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Forum Review

Dual Role of VEGF-Induced Heme-Oxygenase-1 in Angiogenesis

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ABSTRACT

Heme-oxygenase-1 (HO-1) is an inducible cytoprotective molecule that displays antioxidant, antiapoptotic, and antiinflammatory effects. In addition, HO-1 appears to have a complex role in angiogenesis. Recent *in vivo* studies report that vascular endothelial growth factor (VEGF) regulates HO-1 expression and activity in vascular endothelial cells (ECs) and that inhibition of HO-1 abrogates VEGF-induced endothelial activation and subsequent angiogenesis, while promoting VEGF-induced monocyte recruitment and inflammatory angiogenesis. HO-1 may also regulate the synthesis and activity of VEGF, resulting in a positive-feedback loop. In contrast, HO-1 activity has the opposite effect on lipopolysaccharide-driven inflammatory angiogenesis, inhibiting leukocyte invasion and preventing subsequent angiogenesis. In this review, we summarize the current understanding of the role of HO-1 in angiogenesis. We conclude that further investigation, using targeted molecular approaches specifically to alter HO-1 activity, are required to develop our understanding of the role of HO-1 and its products, carbon monoxide, biliverdin, bilirubin, and free iron in angiogenesis. We propose that during chronic inflammation, HO-1 has two roles, first an antiinflammatory action inhibiting leukocyte infiltration, and second, promotion of VEGF-driven noninflammatory angiogenesis, which facilitates tissue repair. Additional studies will help determine whether modulating the activity of HO-1 and/or its products has therapeutic potential in chronic inflammatory diseases. *Antioxid. Redox Signal.* 8, 1153–1163.

VASCULAR BIOLOGY OF HO-1

Heme oxygenases (HOs) are rate-limiting enzymes that catalyze the conversion of heme into carbon monoxide (CO), free iron (Fe^{2+}), and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (Fig. 1). The presence of intracellular Fe^{2+} induces expression of the iron-binding protein ferritin, as well as the opening of Fe^{2+}export channels. Human HO, which has antioxidant, antiinflammatory, and antiapoptotic effects, exists in three isoforms, HO-1 (inducible), HO-2, and HO-3 (constitutive). HO-1 is widely distributed and induced by a range of stimuli including shear stress, oxidative stress, nitric oxide (NO), and hypoxia (1).

The purpose of this review is to explore in detail the emerging role for HO-1 in angiogenesis. After discussion of the antiinflammatory and vasculoprotective actions of HO-1 and its products, the remainder of the article focuses on the complex relation between HO-1 and the angiogenic process. In particular, the mechanisms by which HO-1 induces vascular endothelial growth factor (VEGF) release from a variety of cell types is discussed, along with evidence showing that VEGF can in turn increase intracellular HO-1. We review available data on the role of bilirubin and CO in angiogenesis and put forward the hypothesis that HO-1 may have a bifunctional role in chronic inflammation, an antiinflammatory action inhibiting leukocyte infiltration combined with promotion of

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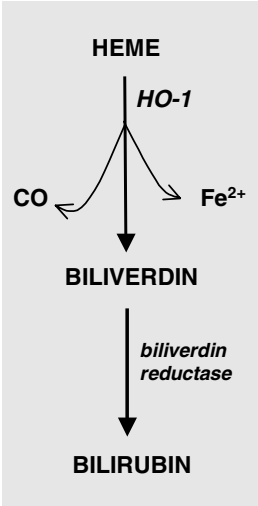

FIG. 1. Hemoxygenase-1–mediated heme degradation. The interaction of HO-1 with heme generates carbon monoxide (CO), biliverdin, and free iron (Fe^{2+}). Biliverdin reductase subsequently catalyzes the conversion of biliverdin to bilirubin.

VEGF-driven noninflammatory angiogenesis, which facilitates tissue repair.

HO-1 and inflammation

HO-1 exerts antiinflammatory effects in several *in vivo* models including cardiac ischemia–reperfusion injury (I/R), pulmonary inflammation, and endotoxic shock (2–5). Compounds such as hemin that induce HO-1 suppress inflammation, whereas HO-1 antagonists exacerbate the inflammatory response (5). Induction of HO-1 has been shown to inhibit leukocyte adhesion to vascular endothelium, at least in part, through downregulation of the expression of P- and E-selectin, VCAM-1, and ICAM-1 (6–9). The mechanisms underlying this remain to be elucidated fully. However, HO-1 expression is associated with inhibition of nuclear factor (NF)-κB activation, which appears to be mediated predominantly by bilirubin (9).

HO-1 and vascular injury

Heme, released from damaged cells, is potently proinflammatory and may be associated with vascular injury after hemolysis, I/R, and rhabdomyolysis (5). The fundamental cytoprotective role of HO-1 in this setting is exemplified by the respective phenotypes of HO-$1^{-/-}$ mice (10) and an HO-1–deficient patient (11), which are characterized by excess free heme, chronic inflammation, and vascular injury. *In vitro*, HO-1 protects endothelial cells (ECs) from hydrogen peroxide–mediated cell death (12) and from tumor necrosis factor (TNF)-α–induced cytotoxicity (13). Further evidence in support of the cytoprotective role of HO-1 comes from the identification of functionally relevant HO-1 promoter polymorphisms such as the human short $(GT)_n$ repeat polymorphism, which is associated with an enhanced HO-1 response and protection against vascular inflammation (14). Furthermore, bilirubin generated by HO-1 may attenuate vascular activation and reverse EC dysfunction (15).

In vivo, HO-1 exerts a potent protective effect against atherogenesis (16), cardiac I/R (17), and both graft rejection and accelerated arteriosclerosis after transplantation (18–21). The products of HO-1, CO (19, 20, 22), bilverdin/bilirubin (7, 15, 23), and in some studies, Fe^{2+}/ferritin (24), have been shown to mimic many of its antiinflammatory and vasculoprotective effects.

VEGF and inflammatory angiogenesis

VEGF is predominantly known as a potent angiogenic factor, whose biologic importance is revealed by the fact that the lack of only one of its alleles is incompatible with life (25, 26). VEGF has effects on endothelial cells (ECs) in all the phases of the angiogenic process including proliferation, migration, and cell organization. The effects of VEGF are not confined to ECs. In particular, VEGF also activates and recruits monocytes and phagocytes *via* the VEGF receptor-1 (27).

Angiogenesis is closely associated with inflammation in several diseases, including rheumatoid arthritis, psoriasis, atherosclerosis, carcinoma, and hematologic malignancies (28, 29). Thus, the combined action of VEGF on inflammatory and ECs is particularly evident in the setting of inflammatory angiogenesis, and hence VEGF may contribute to the progression of a variety of inflammatory diseases (30–33).

Several articles report a role for VEGF in inflammatory angiogenesis. Overexpression of VEGF in murine skin resulted in lesions very similar to those observed in human psoriasis (34). In addition, VEGF was shown to mediate pathologic neovascularization in a model of hypoxia-induced retinal neovascularization in rats (35). Moreover, infusion of VEGF into the neocortex of normal rats resulted in increased vascular density accompanied by an early and marked dose-dependent extravasation of immunoglobulin G (IgG) and leukocytes (36, 37). Taking into account the differential roles of VEGF receptors (VEGF-R2 in EC proliferation and VEGFR-1 in monocyte recruitment), blockade of VEGFR-1 has been proposed as a therapeutic tool for the reduction of inflammatory angiogenesis. In ischemic retinopathy and rheumatoid arthritis, inhibition of VEGFR-1, but not VEGFR-2, was effective in reducing inflammatory angiogenesis, by acting predominantly on inflammatory and hematopoietic stem/progenitor cells [reviewed in (38)]. To this end, we have shown that binding of VEGF to VEGFR-1 inhibits EC proliferation *via* release of nitric oxide (NO) by ECs and activation of cyclic GMP, which initiates a program of EC differentiation (39). However, the precise mechanisms underlying these events remain to be determined. We have also recently reported that HO-1 plays a unique role in the modulation of the angiogenic versus inflammatory actions of VEGF (40). However, the VEGF receptor involved in HO-1 activation remains to be determined.

HO-1 AND ANGIOGENESIS

The role of HO-1 in angiogenesis is complex, intriguing, and remains to be fully understood. Data to date suggest that HO-1 may influence the angiogenic process at various levels. However, the precise mechanisms through which HO-1 exerts its effects and the role of CO, biliverdin/bilirubin, and iron remain to be determined (41).

The use of both pharmacologic agonists and antagonists for HO-1 has revealed differential effects for HO-1 on the proliferation of vascular smooth muscle cells (VSMCs) and ECs, with HO-1 increasing EC cycle progression while inhibiting that of VSMCs (42). Overexpression of HO-1 induced HO-1 activity in coronary microvascular ECs and increased EC proliferation and formation of capillary-like structures in a 2-D Matrigel assay (43). In contrast, the same approach induced apoptosis in VSMCs (44). A recent study extended these results with the demonstration that retroviral expression of antisense HO-1 inhibits cell-cycle progression, EC proliferation, and capillary formation (45). These responses were significantly reversed by CO but not by bilirubin. Furthermore, murine aortic ECs, isolated from HO-$1^{-/-}$ mice, demonstrated reduced proliferation when compared with ECs from matched HO-$1^{+/+}$ littermates (46). In VSMCs, CO activates p38β MAPK and upregulates caveolin-1, which in turn inhibits cell proliferation (47).

We overexpressed HO-1 in human umbilical vein ECs, by using an adenoviral approach, and observed increased EC proliferation (Fig. 2A). Experiments are under way to explore the role of CO and bilirubin in this response, and our initial studies have shown that treatment of EC with bilirubin induces a modest, but significant, increase in EC proliferation (Fig. 2B). However, it is important to note that in addition to its influence on the cell cycle, HO-1 may also protect against EC apoptosis (48), indeed HO-$1^{-/-}$ ECs are more susceptible to apoptotic stimuli and have increased caspase-3 activity compared with HO-$1^{+/+}$ EC (46). Thus, in addition to EC proliferation, a reduced rate of apoptosis may also contribute to the increased cell numbers seen after overexpression of HO-1 or treatment with bilirubin or CO.

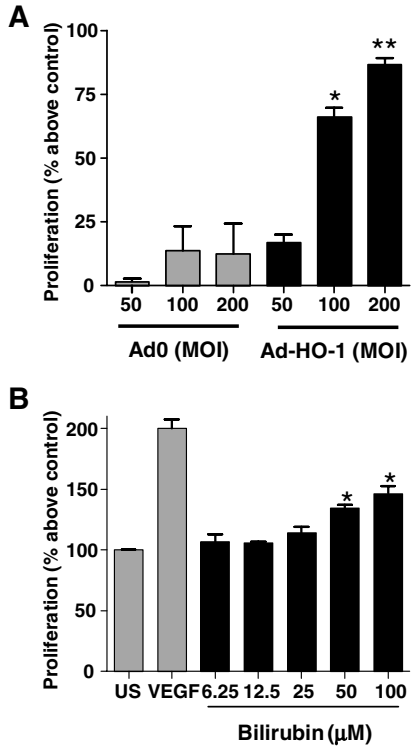

FIG. 2. (A) Human umbilical vein endothelial cells (HUVECs) were infected, at the multiplicity of infection (MOI, number of virus particles per cell) shown, with an adenovirus expressing HO-1 or a control adenovirus (AdO) for 2 h in serum-free M199 and were then cultured for a further 72 h in M199/5% FBS. HUVEC proliferation was quantified by using the CellTiter96 MTT assay (Promega), and the data are expressed as the percentage above that seen in HUVECs not infected with adenovirus. (**B**) HUVECs were cultured for 72 h in M199/5% FBS (US) in the presence of bilirubin (at the concentrations shown) or VEGF (25 ng/ml). Proliferation was quantified with the MTT assay, and data are expressed as percentage above the US control. *$p < 0.01$. **$p < 0.001$.

HO-1 and VEGF synthesis

In addition to direct effects on angiogenesis, HO-1 may have indirect effects through its ability to increase VEGF synthesis (41). Inhibition of HO activity with the protoporphyrins SnPP and ZnPP results in a dose-dependent reduction of the synthesis of VEGF induced by hypoxia and prostaglandin J_2 (49–51). These data have been confirmed by overexpression of HO-1 in VSMCs and ECs, which resulted in VEGF biosynthesis (49–52). In addition, ECs obtained from HO-$1^{-/-}$ mice demonstrated lower basal and H_2O_2-induced VEGF production (53). *In vivo*, overexpression of HO-1 promoted VEGF synthesis in rat placenta as well as in a rat hindlimb ischemia model, resulting in improved local blood flow (54, 55). In the latter study, the role of HO-1 was further supported by the observation that treatment with ZnPP inhibited the beneficial effects associated with the HO-1 adenovirus (55).

The role of CO in HO-1–induced VEGF biosynthesis remains to be established. Low concentrations of CO are protective to ECs and inhibit apoptosis through a p38 MAPK–dependent pathway (48, 56). Treatment of EC with PGJ_2 led to release of CO and a concomitant increase in VEGF synthesis, which was inhibited by the CO scavenger oxyhemoglobin (50). Contrasting results have been reported for the effects of CO on VEGF synthesis by VSMCs. This may relate to the different concentrations used, as low-dose CO (1%) increased the release of VEGF, whereas 5% CO suppressed hypoxia-induced VEGF synthesis (57).

Even less is known about the actions of biliverdin and bilirubin in this setting. Both may protect ECs against oxidative stress (58), and the ability of heme-induced HO-1 to protect against EC dysfunction in low density lipoprotein (LDL)-receptor knockout mice was reproduced by bilirubin but not CO (15). However, little is known about their role in angiogenesis, and it has been proposed that their action to protect against oxidative stress may reduce VEGF synthesis (41). Furthermore, HO-1 has been shown to participate in a molecular cascade in human ECs, which includes NO–HO-1–VEGF–IL-8 in sequence, and this may be involved in the amplification of angiogenesis (59).

CO activates soluble guanylate cyclase, leading to an increase in intracellular cyclic guanosine monophosphate (cGMP) (60). Activation of guanylate cyclase may also be induced by NO, the major secondary mediator of VEGF effects (61). It is therefore possible that HO-1 and NO synthase exert

a synergistic effect during VEGF-induced angiogenesis. Moreover, HO-1 may play a role in different angiogenic settings, such as during hypoxia, in which NO production and endothelial NOS expression are suppressed (62). Indeed, a role for HO-1 in angiogenesis during hypoxia has been suggested by the observation that HO-1 activation enhances VEGF production and that VEGF synthesis induced by hypoxia was downregulated by HO-1 inhibitors but not by inhibitors of NO synthase (49).

Modulatory effect of HO-1 in angiogenesis in vitro

We evaluated the role of HO-1 induction in VEGF-driven angiogenesis *in vitro*. Treatment of human umbilical vein endothelial cells (HUVECs; not shown) and human microvascular ECs with VEGF increased HO-1 expression, as assessed by Western blotting (Fig. 3A), and this was associated with increased enzymatic activity (40). With the chorioallantoic membrane model (CAM), Fernandez *et al.* (63) also demonstrated upregulation of HO-1 in ECs, after treatment with VEGF for 48 h. HO-1 induction was inhibited by staurosporine and a calcium chelator, indicating a potential role for protein kinase C and increased cytosolic calcium, respectively. Moreover, VEGF-induced angiogenesis was inhibited by HO-1 antagonist ZnMP. In contrast to VEGF, acidic-fibroblast growth factor (aFGF), at a concentration sufficient to induce EC proliferation, failed to induce HO-1 expression (Fig. 3B) (40). These data support previous studies showing that VEGF and FGF induce angiogenesis through distinct pathways (64).

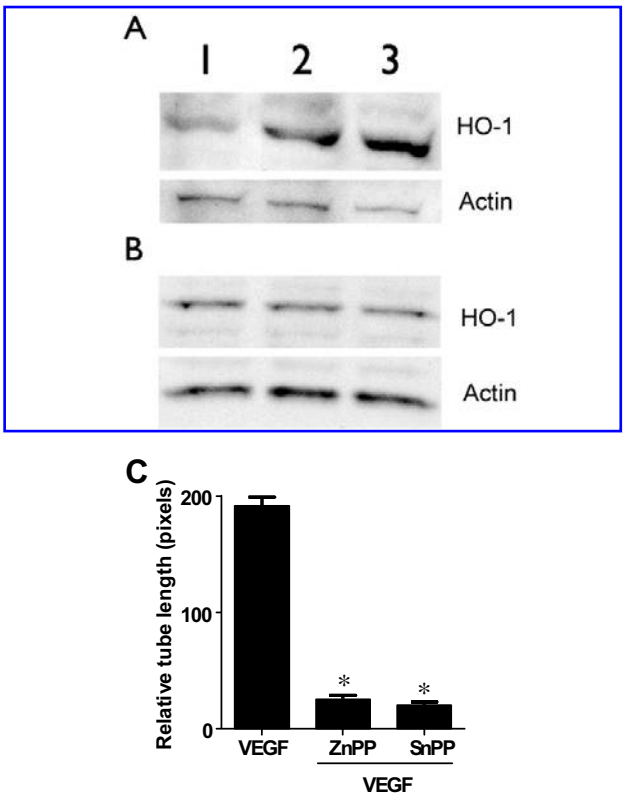

FIG. 3. Lysates were prepared from human microvascular endothelial cells (ECs) treated with (A) vascular endothelial growth factor (VEGF; 25 ng/ml) or (B) acidic fibroblast growth factor (aFGF; 100 ng/ml) for #48 h, and proteins were separated by SDS PAGE and transblotted onto nitrocellulose membranes. Immunoblots were probed with a polyclonal Ab against HO-1 and mAb C-2 against actin as a loading control. (**A**) Lane **1**. unstimulated ECs; **2.** VEGF 24 h; **3.** VEGF 48 h. (**B**) Lane **1**. unstimulated ECs; **2.** aFGF 24 h; **3.** aFGF 48 h. (**C**) Murine abdominal muscle was implanted in Matrigel and cultured in DMEM supplemented with 10% FBS and heparin (10 U/ml). VEGF (25 ng/ml) was added to the medium in the absence and presence of SnPP or ZnPP (20 μ*M*). On day 9, EC tube length was measured by image analysis. Data are presented as mean + SD tube length ($n = 3$). *$p < 0.01$.

Inhibition of HO-1 with pharmacologic antagonists, SnPP or ZnPP, significantly inhibited VEGF-induced EC proliferation in a dose-dependent manner. Moreover, induction of HO-1 with CoPP alone resulted in EC proliferation comparable to that seen with VEGF (40). To investigate this further, murine abdominal muscle was implanted in Matrigel, and the capillary sprouting and neovessel development in response to VEGF were analyzed. As seen in Fig. 3C, inhibition of HO-1 with both SnPP and ZnPP completely inhibited the development of VEGF-induced neovessels. Moreover, our observation that VEGF-induced expression of HO-1 is coupled with enhanced HO-1 activity (40) leads to the speculation that, as VEGF induces HO-1 expression and HO-1 enhances VEGF synthesis in ECs, HO-1 acts as an endogenous amplifying factor for angiogenesis in vascular endothelium (65).

Modulatory effect of HO-1 in noninflammatory angiogenesis

To test the possible role of HO-1 in VEGF induced *in vivo*, we used a murine Matrigel model of angiogenesis and inhibited HO-1 activity by using SnPP or ZnPP. In this model, VEGF induces a rapid angiogenic process within Matrigel in 6 days, with development of several neovessels connected to the murine vasculature and containing erythrocytes (66). Surprisingly, HO-1 inactivation by SnPP (20 μ*M*) increased the angiogenic effect induced by VEGF (Fig. 4). Histologic examination of the implants containing VEGF plus SnPP or ZnPP showed the presence of dilated hemorrhagic vessels (Fig. 5). This effect appeared contrary to the role of HO-1 in ECs observed *in vitro* (see earlier). We therefore tested the effect of SnPP on angiogenesis induced in Matrigel by other known proangiogenic factors. Addition to Matrigel of SnPP did not affect the angiogenic effect of angiopoietin-1 or aFGF (Fig. 4). In addition, SnPP significantly decreased angiogenesis induced by the agonistic Ab against CD40 (67) (Fig. 4). These data exclude a nonspecific effect of SnPP in the murine Matrigel model of angiogenesis and indicate that HO-1 plays a complex and multifaceted role in the setting of VEGF-induced angiogenesis *in vivo*.

Histological examination of the implants containing VEGF plus the HO-1 inhibitors SnPP or ZnPP highlighted the presence of a dense inflammatory infiltrate within implants treated with VEGF + SnPP or ZnPP (Fig. 5A–D). The presence of inflammatory cells was confirmed by immunofluorescence staining for myeloperoxidase (Fig. 5E), which revealed a significant leukocytic infiltrate in Matrigel plugs

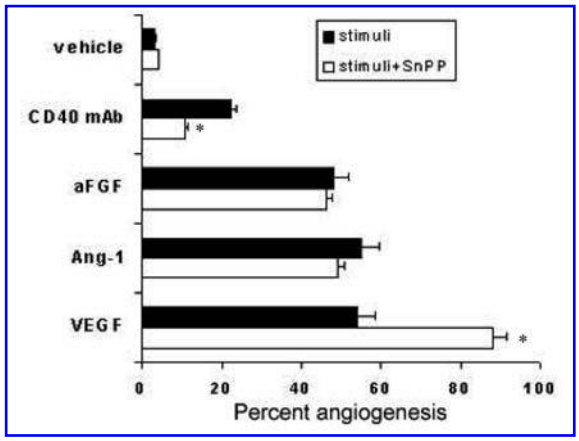

FIG. 4. Role of HO-1 during *in vivo* angiogenesis. Quantitative evaluation of neovessels infiltrating Matrigel incorporating agonist rat anti-mouse CD40 mAb (40 μg/ml), aFGF (50 ng/ml), angiopoietin-1 (100 ng/ml) (Ang-1),VEGF (40 ng/ml), or vehicle alone, in the absence or presence of SnPP (20 μ*M*). A significant increase in angiogenesis was observed in Matrigel plugs containing VEGF and SnPP, whereas SnPP had no effect on angiopoietin-1– or aFGF-induced angiogenesis. In contrast, SnPP significantly inhibited angiogenesis induced by CD40 stimulation. The results are expressed as percentage ± SEM of the vessel area to the total Matrigel area. Six mice were used per condition in each experiment. *$p < 0.05$.

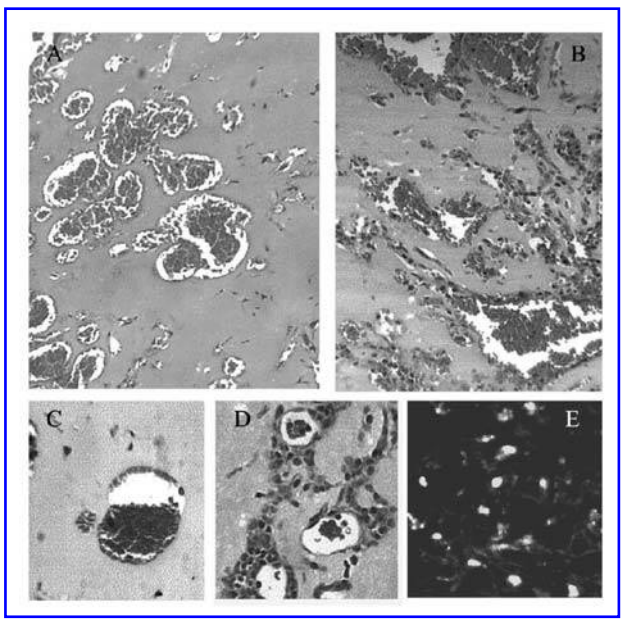

FIG. 5. Histologic and immunohistochemical analysis of Matrigel implants containing VEGF or VEGF plus SnPP. Representative hematoxylin and eosin–stained sections of Matrigel containing VEGF (**A** and **C**) or VEGF plus SnPP (**B** and **D**), implanted in mice and excised 6 days after injection. A dense inflammatory infiltrate around vessels was observed in plugs containing VEGF plus SnPP when compared with VEGF alone (original magnification: **A, B**, ×160; **C, D**: ×250). (**E**) Immunofluorescence staining for myeloperoxidase, showing the presence of leukocytes around neovessels in Matrigel plugs incorporating VEGF plus SnPP (original magnification, ×250).

treated with a combination of VEGF and either SnPP or ZnPP, when compared with VEGF alone (40). Interestingly, in a recent study of wound healing, heme-induced influx of leukocytes was also significantly elevated after pharmacologic inhibition of HO-1 (5).

It has been suggested that HO-1 may be a potentially important regulator of inflammatory cell trafficking within the vessel wall, most likely by inhibiting the expression of endothelial-leukocyte adhesion molecules such as E-selectin and vascular adhesion molecule-1 and the production of certain proinflammatory chemokines including MCP-1 (9, 59). Considering this role of HO-1, the observed effect of HO-1 inhibition on VEGF-induced angiogenesis could be ascribed to an increase in inflammatory angiogenesis rather than endothelial-dependent angiogenesis (40). This was confirmed by experiments in which inflammatory leukocytes were depleted by treatment with an anti-β2 integrin Ab. In the absence of leukocyte infiltration into Matrigel plugs, SnPP failed to enhance VEGF-induced angiogenesis. Furthermore, in the absence of leukocyte infiltration, ZnPP completely inhibited VEGF-induced angiogenesis (40). These data support the hypothesis that HO-1 has a dual role in VEGF-dependent angiogenesis: HO-1 activation promotes angiogenesis, while inhibiting the local recruitment of leukocytes. HO-1 inhibition, conversely, blocks physiologic angiogenesis and favors inflammatory angiogenesis because of leukocyte recruitment.

Modulatory effect of HO-1 in inflammatory angiogenesis

As a potential therapeutic role for HO-1 manipulation is emerging (68), we evaluated the efficacy of HO-1 induction in the prevention of inflammatory angiogenesis. We used the lipopolysaccharide (LPS)-induced model of angiogenesis, which is directly dependent on leukocyte migration and is characterized by an intense leukocytic infiltration and associated angiogenic response (69). The inclusion of LPS alone resulted in significant angiogenesis with an associated intense leukocytic infiltrate (Fig. 6A). HO-1 activation induced by CoPP significantly inhibited leukocyte-induced angiogenesis (Fig. 6B), suggesting that HO-1 may act to suppress inflammatory angiogenesis *in vivo*. The induction of HO-1 has been previously shown to inhibit monocyte chemotaxis (70). Moreover, increased leukocyte adhesion to the vessel wall and spontaneous perivascular infiltration of leukocytes into the liver, lungs, and kidneys was observed in HO-1–deficient mice (10). Therefore, HO-1–mediated inhibition of leukocyte recruitment represents an important mechanism for the control of angiogenesis associated with inflammation.

CONCLUSIONS

HO-1 is a cytoprotective gene that acts, in the face of a variety of noxious stimuli, to exert antiapoptotic, anti-inflammatory, and antiproliferative effects (71). However, the actions of HO-1 may be cell-type specific, and the proproliferative effects of HO-1 in ECs, and its modulatory actions during angiogenesis, suggest that HO-1 may also have a reparative role through generation of neovessels. Thus, after

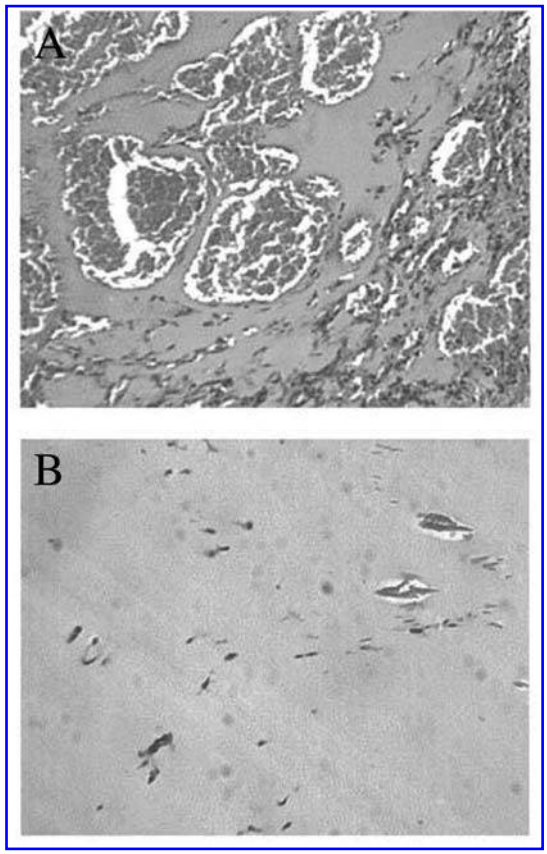

FIG. 6. Effect of HO-1 on inflammatory angiogenesis. Matrigel containing LPS (10 ng/ml) or LPS plus the HO-1 activator CoPP (25 μ*M*) was injected subcutaneously into C57BL/6 mice. CoPP was also injected intraperitoneally (5 mg/kg) on day 0. After 6 days, plugs were explanted, fixed in formalin, and paraffin embedded. Representative hematoxylin and eosin–stained sections of Matrigel containing LPS (**A**) or LPS in the presence of CoPP (**B**) are shown. Activation of HO-1 by CoPP completely prevented leukocyte-dependent angiogenesis (original magnification, ×160).

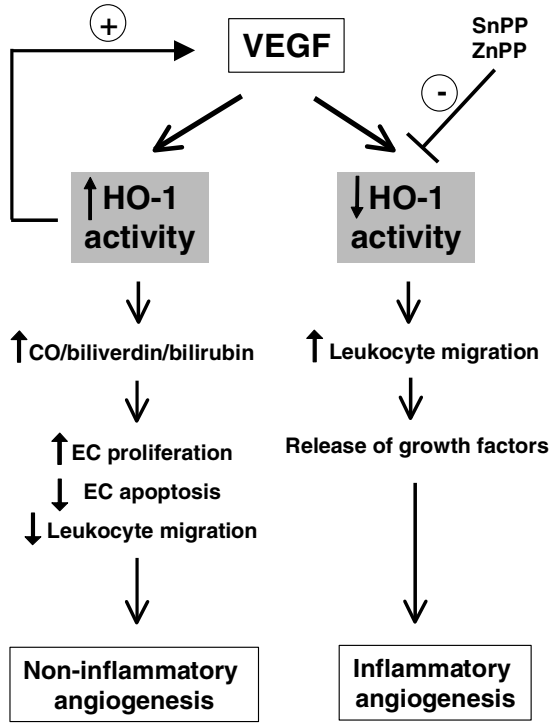

FIG. 7. Potential actions of VEGF-induced HO-1 during angiogenesis *in vivo*. HO-1 activation by VEGF favors endothelial cell (EC) proliferation and prevents EC apoptosis, while inhibiting leukocyte migration, thus resulting in predominantly noninflammatory angiogenesis. In addition, HO-1 activates a positive-feedback loop, increasing VEGF synthesis. The relative contributions of carbon monoxide (CO), biliverdin, and bilirubin in these mechanisms remain to be determined. In contrast, when HO-1 activation by VEGF is inhibited, increased leukocyte migration occurs with subsequent local release of growth factors and induction of inflammatory angiogenesis.

vascular injury, VEGF may induce HO-1, which acts to enhance apoptosis and inhibit proliferation of VSMCs, while increasing EC proliferation and encouraging reendothelialization (65)

VEGF may act both as a potent proangiogenic factor and as a leukocyte chemoattractant. Based on data obtained from two murine models: (a) angiogenesis initiated by addition of VEGF to Matrigel and (b) an LPS-induced model of inflammatory angiogenesis in which angiogenesis is secondary to leukocyte invasion, we propose that during chronic inflammation, HO-1 has two roles, first an antiinflammatory action inhibiting leukocyte infiltration, and second, promotion of VEGF-driven noninflammatory angiogenesis that facilitates tissue repair (Fig. 7). However, HO-1 induction may be impaired during inflammation, as recently reported in human chronic graft rejection (72). Thus, therapeutic induction of HO-1, or delivery of CO or bilirubin, may be beneficial in the treatment of chronic inflammatory diseases.

Notwithstanding this, therapeutic administration of SnPP in a rat adjuvant arthritis model significantly inhibited the development of arthritis, implicating HO-1 in disease activity (73). However, SnPP is not a specific antagonist of HO-1, and it is of note that it also reduced the levels of TNF-α (73), a critical cytokine in the development of inflammatory arthritis (74). Moreover, inhibition of cyclooxygenase-2 (COX-2) increased biosynthesis of interleukin (IL)-10 and HO-1 by chondrocytes (75), and overexpression of HO-1 in ECs is associated with decreased prostaglandin production and COX-2 activity (76), suggesting that HO-1 may have an antiinflammatory chondroprotective role (77). Further studies aimed at increasing HO-1 and its products in this setting are awaited with interest.

The apparently complex and diverse nature of the effects of HO-1 may relate in part to its ability to mediate the functions of a variety of stimuli. Thus, Fritz Bach (68, 71) has referred to HO-1 functioning as a "therapeutic funnel." The data reviewed herein suggest that VEGF can be added to the list of molecules that use HO-1 in this way. Moreover, it has been proposed that HO-1 may amplify the therapeutic effect through a positive-feedback loop in which VEGF upregulates

HO-1, which in turn increases VEGF synthesis (65, 68) (Fig. 7). This is analogous to the role of HO-1 in mediating the antiinflammatory effects of IL-10 (65, 68). IL-10 induces HO-1 expression in macrophages (78, 79), which mediates the antiinflammatory effects of IL-10, while also increasing IL-10 synthesis (4).

The importance of these observations principally relates to their clinical potential. The amplifying positive-feedback loops offer an explanation for the prolonged efficacy of a brief exposure to CO. Thus, exposure to exogenous CO, biliverdin, or bilirubin for as little as 1 h inhibited the development of restenosis after balloon angioplasty–mediated vascular injury (21, 80). This suggests that the frequency of CO, biliverdin, or bilirubin treatment required maybe less than anticipated (68). If so, this would also reduce any toxicity associated with the therapeutic use of these products of HO-1.

These studies have led to interest in CO-releasing molecules (CORMs) as potential therapeutic agents. CORMs release CO at physiologic pH and in amounts sufficient to exert biologic effects. Roberto Motterlini and colleagues (81) reported on two water-soluble CORMs, CORM-3 [tricarbonylchloro(glycinato)ruthenium (II)] and CORM-A1 (sodium boranocarbonate) (82). *In vitro*, CORM-3 protected cardiac cells against injury after exposure to oxidative stress or hypoxia–reoxygenation and *in vivo* prolonged the survival rate of cardiac allografts in a murine model (81). Moreover, pretreatment with CORM-3 resulted in a protective effect similar to that induced by ischemic preconditioning, and most important, this effect was prolonged for $\leq$72 h (83), supporting the view that only a relatively short exposure to CO is required.

CORMs are not the only way in which CO can be delivered, and considerable success has also been achieved by using an inhalation approach. Exposure to low-dose inhaled CO (250 ppm) for 1 hr before and 24 h after intestinal transplant surgery in rats resulted in significantly improved survival compared with room air–treated controls of 92% versus 58%, respectively (84). Immunohistochemical analysis revealed that CO reduced adhesion molecule and cytokine expression in the grafts and also exerted antiapoptotic effects through reduction of Bax and induction of Bcl-2 mRNA. A lower dose of CO (20 ppm) for 30 days after transplantation abrogated the chronic inflammatory changes associated with chronic allograft nephropathy and improved long-term renal function in a rat model (85). Furthermore, treatment of donor pancreatic islets alone has been shown to enhance cytoprotection and improve allogeneic islet survival (86). Of note, a recent study using rat renal and cardiac transplant models suggested that combination therapy with CO and biliverdin is more effective than monotherapy in protecting against I/R injury (87). The added benefit of dual therapy may, at least in part, reflect the use of distinct mechanisms by these two products of HO-1.

PERSPECTIVES

Despite considerable progress made in defining the functions of HO-1 in the vasculature, further studies are required in which specific inhibition of HO-1, such as that afforded by siRNA or antisense oligonucleotides, is used. HO-1–deficient mice and the development of tissue-specific or inducible knockout models will also help to establish the functions of HO-1. Likewise, further studies are required to identify the signaling pathways involved in the regulation of HO-1 expression and those used by its products. The regulation of HO-1 is complex, and data to date suggest that MAPK pathways have opposing effects in different cell types and specifically that inhibition of p38 MAPK and ERK1/2 in rat ECs may enhance HO-1 expression in hypoxia (88, 89). The study of EC apoptosis has suggested that CO activates an MKK3/p38α MAPK–dependent pathway (48, 56, 90). A recent study has extended this by showing that antiapoptotic effects of CO depend on PI-3K/Akt and p38 MAPK activity, which in turn activates STAT3 (91). Additional studies are required to confirm these findings and to see how they relate to other CO-mediated changes in EC function. Likewise, the mechanisms regulating the effects of bilirubin must be defined. Thus, it is of note that HO-1 can inhibit NF-κB activation induced by TNF-α and that bilirubin, but not CO, seems to mediate this effect (9). The precise definition of the respective roles of the products of HO-1 and the signaling mechanisms through which they act may allow the development of novel therapeutic approaches for the treatment of chronic inflammatory diseases. However, this will not be straightforward, as evidenced by a recent study of a murine inflammatory arthritis in which both induction of HO-1 with CoPP and inhibition with SnPP had some beneficial effects (92). In contrast, a second study using different models suggested that induction of HO-1 suppressed osteoclastogenesis and bone destruction in TNF-mediated arthritis and that serum levels of bilirubin were elevated in RA patients without erosive damage (93).

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ABBREVIATIONS

aFGF, acidic fibroblast growth factor; CO, carbon monoxide; CoPP, cobalt protoporphyrin; CORM, carbon monoxide releasing molecule; COX-2, cyclooxygenase-2; Fe^{2+}, free iron; HO, heme oxygenase; HMECs, human microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; I/R, ischemia–reperfusion; IL-10, interleukin-10; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NO, nitric oxide; PG, prostaglandin; SnPP, tin protoporphyrin; STATs, signal transducers and activators of transcription; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VSMCs, vascular smooth muscle cells; ZnPP, zinc protoporphyrin.

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