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Heme Oxygenase and Angiogenic Activity of Endothelial Cells: Stimulation by Carbon Monoxide and Inhibition by Tin Protoporphyrin-IX

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ABSTRACT

The activity of heme oxygenase enzymes (HOs) is responsible for the endogenous source of carbon monoxide (CO). Their activities can be inhibited by tin protoporphyrin-IX (SnPPIX). Recent data indicate the involvement of HOs in the regulation of angiogenesis. Here, we investigated the role of the HO pathway in the production and angiogenic activity of vascular endothelial growth factor (VEGF) in endothelial cells treated with SnPPIX, or cultured in the presence of a CO-releasing molecule (CO-RM). Addition of CO-RM or induction of HO-1 by hemin resulted in a threefold elevation in CO production in culture medium (up to 20.3 μg/L) and was associated with a 30% increase in VEGF synthesis. Much higher levels of CO (up to 60 μg/L) and a further increase in VEGF production (by 277%) were measured in cells treated with prostaglandin-J_2, a potent activator of HO-1. SnPPIX prevented the induction of CO generation and inhibited VEGF synthesis. Moreover, SnPPIX reduced the VEGF-elicited angiogenic activities of endothelial cells by decreasing their proliferation (by 26%), migration (by 46%), formation of tubes on Matrigel (by 48%), and outgrowth of capillaries from endothelial spheroids (by 30%). In contrast, overexpression of HO-1 or incubation of cells with CO-RM led to an increase in capillary sprouting. Thus, HO activity up-regulates VEGF production and augments the capability of endothelial cells to respond to exogenous stimulation. *Antioxid. Redox Signal.* 5, 155–162.

INTRODUCTION

HEME OXYGENASES (HOs) are enzymes that catalyze the oxidation of heme to biologically active molecules: ferrous iron, carbon monoxide (CO), and biliverdin, the latter being converted to bilirubin (for review, see 22). Two distinct variants of HOs have been described in human, each encoded by a different gene. HO-2 is constitutively expressed, whereas HO-1 can be potently and rapidly induced by many compounds including heme, prostaglandin-J_2, oxidized lipoproteins, inflammatory cytokines, nitric oxide (NO), or heavy metals (8, 10, 13, 22, 23, 32). Both HO-1 and HO-2 have been detected in endothelial cells (10, 15, 22, 34).

Several lines of evidence suggest that HO-1 plays an important protective role in the vessels by reduction of oxidative stress, diminution of vascular constriction, attenuation of inflammation, decrease in vascular smooth muscle cell proliferation, or inhibition of endothelial cell apoptosis (8, 35). Its significance is illustrated by disturbed growth of HO-1 knockout mice and by the fatal consequences of HO-1 deficiency in humans. In both cases, lack of HO-1 activity results in extreme vulnerability of vessels to common stressful stim-

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uli, leading to severe vascular endothelial damage and detachment, thus pointing to the essential role of HO-1 in endothelium (16, 28, 37). Accordingly, up-regulation of HO-1 significantly reduces neointima development after balloon angioplasty, protects against the chronic rejection of transplants, and inhibits progression of atherosclerosis in animal models (35).

Many of those effects are mediated by HO-derived CO, a cellular messenger, with the signaling functions resembling that of NO. It exerts antiinflammatory effects by inhibition of tumor necrosis factor-α, interleukin-1β, or macrophage inflammatory protein-1β, and by up-regulation of interleukin-10 (27). Like NO, CO induces soluble guanylyl cyclase (sGC) and thereby inhibits platelet aggregation, decreases leukocyte adhesion, improves endothelial cell survival, and promotes vasodilatation, working synergistically with NO produced by endothelial NO synthase (eNOS) (8, 32).

It is well established that eNOS-derived NO plays a fundamental, permissive role in the neovascularization induced in response to vascular endothelial growth factor (VEGF). Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is increased during embryogenesis and in pathological events, such as hypoxia, ischemia, inflammation, tumor growth, and wound healing (4, 29). The same conditions simultaneously induce HO-1 expression (1, 22). Furthermore, the promoter of the HO-1 gene contains the regulatory sequences for ETS-1, FLI-1, and ERG, the transcription factors associated with endothelial cell proliferation and differentiation (7). Altogether, these observations suggest the involvement of HO-1, and possibly HO-derived CO, in the regulation of angiogenesis.

Indeed, in earlier experiments we have shown that activation or overexpression of HO-1 leads to the up-regulation of VEGF synthesis (9, 15). Our results, conflicting with a previous report suggesting the inhibitory effect of CO (21), have been supported very recently in other models (2, 19). Additionally, it has been reported that HO-1 activity augments some angiogenic events in endothelial cells (6). Nevertheless, understanding the role played in angiogenesis by HO-1 and especially by HO-derived CO is only fragmentary and based on contradictory results. One of the causes impeding research in the field of CO was the lack of available compounds that can release and deliver CO into biological systems. However, first such molecules have been discovered very recently, and their capability of eliciting specific vascular activities that are reminiscent of those mediated by the HO-1/CO pathway has been reported (24). In the present study, we examined the effect of modulation of CO synthesis and the influence of a CO-releasing compound on the VEGF production and angiogenic activities in endothelial cells.

MATERIALS AND METHODS

Reagents

15-Deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) was obtained from Biomol. Hemin was purchased from Fluka, and both tin protoporphyrin-IX (SnPPIX) and copper protoporphyrin-IX (CuPPIX) were from Porphyrin Products. Oxyhemoglobin was from Calzyme Laboratories, whereas L-glutamine, epidermal growth factor, hydrocortisone, 1*H*-[1,2,4]oxydiazole[4,3-a]quinoxalin-1-one (ODQ), carboxymethylcellulose, and tricarbonyldichlororuthenium (II) dimer ($[Ru(CO)_3Cl_2]_2$), a specific CO-releasing molecule (CO-RM) (24), were purchased from Sigma. Fetal calf serum (FCS) was purchased from PromoCell and pcDNA3.1+ expression plasmid from Invitrogen. The immunoenzymatic assay for 3′,5′-cyclic guanosine monophosphate (cGMP) measurement was bought from Amersham. CytoTox-96 assay and control pSVβgal plasmid were obtained from Promega. SuperFect oligodenrimers and Maxiprep QIAfilter Plasmid Isolation Kit were purchased from Qiagen. Human VEGF enzyme-linked immunosorbent assay (ELISA) kits for human VEGF protein were obtained from R&D Systems. The cell proliferation ELISA was obtained from Roche Diagnostic. Quantitative Cell Migration Assay and In Vitro Angiogenesis ECMatrix™ Kit were from Chemicon. All others reagents were obtained from Gibco BRL.

Cell culture and incubation experiments

Human microvascular endothelial cells (HMEC-1) were purchased from Centers for Disease Control and Prevention (Atlanta, GA, U.S.A.) and cultured in Dulbecco's modified Eagle's medium/F-12 medium containing 10% FCS, L-glutamine (2 m*M*), epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), penicillin (100 U/ml), and streptomycin (10 μg/ml).

Human umbilical vein endothelial cells (HUVEC) were freshly isolated from umbilical veins of newborn babies by collagenase digestion. Cells were cultured in M-199 medium supplemented with FCS (10%), endothelial cell grow supplement (ECGF), HEPES, heparin, L-glutamine, and antibiotics. Experiments were performed on confluent cell cultures at second or third passages. The same medium was used for HMEC-1 for measurement of CO production.

Angiogenic activities were stimulated by supplementation of cells with $VEGF_{165}$ (30 ng/ml). HO-1 was induced using hemin (1 μ*M* for 2 h) or 15d-PGJ_2 (10 μ*M*, all the time with cells). To inhibit HO-1/HO-2 or eNOS activities, cells were preincubated for 1 h with SnPPIX (1–10 μ*M*) or N^{ω}-nitro-L-arginine methyl ester (L-NAME; 2 m*M*), respectively.

CO concentration

HMEC-1 were incubated in six-well plates in 3.5 ml of medium. After 24 h, the media were collected and frozen at −80ºC. The CO concentration in the medium was determined by colorimetric measurement of carboxyhemoglobin, as described earlier (34).

VEGF protein concentrations

HMEC-1 were cultured in 24-well plates containing 1 ml of medium per well. Concentrations of VEGF protein in the culture media were quantified using the sandwich ELISA, following the manufacturer's instructions.

cGMP measurements

Total production of cGMP in HMEC-1 was quantified by immunoenzymatic assay in cell lysates and culture media, ac-

cording to the vendor's protocol. Samples were acetylated prior to measurements.

Proliferation assay

Experiments were performed on HUVEC cultured in medium with 10% FCS, but without ECGF. After a 48-h incubation period, bromodeoxyuridine was added for 2 h and proliferation was measured by bromodeoxyuridine incorporation assay.

Migration assay

Experiments were performed on HUVEC in medium without FCS and ECGF and supplemented with 5% bovine serum albumin. Migration was tested using a modified Boyden chamber (diameter of pores, 8 μm) coated with vitronectin. The assay was performed 24 h after stimulation, according to the manufacturer's protocol.

Morphogenesis

Experiments were conducted using medium containing 10% FCS, but without ECGF. HUVEC were seeded on EC-Matrix (Matrigel, a solid gel of basement proteins from Engelbreth Holm–Swarm mouse tumor) and assayed according to the vendor's instruction. Tube formation was inspected under an inverted microscope 24 h after the beginning of the experiment.

Capillary sprouting

Experiments were performed as previously described (14) according to the procedure established by Korff and Augustin (17, 18) using medium containing 10% FCS, but without ECGF. In order to generate HUVEC spheroids, 750 cells were suspended in culture medium containing 0.25% (wt/vol) carboxymethylcellulose. During the first 24 h of culture, all the suspended cells contributed to the formation of a single spheroid, which was then embedded in a collagen gel. Under such conditions, spheroids formed capillary-like sprouts, which were measured in the following 24 h of culture using a digitized imaging system connected to an inverted microscope.

Transient transfection

Rat HO-1 cDNA (kindly provided by Dr. Mahin Maines, Rochester, NY, U.S.A.) was cloned into a pcDNA3.1+ expression plasmid. The resulting construct (pcDNA-HO1), as well as a control plasmid pSV-βgal (bacterial β-galactosidase gene under the control of an SV40 promoter) were prepared as described previously (15). HUVEC grown to 80% confluence were transfected in 24-well plates with 0.25 μg of plasmid DNA and 1.25 μl of SuperFect per well. After 2 h, the cells were washed, overlaid with routine culture medium for 24 h, and then used for preparation of endothelial spheroids.

Cell viability assay

Cell viability was assessed by colorimetric measurement of lactate dehydrogenase release.

Statistical analysis

All experiments were performed in duplicate and were repeated three to five times. Data are presented as means ± SD. Statistical evaluation was done with Student's t test or with ANOVA followed by Tukey test. Differences were accepted as statistically significant at $p < 0.05$.

RESULTS

SnPPIX reduces cGMP production in endothelial cells

The average concentration of cGMP in media and cell lysates prepared from resting endothelial cells was 11.1 fmol/well. We have found that SnPPIX decreased the cGMP production in HMEC-1 by 45%, whereas L-NAME, an inhibitor of NO synthase (NOS), reduced it by 33% (Fig. 1). Treatment of cells with both blockers resulted in an additive inhibitory effect and diminished cGMP concentration by ~80%.

SnPPIX decreases the generation of VEGF

This set of experiments was performed on HMEC-1, endothelial cells that spontaneously produce VEGF and after a 24-h incubation period release ~20 pg/ml VEGF protein (15). All the compounds used for the treatments did not impair cell viability as lactate dehydrogenase release was not affected (data not shown). Addition of CO-RM (10 μ*M*) resulted in about threefold elevation of CO concentration in the culture medium, up to 20.3 μg/L. Similar amounts of CO were measured after induction of HO-1 by hemin (Fig. 2A). In both cases, the augmented levels of CO were associated with 30% increase in the production of VEGF by HMEC-1 (Fig. 2B). SnPPIX, which prevented induction of CO synthesis in hemin-treated cells, inhibited the hemin-induced VEGF production as well (Fig. 2).

A much higher concentration of CO (up to 60 μg/L) was measured in cells treated with 15d-PGJ_2 (Fig. 3A), one of the

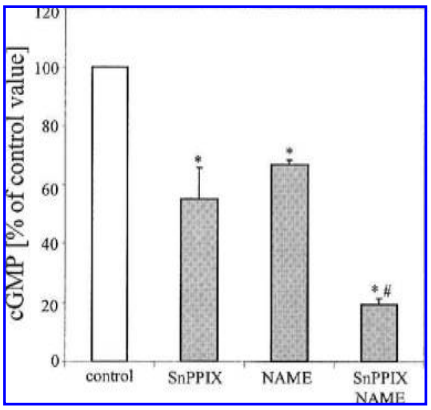

FIG. 1. Effect of SnPPIX (10 μ*M*) and L-NAME (2 m*M*) on cGMP synthesis in HMEC-1. Each column represents the mean ± SD of five experiments. *$p < 0.05$ in comparison with control, #$p < 0.05$ in comparison with SnPPIX.

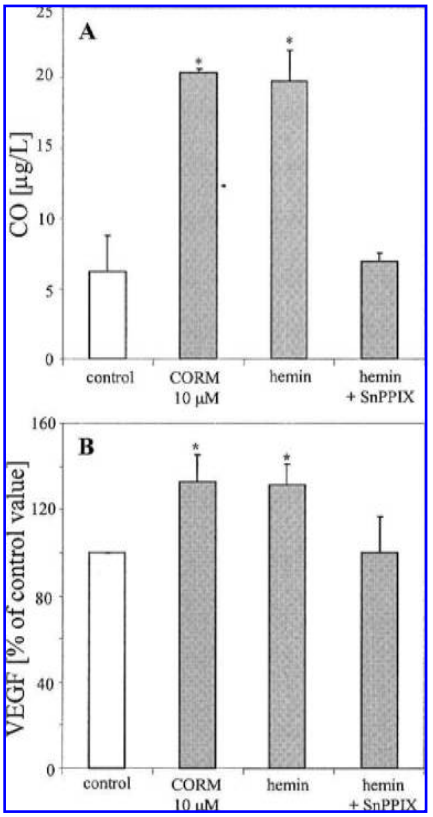

FIG. 2. Effect of CO-RM (10 µ*M*), hemin (1 µ*M*), and SnPPIX (10 µ*M*) on the concentration of CO in media from HMEC-1 cultures (A) and on the VEGF synthesis in HMEC-1 (B). Each column represents the mean ± SD of three or four experiments. *$p < 0.05$ in comparison with control.

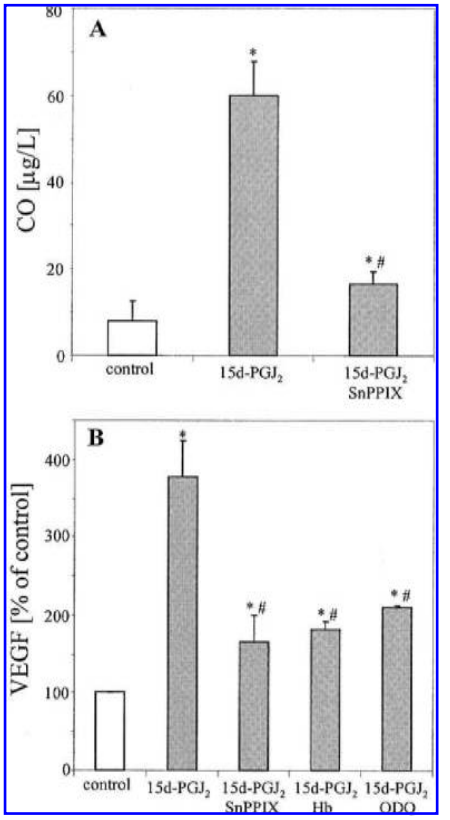

FIG. 3. (**A**) Concentrations of CO in culture media from HMEC-1 treated with 15d-PGJ_2 (10 µ*M*) and SnPPIX (10 µ*M*). (**B**) Effect of 15d-PGJ_2, SnPPIX, oxyhemoglobin (Hb; 5 µ*M*), and ODQ (5 µg/ml) on the synthesis of VEGF in HMEC-1. Each column represents the mean ± SD of three experiments. *$p < 0.05$ in comparison with control; #$p < 0.05$ in comparison with 15d-PGJ_2.

strongest activators of HO-1 in endothelial cells (15). Accordingly, 15d-PGJ_2 induced the synthesis of VEGF in HMEC-1 much more efficiently (by 277%) (Fig. 3B). Noteworthy, this effect was significantly inhibited not only by SnPPIX, but also by oxyhemoglobin (5 µ*M*, a CO scavenger) and ODQ (5 µg/ml, an inhibitor of cGMP synthesis), suggesting that the effector molecule responsible for induction of VEGF synthesis in endothelial cells is HO-derived CO, acting through the elevation of cGMP production (Fig. 3B).

SnPPIX decreases the angiogenic activities of endothelial cells

This set of experiments was performed on HUVEC. These cells do not release detectable quantities of VEGF and display a low spontaneous angiogenesis, but respond well to exogenous stimulation. SnPPIX, but not CuPPIX, significantly inhibited all the VEGF-induced angiogenic activities tested. The proliferation of endothelial cells was reduced in response to SnPPIX by 26% (Fig. 4). Surprisingly, the effect of L-NAME on the angiogenic activities was weak, and simultaneous inhibition of NOS and HO activities by L-NAME and SnPPIX did not strengthen significantly the effect of SnPPIX alone.

SnPPIX strongly inhibited cell motility (measured in modified Boyden chambers coated with vitronectin) and morphogenesis (assessed by formation of tube-like structures on Matrigel). VEGF-induced migration of HUVEC was attenuated in the presence of the HO inhibitor by 46%, whereas L-NAME decreased it by 65% (Fig. 5). We did not observe any additive effect by both inhibitors. On the other hand, the HUVEC mor-

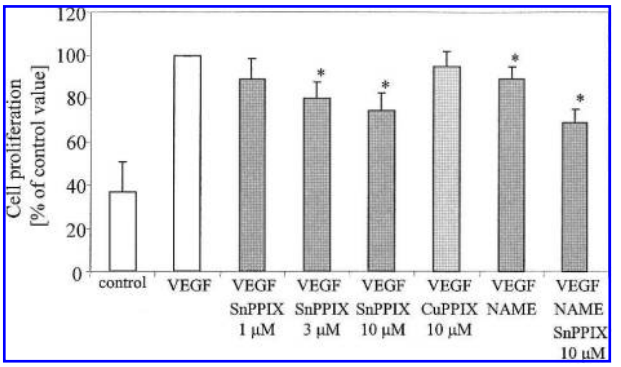

FIG. 4. Effect of SnPPIX (1–10 µ*M*), CuPPIX (10 µ*M*), and L-NAME (2 m*M*) on the VEGF-induced proliferation of HUVEC. Each column represents the mean ± SD of five experiments. *$p < 0.05$ in comparison with VEGF.

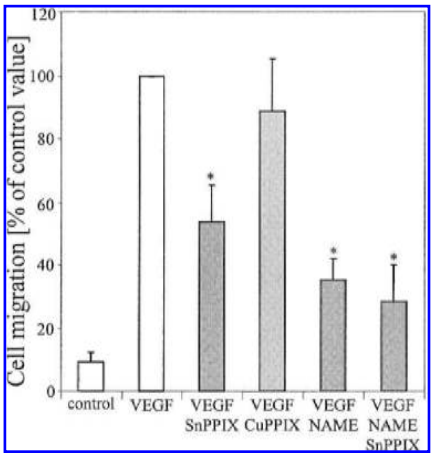

FIG. 5. Effect of SnPPIX (10 μ*M*), CuPPIX (10 μ*M*), and L-NAME (2 m*M*) on the VEGF-induced migration of HUVEC. Each column represents the mean ± SD of five experiments. **p* < 0.05 in comparison with VEGF.

phogenesis (Fig. 6) was comparably reduced by SnPPIX (by 48%) and L-NAME (by 59%), and both compounds displayed a strong additive effect.

SnPPIX decreases outgrowth of capillaries

SnPPIX attenuated not only the VEGF-induced activities of HUVEC, but decreased also a much more complex angiogenic response, namely the outgrowth of capillaries from endothelial spheroids embedded in collagen gel (Fig. 7). In accordance, the opposite effect—augmentation of capillary sprouting—was observed in spheroids overexpressing HO-1 after transfection with pcDNA-HO1 plasmid (Fig. 8). Control pSV-βgal plasmid did not exert any influence. Furthermore, the effect of HO-1 overexpression was mimicked by delivering CO into the cell system, as the augmentation of capillary outgrowth was demonstrated in HUVEC spheroids incubated with CO-RM. This suggests that the observed stimulation of angiogenesis by HO-1 may result from the increased CO production, whereas its inhibition by SnPPIX may be a consequence of the decrease in endogenous CO levels.

DISCUSSION

In earlier experiments, we demonstrated that induction or overexpression of HO-1 leads to the transcriptional activation of VEGF promoter, followed by the increased generation of VEGF mRNA and protein both in vascular smooth muscle cells and in microvascular endothelium (9, 15). These results, discrepant with a previous report (21), have been supported very recently in other experimental models (2, 19).

The present study confirms and further extends our earlier findings. We validated that induction of HO-1 by hemin or by 15d-PGJ_2 leads to increased synthesis of VEGF in HMEC-1, whereas inhibition of HO activity by SnPPIX attenuates VEGF generation. Most importantly, the changes in VEGF levels were proportional to the CO production in stimulated cells. Furthermore, incubation of HMEC-1 with $[Ru(CO)_3Cl_2]_2$ resulted

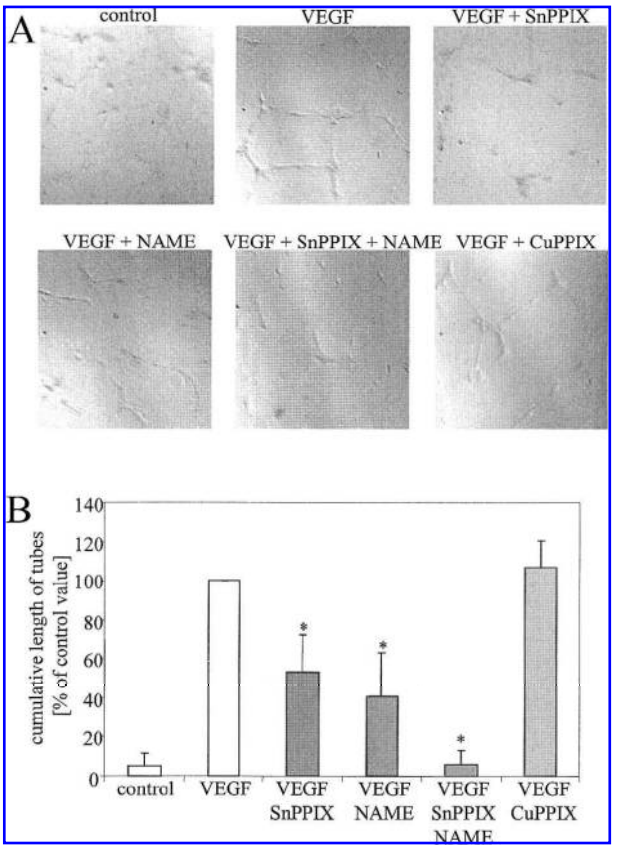

FIG. 6. Effect of SnPPIX (10 μ*M*), CuPPIX (10 μ*M*), and L-NAME (2 m*M*) on the VEGF-induced formation of tube-like structures by HUVEC seeded on Matrigel. (**A**) Representative pictures. (**B**) Quantitative analysis. Each column represents the mean ± SD of three experiments. **p* < 0.05 in comparison with VEGF.

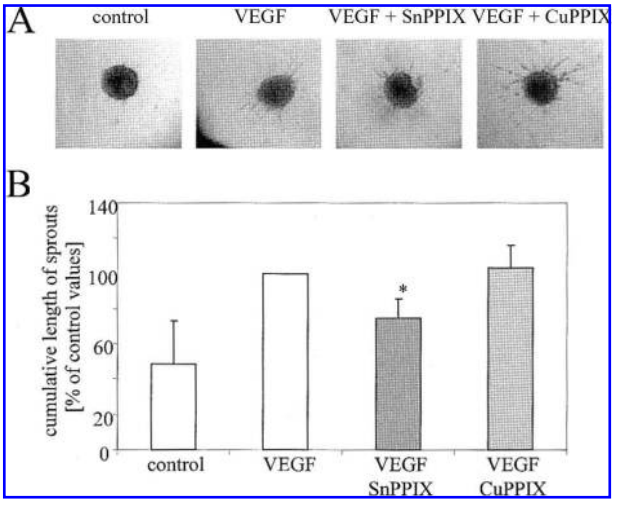

FIG. 7. Effect of SnPPIX (10 μ*M*), and CuPPIX (10 μ*M*) on the VEGF-induced outgrowth of capillaries from HUVEC spheroids. (**A**) Representative pictures. (**B**) Quantitative analysis. Each column represents the mean ± SD of three experiments. **p* < 0.05 in comparison with VEGF.

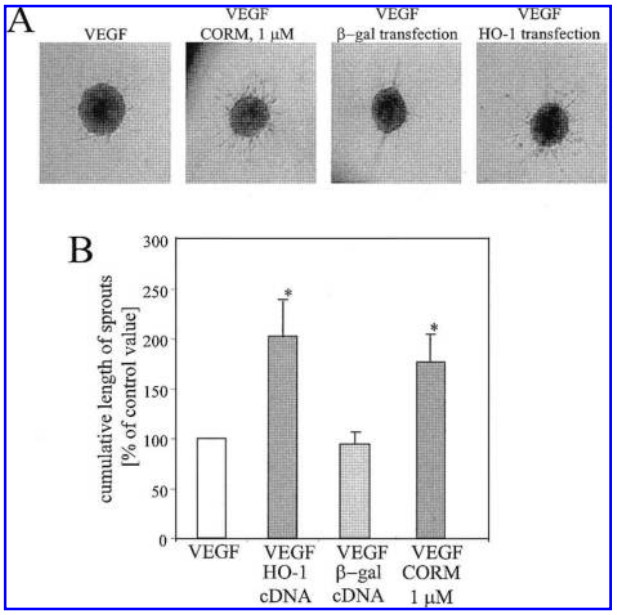

FIG. 8. Effects of CO-RM (1 μ*M*) and overexpression of HO-1 on the VEGF-induced outgrowth of capillaries from HUVEC spheroids. (**A**) Representative pictures. (**B**) Quantitative analysis. Each column represents the mean ± SD of three experiments. $*p < 0.05$ in comparison with VEGF. β-gal DNA, control plasmid.

both in increased CO concentration and in augmented VEGF synthesis, on a scale comparable to that after stimulation of cells with hemin. $[Ru(CO)_3Cl_2]_2$ has the ability to deliver CO into biological systems and has been demonstrated to act as a CO-RM (24). Finally, the stimulatory effect of HO-1 induction was reversed not only by SnPPIX, but also by hemoglobin (a CO scavenger) and by ODQ (inhibitor of cGMP synthesis). Taken together, our results suggest that in endothelial cells the effector molecule in HO-1-induced VEGF up-regulation is CO and its effect is mediated by cGMP elevation.

It is well known that, similarly to NO, CO leads to enzymatic activation of sGC enhancing the cGMP production. In experiments on purified enzymes, the potency of CO is much weaker compared with that of NO, and therefore some reports have questioned whether the sGC is a physiological target (32). It seems possible, however, that in intact tissues sGC is considerably more sensitive to CO (11). This supposition has been supported by the finding that cGMP levels are reduced in HO2−/− mice (20). Our results are in line with these suggestions, showing that incubation with SnPPIX decreased cGMP production in microvascular endothelial cells comparably to the effect of NO inhibition. Furthermore, simultaneous treatment of HMEC-1 with SnPPIX and L-NAME led to an additive inhibition, suggesting the independent pathways of HO- and NOS-mediated sGC activation.

Angiogenesis is characterized by a complex morphogenic cascade of events during which quiescent resting endothelial cells become activated to degrade extracellular matrix, migrate toward the angiogenic stimulus, proliferate, and align into new three-dimensional capillary networks (29). It is commonly accepted that VEGF-elicited neovascularization strongly depends on the generation of NO, as NOS inhibitors reduce the angiogenic potential of endothelial cells (for references, see 5).

Our experiments demonstrate that attenuation of HO activities exerts a similar antiangiogenic effect. We found that SnPPIX significantly decreased HUVEC proliferation, migration, and formation of tube-like structures on Matrigel. Importantly, the same doses of CuPPIX, a closely related compound that does not influence the HO pathway, did not change the VEGF-induced angiogenic response, supporting that the effect of SnPPIX is indeed associated with HO inhibition.

These results are in agreement with an earlier report showing that overexpression of HO-1 augments the angiogenic potential of the endothelium (6). However, in the previous study, only some angiogenic events were tested, namely cell proliferation and formation of tube like structures (6). The latter assay is the most commonly used model for testing angiogenesis *in vitro*. Nevertheless, as an angiogenic test, it appears to have some limitations, because tube formation on Matrigel has also been observed in nonendothelial cells (36). Therefore, in the present work, we used also another angiogenic model, *i.e.,* HUVEC spheroids. Spheroidal aggregation stabilizes endothelial cells, decreases their proliferation, and upregulates surface molecules typical for endothelium *in vivo*. Embedding of spheroids in collagen gel leads to radial capillary sprouting by invasion into the extracellular matrix. This process is very much in contrast to the alignment of a large number of separated endothelial cells into tubular structures and appears to be the closest *in vitro* representation of the angiogenic invasion that occurs *in vivo* (17).

Using the spheroid culture, we have shown that SnPPIX influences also the complex angiogenic response and decreases the VEGF-induced outgrowth of capillaries by ~30%. Once again, CuPPIX did not exert any significant effect, indicating that SnPPIX acts through the inhibition of HO. This finding is further strengthened by the observation that outgrowth of capillaries is strongly enhanced from spheroids overexpressing HO-1. Notably, a similar augmentation was induced when spheroids were incubated with a CO-RM. Altogether these results suggest that HO activity plays an important and permissive role in VEGF-induced angiogenesis and that CO is likely the molecule responsible for this effect.

Several angiogenic or proinflammatory mediators can activate HO-1 under pathological conditions, suggesting that this enzyme may be involved in the regulation of angiogenesis elicited by many inducers (6, 25, 31, 33). For instance, it appears that HO-1 expression correlates with increased vascular density in some tumors (12, 26, 30). In our study, the induction of VEGF in endothelial cells treated with hemin or 15d-PGJ_2 is mediated by HO-1. However, on the basis of the present experiments, we cannot discriminate between the effects of HO-1 and HO-2 in augmentation of VEGF-induced angiogenic responses, as SnPPIX inhibits both HO isozymes.

The role of HOs in angiogenesis resembles the function of NOSs (3, 5, 38). Comparison of the effects of L-NAME and SnPPIX suggests that the level of reduction of angiogenic potential is similar after blockage of HOs and NOSs. Interestingly, only HO seems to be involved in the regulation of HUVEC proliferation. The effect of L-NAME is weaker and is likely to be NO-independent (Józkowicz *et al.,* in prepara-

tion). On the other hand, cell motility and morphogenesis are regulated by both HO and NOS. L-NAME and SnPPIX did not exhibit an additive effect on cell migration, suggesting that NO and CO regulate the same pathway. In contrast, in the tube formation assay, L-NAME and SnPPIX displayed a strong additive effect, implying the existence of independent mechanisms regulating this complex process.

In summary, we postulate that HOs play an important and permissive role in angiogenesis. Inhibition of HO activities by SnPPIX can significantly reduce both the synthesis of VEGF and the capability of endothelial cells to respond to exogenous stimulation. These effects are mediated, at least in part, by changes in CO production.

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ABBREVIATIONS

cGMP, 3′5′-cyclic guanosine monophosphate; CO, carbon monoxide; CO-RM, carbon monoxide-releasing molecule; CuPPIX, copper protoporphyrin IX; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$prostaglandin-J_2; ECGF, endothelium cell growth supplement; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; FCS, fetal calf serum; HMEC-1, human microvascular endothelial cells; HO, heme oxygenase; HUVEC, human umbilical vein endothelial cells; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxydiazole[4,3-a]quinoxalin-1-one; sGC, soluble guanylyl cyclase; SnPPIX, tin protoporphyrin IX; VEGF, vascular endothelial growth factor.

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