig. 1. A, representative gels depicting genotyping (top), HO-2 mRNA (middle), and protein (bottom) in HO-2(−/−) (KO) cells. B, representative blot and densitometry analysis of HO-1 in WT and HO-2(−/−) (KO) mAECS (*n* = 4; *, *p* < 0.05). C, HO-dependent CO levels in WT and KO cells (*n* = 3; *, *p* < 0.05). D, representative blot and densitometry analysis of HO-1 protein in nuclear (N) and cytosolic (C) extracts from mAECS (*n* = 4; **, *p* < 0.01); glyceraldehyde-3-phosphate dehydrogenase and histone h3 were used to assess purity of cytosolic and nuclear fractions, respectively.

Fig. 2. A, representative images of DHE staining of WT and HO-2(−/−) (KO) mAECS in the absence and presence of Tiron (10 mM) or biliverdin (10 μ M). B, quantitative analysis of DHE fluorescence intensity (arbitrary units). Results are expressed as the Tiron-sensitive DHE fluorescence corrected for the number of cells (*n* = 5–9; ***, *p* < 0.001 compared with WT; ‡, *p* < 0.01 to control).

2(−/−) cells compared with WT cells (Fig. 1C). It is interesting to note that a larger percentage of HO-1 in HO-2(−/−) cells was present in the nuclear fraction (Fig. 1D).

Increased Oxidative Stress in HO-2(−/−) Cells. Reduction in HO activity in cells and tissues is frequently associated with increased oxidative stress. Assessment of superoxide levels using DHE fluorescence indicated higher levels in HO-2(−/−) cells compared with WT cells. Treatment of cells with Tiron, a superoxide-specific scavenger, markedly reduced DHE fluorescence (Fig. 2A). Quantitative analysis of DHE fluorescence intensity corrected for Tiron suggested that HO-2(−/−) mAEC produced 2-fold more superoxide anion than WT cells (Fig. 2B). Moreover, treatment of HO-2(−/−) mAEC with biliverdin significantly reduced DHE fluorescence by $68.5 \pm 13.3\%$ (Fig. 2B).

NAD(P)H oxidase is a major source of superoxide in vascular cells, including endothelial cells. Among Nox homologs, gp91phox/Nox2 and Nox4 are the major ones expressed in endothelial cells (Ago et al., 2004). Real-time PCR analysis revealed that gp91phox/Nox2 mRNA levels were 2-fold higher in HO-2-deficient mAECs compared with WT cells (Fig. 3A), whereas Nox4 mRNA levels were unchanged (Fig.

3B). Western blot analysis showed a significant 60% increase in the protein levels of gp91phox/Nox2 in mAECs from HO-2(−/−) mice compared with cells from WT mice (Fig. 3C). In contrast, protein levels of EC-SOD, a major antioxidant enzyme, were significantly lower in HO-2(−/−) cells compared with WT cells (Fig. 3D). Uncoupled eNOS presents another source for reactive oxygen species (ROS) (Vásquez-Vivar et al., 1998). In HO-2-deficient cells, levels of eNOS protein were significantly lower compared with WT cells, as were the levels of phosphorylated eNOS (peNOS), leading to a significant decrease in the peNOS/eNOS ratio and suggesting a reduced eNOS activity (Fig. 3E).

NF- κ B Is Activated in HO-2(−/−) Endothelial Cells. NF- κ B is a key transcriptional factor whose activation is the driving force of increased inflammatory gene expression. As seen in Fig. 4, nuclear expression of NF- κ B in HO-2(−/−) cells measured by immunofluorescence was greater than that in WT cells. Quantitative image analysis, which measured the number of cells with nuclear NF- κ B (p65) immunofluorescence relative to the number of cells in a given field, indicated an increase of $76 \pm 9\%$ in nuclear localization of NF- κ B (p65). This was further confirmed by Western blot analysis showing that the relative nuclear to cytoplasm expression of phospho-NF- κ B (p65) alone and normalized to levels of NF- κ B (p65) was 50% and 2-fold higher, respectively, in HO-2(−/−) cells compared with WT cells (Fig. 5). The increased inflammatory phenotype in the HO-2(−/−)

Fig. 3. Expression of NOX-2 (gp91phox) (A) and NOX-4 (B) mRNA in HO-2(−/−) (KO) and WT mAECs ($n = 4-11$; *, $p < 0.05$). Western blots and densitometry analysis of gp91phox (C), EC-SOD (D), and eNOS and peNOS (E) at serine 1177 ($n = 3-4$; *, $p < 0.05$; **, $p < 0.01$).

Fig. 4. NF- κ B activation in WT and HO-2(−/−) (KO) mAECs. Representative photographs showing the cellular localization of NF- κ B (red). Nuclei were stained with 4',6-diamino-2-phenylindole (blue). Quantitative image analysis presents the percentage of cells with nuclear NF- κ B expression ($n = 4$; *, $p < 0.05$).

Fig. 5. NF- κ B expression in cytosolic and nuclear extracts of mAECs from HO-2(-/-) (KO) and WT mice. Representative Western blot of NF- κ B and its phosphorylated form and densitometry analysis showing the nuclear to cytosol ratio of phosphorylated NF- κ B and the phosphorylated NF- κ B to NF- κ B ratio ($n = 3$; *, $p < 0.05$).

mice was also evident in vivo. At the time of mAEC isolation, serum levels of inflammatory cytokines, including tumor necrosis factor- α , interleukin (IL)-1 α , and IL-6, were several-fold higher in HO-2(-/-) mice compared with WT mice

(Fig. 6, A–C). Consequently, HO-2(-/-) mAECs expressed 2-fold higher mRNA levels of IL-1 α and IL-6 than WT mAECs (Fig. 6, D and E). Moreover, the expression level of VCAM-1, a key adhesion molecule that typified endothelial activation, was 2-fold higher in HO-2(-/-) mAECs compared with WT cells (Fig. 6F).

Expression of VEGFs and VEGFRs. VEGF is a key angiogenic factor, and its expression levels influence the activation state of endothelial cells. It is possible that the angiogenic phenotype of the HO-2(-/-) cells is driven by increased expression of the VEGF and/or its receptors. As seen in Fig. 7, mRNA levels of VEGFR-1 were 3-fold higher ($p < 0.01$) in HO-2(-/-) cells compared with WT cells. In contrast, both VEGFR-2 and VEGFR-3 mRNA levels were significantly lower ($p < 0.05$) in HO-2(-/-) mice by 50% and 40-fold, respectively. The mRNA expression levels of VEGF-A and -D were largely unchanged, whereas levels of VEGF-C were lower ($p < 0.01$) in HO-2(-/-) cells compared with WT cells (Fig. 7).

HO-2-Deficient Endothelial Cells Exhibit a Pronounced Angiogenic Phenotype. As seen in Fig. 8A, HO-2-deficient endothelial cells were more angiogenic than cells isolated from WT mice when placed onto Matrigel-coated plates (Fig. 8A). Quantitative analysis indicated that HO-2(-/-) cells were more ($p < 0.001$) angiogenic, displaying longer capillary length and higher number of lumen than WT

Fig. 6. Inflammatory markers in serum and mAECs from WT and HO-2(-/-) (KO) mice. Serum levels of tumor necrosis factor- α (A), IL-1 α (B), and IL-6 (C) ($n = 4$; *, $p < 0.05$ and **, $p < 0.01$). Real-time PCR analysis of IL-1 α (D) and IL-6 (E) mRNA ($n = 11$; *, $p < 0.05$). F, Western blot and densitometry analysis of VCAM-1 ($n = 3$).

Fig. 7. Expression of VEGF-A, -C, and -D and VEGFR-1, -2, and -3 in mAECs from HO-2(-/-) (KO) and WT mice. Real-time PCR analysis is given as relative expression of VEGFR-1, -2, and -3 and VEGF-A, -C, and -D mRNA ($n = 4-6$; *, $p < 0.05$; **, $p < 0.01$).

cells (Fig. 8B). Addition of VEGF increased the angiogenic response of WT cells but had no significant effect on HO-2(-/-) cells (Fig. 8B). It is noteworthy that treatment of HO-2(-/-) cells with biliverdin significantly reduced the angiogenic activity (Fig. 8B), suggesting that deficiency in HO activity contributes to the angiogenic phenotype of the HO-2(-/-) cells. The angiogenic activity in HO-2(-/-) cells was also attenuated by treatment with the NF- κ B inhibitor, SN50, and by VEGFR1 inhibitory antibody (Fig. 8C), suggesting that both pathways play a part in this excessive angiogenesis.

Discussion

Overexpression of HO-1 enhances endothelial cell proliferation, thereby linking the HO system to angiogenesis. Recent studies confirmed that HO-1 promotes endothelial cell cycle progression and showed that suppression of HO-1 expression decreased endothelial cell proliferation and capillary formation in vitro (Li Volti et al., 2005). Others showed that angiogenic stimuli induced HO-1 expression, whereas inhibition of HO activity prevented the angiogenic response to these stimuli. The effect of HO-1 on angiogenesis in vivo is dependent on the state of inflammation (Loboda et al., 2008). HO-1 is proangiogenic in VEGF-driven, inflammation-inde-

pendent angiogenesis, but it blocks inflammatory angiogenesis (Bussolati et al., 2004), suggesting that a deficiency in HO activity can promote inflammation-driven angiogenesis. Our laboratory clearly showed that a deficiency in HO activity in HO-2(-/-) mice promotes massive inflammation-induced neovascularization of the cornea (Seta et al., 2006; Bellner et al., 2008). This study provides additional evidence to support a role for the HO system in the control of inflammatory angiogenesis. Highly angiogenic endothelial cells isolated from the aorta of HO-2(-/-) mice experienced a 2.7-fold reduction in HO activity compared with corresponding cells from WT mice. However, the angiogenic activity of the HO-2(-/-) cells was significantly attenuated after addition of biliverdin, indicating that deficiency in HO activity in the absence of the HO-2 gene contributes to angiogenesis in vitro and neovascularization in vivo (Seta et al., 2006; Bellner et al., 2008).

Spontaneous angiogenic activity was accompanied by alteration in the VEGF pathway, including changes in the expression of VEGFRs and VEGFs. Compared with WT cells, endothelial cells from HO-2(-/-) mice displayed a 2.5-fold increase in the mRNA levels of VEGFR-1, whereas mRNA levels of VEGFR-2 and -3 were markedly decreased. VEGFR-1 has been implicated as a positive regulator of macrophage activation and a stimulator of inflammatory angiogenesis; increased VEGFR1 expression is associated with chronic inflammatory angiogenic disease (Shibuya, 2006b). Whereas VEGFR-2 and -3 have been shown to promote pathologic neovascularization (Olsson et al., 2006; Tammela et al., 2008) in vivo, their expression levels were decreased in the absence of HO-2. A decrease in VEGFR-2 may be explained by findings that, under certain conditions, VEGFR-1 exerts negative regulation on VEGFR-2 (Shibuya, 2006a). However, the cause for and the significance of the marked decrease in VEGFR-3 mRNA in the HO-2-deficient mice are unclear. The finding that the expression of VEGF-A, the primary ligand for VEGFR-1, was largely unchanged whereas the expression of VEGF-C and -D, ligands for VEGFR-2 and -3, was significantly reduced suggests that the VEGFR-1/VEGF-A pathway contributed to the increased inflammatory and angiogenic phenotype of the HO-2(-/-) cells. Indeed, addition of VEGFR-1 inhibitory protein significantly attenuated the angiogenic activity of the HO-2(-/-) cells.

The HO-2(-/-) mice displayed an inflammatory state as evidenced by increased serum levels of inflammatory cytokines. This increased inflammatory state was effectively transferred to endothelial cells: HO-2(-/-) endothelial cells expressed higher levels of inflammatory cytokines than those isolated from WT mice, and nuclear expression of NF- κ B was higher in HO-2(-/-) cells. The expression of many inflammatory cytokines is driven by NF- κ B activation. The increased levels of nuclear NF- κ B signifies increased NF- κ B transcriptional activity and suggests that it contributes to the activated, inflammatory phenotype of endothelial cells derived from the HO-2(-/-) mice and to the increased angiogenic activity as inflammation is tightly linked to angiogenesis. Inhibition of NF- κ B activation has been shown to attenuate angiogenesis in vitro (Stoltz et al., 1996) and in vivo (Tabruyn and Griffioen, 2008). Indeed, addition of an NF- κ B inhibitor significantly attenuated the angiogenic activity of the HO-2(-/-) cells. Given the reported NF- κ B

Fig. 8. In vitro angiogenesis in mAECs from HO-2(-/-) (KO) and WT mice. Cells plated onto Matrigel were cultured in the presence and absence of biliverdin (10 μ M), SN50 (10 μ M), or VEGFR-1 inhibitory antibody (20 μ g/ml) and assayed for capillary length and lumen formation. A, representative photographs of WT and KO mAECs. B, effect of VEGF and biliverdin on total length of network and number of lumen in KO and WT cells ($n = 8$; *, $p < 0.05$ and ***, $p < 0.001$ compared with untreated; †, $p < 0.05$ compared with WT untreated). C, effect of SN50 and VEGFR-1 inhibitory antibody on total length of network and number of lumen in KO and WT cells ($n = 6$; *, $p < 0.05$ compared with untreated).

inhibitory activity of biliverdin (Gibbs and Maines, 2007), it is possible this bioactivity contributed to the antiangiogenic effect of biliverdin seen in this study. Inflammatory and angiogenic conditions are also characterized by increased levels of ROS. The major source of ROS in endothelial cells is the NADPH oxidase system (Frey et al., 2009). HO-2(-/-) cells expressed higher levels of NOX-2/gp91phox, a component of the NADPH oxidase system, and produced more superoxide while displaying lower expression levels of antioxidant systems such as EC-SOD, indicating that cells lacking the HO-2 gene experience oxidative stress perhaps as a result of the decrease in HO activity and consequently decreased levels of biliverdin/bilirubin, which are major endogenous antioxidants (Stocker et al., 1987) and may, by virtue of their ROS-scavenging mechanisms, attenuate the expression of many proinflammatory genes that are known to be redox-sensitive (Chen and Kunsch, 2004). Uncoupled eNOS

may also contribute to the increased oxidative stress. The HO-2(-/-) cells show lower levels of both eNOS and peNOS at serine 1177, as well as a lower peNOS/eNOS ratio compared with WT cells. Whether this contributes to the proinflammatory state seen in the HO-2(-/-) cells remains to be determined. Inflammation and angiogenesis are promoted primarily by activating redox-sensitive transcriptional factors including NF- κ B. ROS have been shown to stimulate induction of VEGF and angiogenesis through their effect on endothelial cell migration, proliferation, and tube formation through an increase in ROS (Ushio-Fukai, 2007). This dual role as a stimulator and a mediator brings into play an amplification process between ROS and VEGF, possibly driven by NF- κ B, which, in the absence of a functioning HO system, goes unchallenged.

In a previous study, we reasoned that the dysregulation of HO-1 induction in response to injury in the HO-2(-/-) mice

led to overall decreased HO activity and diminished levels of biliverdin and CO, thus weakening the capacity of the HO system to provide cytoprotection. The findings in this study suggest a similar scenario in endothelial cells lacking the *HO-2* gene. HO activity, measured as HO-dependent CO production, was three times lower in *HO-2*($-/-$) cells than in WT cells. This decrease in HO activity was despite an increase in HO-1 protein levels, an apparent discrepancy that has been observed in conditions of oxidative stress such as diabetes and hypertension (Kruger et al., 2006). It is well recognized that although oxidative stress induces HO-1 expression and protein levels, ROS inhibits HO activity (Kruger et al., 2006). Hence, the increased oxidative stress documented in the *HO-2*($-/-$) cells may explain the reduced HO activity in the face of increased HO-1 protein. The observation that HO-1 protein in the *HO-2*($-/-$) cells was associated primarily with the nuclear fraction may also explain, in part, the apparent discrepancy between protein levels and activity. A report (Lin et al., 2007) showed that localization of HO-1 to the nucleus is associated with a loss of total HO activity and suggested that nuclear HO-1 is, by and large, enzymatically inactive.

In summary, deletion of the *HO-2* gene is associated with oxidative stress, inflammation, and angiogenesis in vivo (Seta et al., 2006; Bellner et al., 2008) and in vitro in endothelial cells. These three processes have been linked to the HO system through the anti-inflammatory and antioxidant properties of its metabolites, CO and biliverdin/bilirubin. The current study suggests, once again, that *HO-2* deletion weakens a key regulatory element that protects the endothelium.

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