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Heme Oxygenase Activity Modulates Vascular Endothelial Growth Factor Synthesis in Vascular Smooth Muscle Cells

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ABSTRACT

Hypoxia, cytokines, and nitric oxide (NO) stimulate the generation of vascular endothelial growth factor (VEGF) and induce heme oxygenase-1 (HO-1) expression in vascular tissue. HO-1 degrades heme to carbon monoxide (CO), iron, and biliverdin, the latter being reduced to bilirubin by biliverdin reductase. In the present study, we investigated the role of HO-1 in the modulation of VEGF synthesis in rat vascular smooth muscle cells (VSMC). In VSMC stimulated with cytokines, inhibition of NO production significantly, but not completely, reduced VEGF release. In contrast, inhibition of HO activity by tin protoporphyrin IX (SnPPIX) totally prevented cytokine-induced increase in VEGF, despite an augmented synthesis of intracellular NO. Stimulation of HO-1 activity by hemin enhanced VEGF production; this effect was abrogated by blockade of the HO pathway. Similarly, VEGF synthesis induced by hypoxia was down-regulated by SnPPIX, but not by inhibitors of NO synthase. To elucidate further a direct involvement of HO-1 in the observed effects, we generated transfected cells that overexpressed the HO-1 gene. Notably, these cells synthesized significantly more VEGF protein than cells transfected with a control gene. Among the products of HO-1, biliverdin and bilirubin showed no effect, whereas iron ions inhibited VEGF synthesis. Exposure of cells to 1% CO resulted in a marked accumulation of VEGF (20-fold increase) over the basal level. Our data indicate that HO-1 activity influences the generation of VEGF in VSMC in both normoxic and hypoxic conditions. As CO and iron, respectively the inducer and the inhibitor of VEGF synthesis, are concomitantly produced during the degradation of heme, these data indicate that HO by-products may differentially modulate VEGF production. Antioxid. Redox Signal. 4, 229–240.

INTRODUCTION

ANGIOGENESIS is crucial to many physiological and pathological processes, including ovulation, bone maturation, wound healing, cancer, and atherosclerosis (for reviews, see 3, 12). Vascular endothelial growth factor (VEGF; VEGF-A), the main stimulus for proliferation of endothelial cells, is a major player in the processes leading to the formation of new capillaries (3, 12). Enhancement of VEGF expression is mediated by hypoxia, inflammatory cytokines, and some growth factors (3, 12).

Besides VEGF, two gaseous molecules are also important determinants of angiogenesis: nitric oxide (NO) and carbon monoxide (CO). NO is continuously synthesized by endothelial cells, and its production seems to be a prerequisite for maintaining a wide array of vascular activities and functions

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(5). NO production can also be markedly increased under inflammatory conditions when inducible NO synthase (iNOS) is activated in numerous cell types (for review, see 18). CO is generated by heme oxygenases (HO) during the degradation of heme to iron and biliverdin, the latter being converted to bilirubin by a cytosolic biliverdin reductase (for review, see 13, 30). Of the three known different HO isoforms (HO-1, HO-2, and HO-3), the stress-inducible enzyme HO-1 is highly activated both under hypoxic conditions and by a variety of inflammatory stimuli (for review, see 13, 30). Recently, it has been demonstrated that transfer of the HO-1 gene resulted in enhancement of endothelial cell proliferation, suggesting an involvement of this enzyme in angiogenesis (6). It has also been described that the number of HO-1-expressing macrophages positively correlated with the extent of angiogenesis in glioma (37). Numerous lines of evidence indicate that HO-1 expression is enhanced by NO and NO-related species (10, 14, 15, 36). The increased expression of HO-1 may represent a refined intracellular system to control iNOS activity (7, 41), as increased HO activity would limit the availability of heme, both a substrate for HO and an important cofactor for NO synthase (NOS) (43).

Among the possible biological actions of endogenously generated NO and CO in the vessel wall is the regulation of VEGF synthesis. In vascular tissue, VEGF is mostly produced by vascular smooth muscle cells (VSMC) (28). Recently, we have shown that up-regulation of VEGF expression by interleukin-1β(IL-1β) is dependent on NO generation in VSMC (8). This observation was further corroborated by showing that VEGF synthesis is enhanced by different NO donors or by overexpression of endothelial NOS (eNOS) or iNOS gene (21, 22). Similarly, the dependence of VEGF synthesis on NO generation has been demonstrated in other cell types (1, 16, 24, 44).

Previous reports suggest, however, that under hypoxic conditions both NO and CO can be inhibitory for VEGF synthesis; this appears to involve a diminished activation of hypoxia-induced factor-1a (19, 29, 39). In view of recent data from our (8, 21, 22) and other laboratories (1, 16, 23, 44) demonstrating the positive influence of NO on VEGF synthesis, we investigated the specific role of the HO pathway in the modulation of VEGF expression. We show here that HO-1 expression and activity positively correlate with enhanced VEGF production both in normoxic and in hypoxic conditions. Among the three products of heme degradation by HO activity, *i.e.,* bilirubin, iron, and CO, the latter two appear to influence VEGF production significantly in VSMC.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium (DMEM) F12 medium and fetal calf serum (FCS) were purchased from GibcoBRL. The various kits used, including total RNA Extraction, Tth DNA Polymerase, and nonradioactive cytotoxic lactate dehydrogenase (LDH) assay, were from Promega (Madison, WI, U.S.A.). Isothiourea (ITU), tin protoporphyrin IX (SnPPIX), copper protoporphyrin IX (CuPPIX), and zinc (II) deutero protoporphyrin IX-2,4-bisethyleneglycol (ZnDPPIX) were from Alexis. Oxyhemoglobin (oxy-Hb; content of methemoglobin < 7%) was purchased from Calzyme Laboratories (San Luis, Obispo, CA, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). VEGF released in the cell culture media was measured from rat or human VSMC using an enzyme-linked immunosorbent assay (ELISA) kit for mouse or human VEGF, respectively (R&D, Abingdon, U.K.). The quantitative measurements of rat VEGF mRNA were done by ELISA mRNA Quantitative Assay (R&D). The protein content in the cell lysate was determined by Bio-Rad DC Protein Assay. Plasmids used for transfection were isolated from transformed HB101 *E. coli* using the EndoFree Plasmid Isolation Kit (Qiagen, Germany).

Cell culture

Rat VSMC were isolated as previously described (8) and cultured in DMEM F-12 supplemented with 10% FCS at 37°C in an atmosphere of air/5% CO_2. VSMC phenotype was determined by positive immunostaining of cells for β-actin. Cells between passage 3 and 10 were used in all experiments. Human coronary artery VSMC were purchased from Clonetics (CellSystem, St. Katharine, Germany) and cultured in smooth muscle basal medium (Clonetics) containing 5% FCS. NIH 3T3 cells (ATCC, CRL-1658) were cultured in high-glucose DMEM supplemented with 5% FCS.

Experimental protocols

Cell culture and treatments. Rat VSMC were cultured to confluence in 10% FCS/DMEM F-12 medium and then placed in medium containing 0.5% FCS for 24 h prior to any treatment. Cells were treated in normoxic conditions (air/5% CO_2) with IL-1β (10 ng/ml) for 24 h in the presence or absence of L-methyl ester L-arginine (L-NAME, 2 m*M*) and/or SnPPIX (1–10 μ*M*), inhibitors of NOS and HO activity, respectively. After 24 h, the medium was collected for determination of VEGF release, and RNA was isolated from the cells. In some experiments, cells were washed twice with phosphate-buffered saline (PBS) and than lysed in PBS buffer (pH 7.4) containing 0.1% Triton. The supernatants containing cytosol proteins were stored at −70°C until analysis. In other experiments, rat VSMC were treated with cytokine or hemin in the presence of ZnDPPIX (10 μ*M*) or CuPPIX (10 μ*M*), two other commonly used inhibitors of HO activity. In another set of experiments, VSMC were pretreated for 2 h with 3–10 μ*M* hemin, an inducer of HO-1 expression. The medium was then replaced with fresh medium, and cells were incubated for an additional 6 h prior to addition of IL-1β and/or SnPPIX for the remaining 18 h. The medium was collected and VEGF determined by ELISA. For the HO activity assay, cells were grown in complete medium, treated with hemin and/or SnPPIX, and HO activity was determined after 6 h in cell lysates according to the method previously described (36).

Human VSMC were treated with hemin and/or SnPPIX and/or oxy-Hb (5 μ*M*) in the same way as rat VSMC, and the medium was collected after 24 h for determination of VEGF

synthesis. To determine the effect of hemin and SnPPIX on VEGF mRNA expression, rat VSMC were also treated with hemin (10 μ*M*) and/or SnPPIX (10 μ*M*) for 6–12 h. Both compounds were incubated with the cells for the entire period of exposure.

In a final set of experiments, rat VSMC were exposed to hypoxia (95% N_2, 5% CO_2; pO_2 = 2 mm Hg) using a hypoxic chamber (Billups–Rothenberg Inc., Del Mar, CA, U.S.A.) as previously described (36). Incubation was performed in the presence or absence of 2 m*M* L-NAME, 50 μ*M* ITU, (a specific iNOS inhibitor), and/or 1–10 μ*M* SnPPIX.

Transfection of cells with HO-1 expression plasmid. Expression plasmids (pcDNA-HO-1) were constructed by cloning rat HO-1 cDNA (kindly provided by Prof. Mahin Maines, Rochester, NY, U.S.A.) to pcDNA3 plasmid. Transfection of either rat or human VSMC was performed in 24-well plates as described previously (8, 22). In brief, different amounts of the plasmid were mixed with Tfx^{50} liposome, at the charge ratio 1:2. Cells were treated with the transfection mixture (200 μl) for 1 h in a medium without FCS, followed by 23 h of incubation in complete medium (DMEM F-12, 10% FCS). At the end of the incubation period, the medium was replaced with a fresh one and cells were cultured for an additional 48 h. Control cells were transfected with pSVβ-gal plasmid (Promega) or pCMVβ-gal plasmid (Clontech, Palo Alto, CA, U.S.A.).

Effect of HO-1 expression on human VEGF promoter activity. Rat VSMC or NIH 3T3 cells were co-transfected with pcDNA-HO-1 or pSVβ-gal plasmids together with VEGF-luciferase plasmid (kindly provided by Dr. Kimura) (24). This plasmid contains the luciferase reporter gene driven by a full sequence of human VEGF promoter. Luciferase activity in the cellular extracts was determined 48 h (NIH 3T3 cells) or 72 h (VSMC) after transfection according to the vendor's protocol (Luciferase Reporter Gene Assay, High Sensitivity, Roche Biochemicals).

Effect of HO by-products on VEGF synthesis. Rat or human VSMC were treated for 24 h with either biliverdin or bilirubin (0.5–10 μ*M*), ferrous sulfate ($FeSO_4$) or ferric sulfate [$Fe_2(SO_4)_3$] (10–100 μ*M*). In additional experiments, VSMC were treated with 100 or 500 μ*M* deferoxamine mesylate, an iron chelator. To investigate the role of CO in VEGF synthesis, rat VSMC were kept for 24 h in 1% CO atmosphere (*i.e.,* with the normal level of oxygen) and compared with cells grown under normoxic conditions (air/5% CO_2). To investigate whether scavenging of CO will influence VEGF synthesis, rat or human VSMC were treated with oxy-Hb in the presence or absence of hemin, as described above.

Biochemical assays. Gene expression was analysed by reverse transcription–polymerase chain reaction (RT-PCR) using specific primers for VEGF, iNOS, HO-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control (housekeeping gene), as described previously (8). In addition, VEGF mRNA expression was quantified by ELISA mRNA assay. NO generation was estimated by the Griess reagent method (8). VEGF synthesis was determined in culture medium or in cell lysate using an ELISA kit. In our hands, both rat and human VSMC generated easily detectable VEGF protein (picograms per milliliter of medium), even at basal conditions and even when cultured in small wells (24-well plates). When the amount of VEGF differed between cell batches, the mean results of different experiments were expressed as percentage of VEGF produced by controls. The HO activity assay was performed as previously described (14, 15). HO-1 protein expression was detected by western blot (36), and cell viability was estimated by determination of the amount of LDH released into the culture media.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Tukey test for multiple group comparison or with the Mann–Whitney *U* test for comparison between two groups. Data are expressed as means ± SD, and differences at $p < 0.05$ were considered as significant. All experiments were repeated at least three times, and studies were done in at least duplicates or triplicates.

RESULTS

Hemin and IL-1β induce HO-1 expression and increase HO activity in VSMC

Basal expression and activity of HO-1 were detected in nonstimulated rat VSMC (Fig. 1A and B). Incubation of cells with hemin, substrate and inducer for HO-1, resulted in increased HO-1 protein synthesis and enhancement of enzymatic activity (threefold increase, Fig. 1A and B). A similar effect was found after treatment of cells with IL-1β (Fig. 1A). Basal and stimulated activities of HO-1 were both significantly diminished by SnPPIX, a well characterized HO inhibitor (Fig. 1B). Interestingly, as reported by others in previous studies (34, 36), SnPPIX enhanced HO-1 protein expression (Fig. 1A).

Increased HO activity is associated with augmented VEGF synthesis

Treatment of VSMC with hemin caused a significant increase in VEGF synthesis, in both untreated and IL-1β-stimulated cells (Fig. 1C); this effect was completely reversed by SnPPIX (Fig. 1C). In addition, ZnDPPIX, another potent inhibitor of HO (4), decreased basal VEGF synthesis (fourfold) and completely abrogated hemin- or cytokine-induced VEGF production (data not shown). CuPPIX, which is known to have a weak effect on HO activity (45), did not significantly affect HO activity and VEGF synthesis. In the presence of 10 μ*M* CuPPIX, HO activity was 86 ± 13% of control values, whereas VEGF synthesis was 113 ± 29% of control.

L-NAME and SnPPIX down-regulate cytokine-induced synthesis of VEGF in normoxic conditions

Stimulation of rat VSMC with IL-1β induced the expression of iNOS and increased the synthesis of both NO and VEGF (Fig. 2A). As shown in our recent studies (8, 21, 22), the present data confirm that inhibition of cytokine-elicited NO

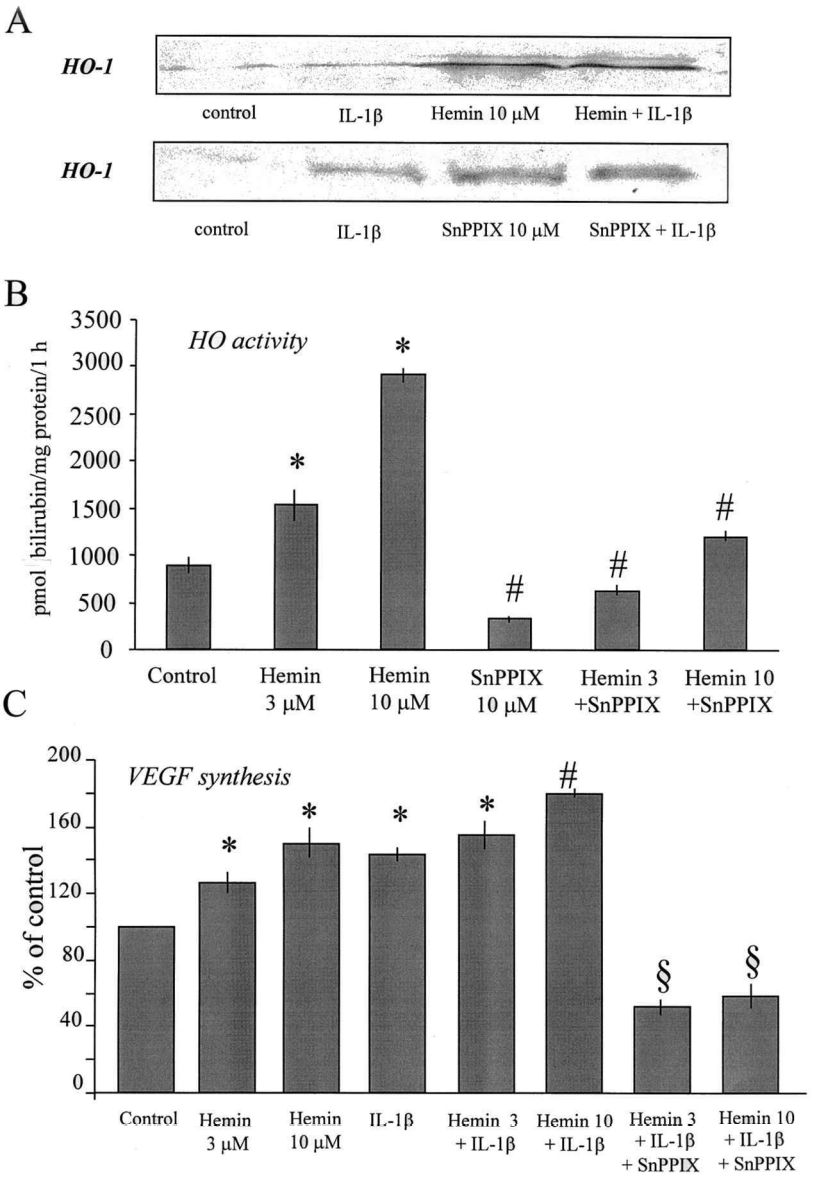

FIG. 1. Modulatory effects by the HO pathway on VEGF synthesis in VSMC. Rat VSMC were treated with hemin, SnPPIX, IL-1β, or a combination of these agents for 24 h. HO-1 protein expression (**A**), HO activity (**B**), and VEGF release in culture media (**C**) were determined as described in Materials and Methods. Results are expressed as means ± SD ($n = 3$). *$p <$ 0.01 vs. control; #$p < 0.01$ vs. IL-1β and vs. hemin 3 μ*M* + IL-1β; §$p < 0.01$ vs. hemin, IL-1β, and control.

generation by L-NAME results in a significant, but not complete, decrease in the production of VEGF (Fig. 2B). In contrast, treatment of VSMC with SnPPIX (10 μ*M*) was accompanied by total inhibition of cytokine-induced VEGF up-regulation (Fig. 2B). The combination of both inhibitors did not further affect VEGF synthesis (Fig. 2B). Incubation of cytokine-treated VSMC with SnPPIX enhanced the generation of NO in a concentration-dependent manner (Fig. 3A). However, at the same time, SnPPIX considerably and dose-dependently inhibited both basal and cytokine-induced release of VEGF into the culture media, as evidenced by ELISA measurements (Fig. 3B). This effect was not the result of an interference by protein secretion because inhibition to a similar extent was also detected in cell lysates (Fig. 4). SnPPIX always decreased VEGF synthesis and was not toxic for the cells at concentrations up to 100 μ*M*, as evidenced by no changes in LDH release (data not shown). In addition, SnPPIX always decreased VEGF production in other cell types, such as human VSMC, human microvascular endothelial cells, or murine macrophages (data not shown; manuscript in preparation).

Interestingly, and similarly to data reported by others (29), we found that SnPPIX increased VEGF mRNA expression. This effect was confirmed by RT-PCR, which demonstrated that 10 μ*M* SnPPIX enhanced VEGF mRNA synthesis in nonstimulated cells (Figs. 3C and 5). However, despite an augmented VEGF mRNA expression, VEGF protein was always inhibited by SnPPIX (Figs. 3–5). VEGF expression could also be increased after hemin treatment, as observed by RT-PCR (Fig. 5A) and determined by measurement with quantitative ELISA mRNA assay (Fig. 5B). This effect reflects the increase in VEGF protein observed after hemin treatment (Fig. 5C).

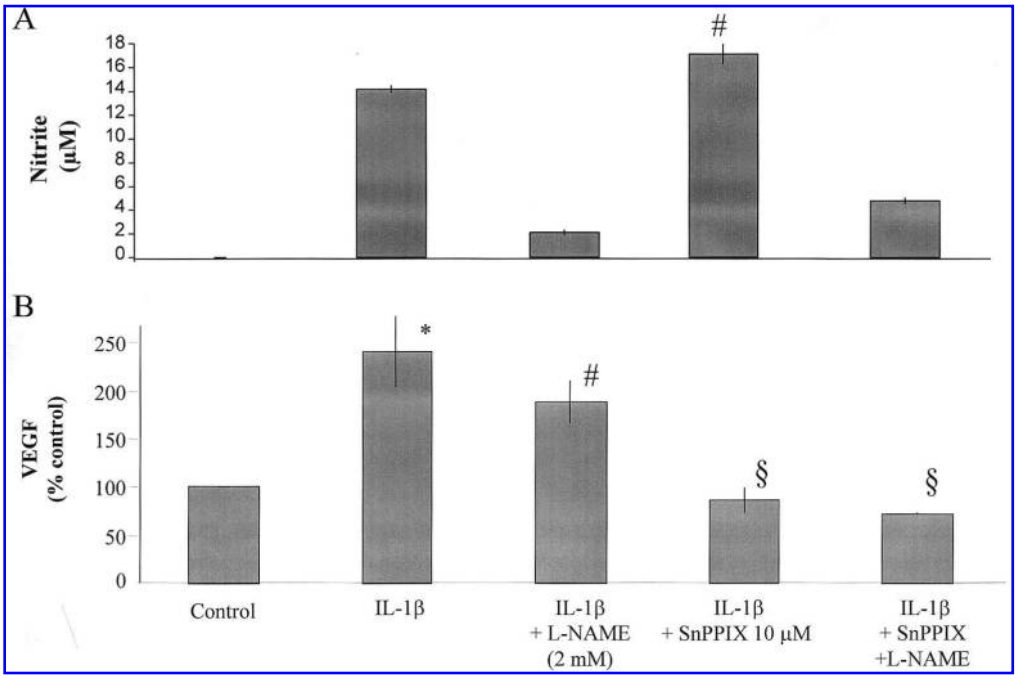

FIG. 2. Effect of NOS and HO inhibitors on VEGF synthesis in normoxic conditions. Rat VSMC were treated with cytokine in the presence or absence of L-NAME or SnPPIX. After 24 h, the media were collected and used for determination of NO generation (Griess method) and VEGF synthesis (ELISA). Results are expressed as means ± SD (n = 5–7). *p < 0.001 vs. control; #p < 0.01 vs. IL-1β; §p < 0.001 vs. IL-1β and IL-1β + L-NAME.

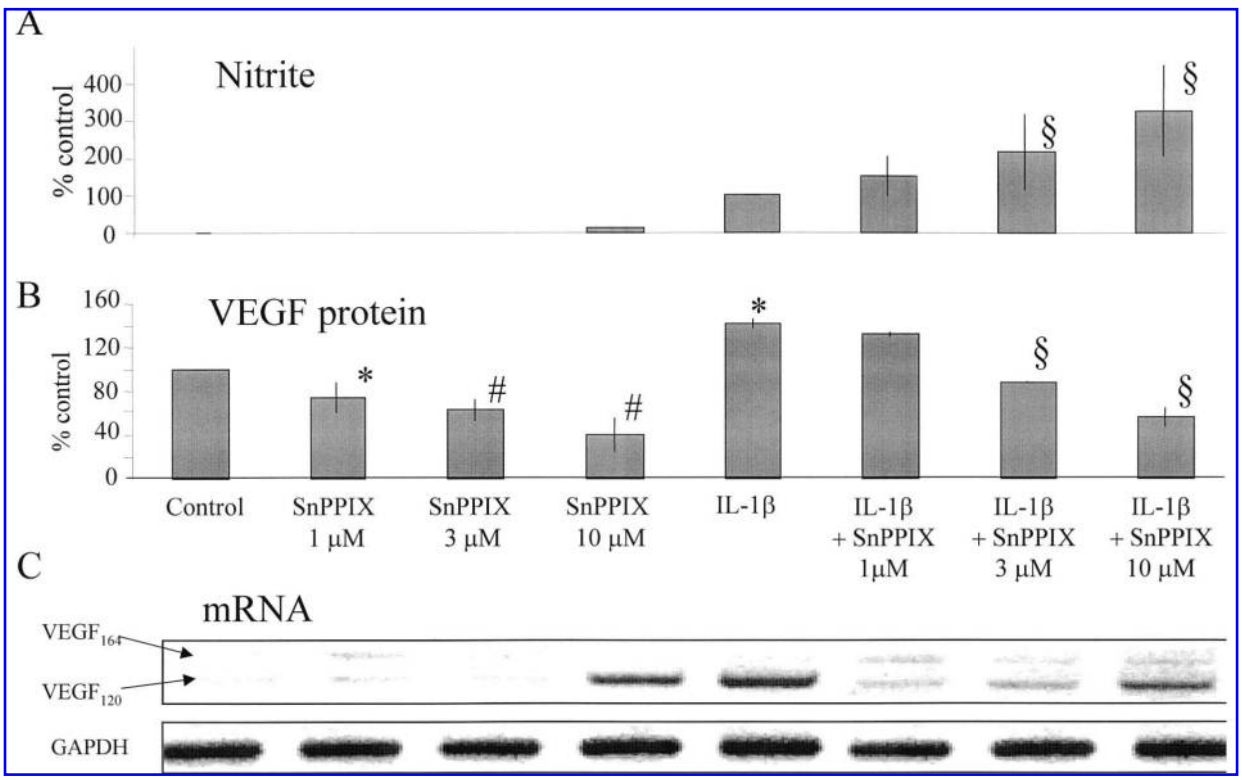

FIG. 3. Regulation of basal and IL-1β-induced VEGF synthesis by HO activity. SnPPIX (1–10 μM) decreased both basal and IL-1β-induced VEGF synthesis (**B**), although at the same time SnPPIX increased IL-1β-induced NO generation (**A**). Interestingly, SnPPIX at the concentration of 10 μM appears to enhance basal VEGF mRNA expression (**C**), and the inhibitory effect of SnPPIX on cytokine-induced VEGF mRNA expression is stronger at lower (1–3 μM) than at higher (10 μM) concentrations (**C**). Results are expressed as means ± SD (n = 5–9). *p < 0.05 vs. control; #p < 0.01 vs. control; §p < 0.05 vs. IL-1β.

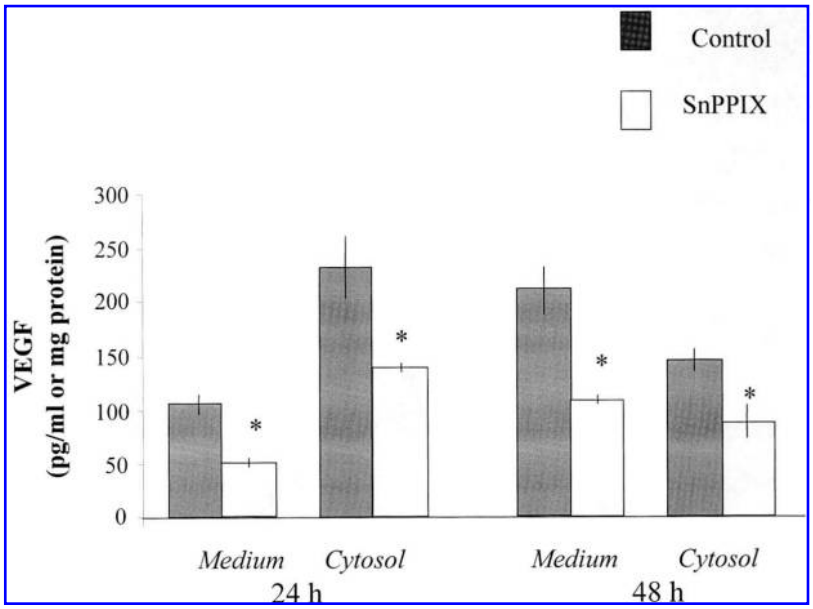

FIG. 4. Effect of SnPPIX on VEGF protein synthesis in VSMC. Rat VSMC were treated with SnPPIX for 24–48 h. At the indicated time points, the media were collected and cells lysed with PBS/0.1% Triton X-100. VEGF was determined in both culture media and cell lysates. The concentration of VEGF in the media is expressed as pg/ml and in the cell lysates as pg/mg of the total cellular protein. Results are expressed as means ± SD (n = 4–6). *p < 0.002 vs. control.

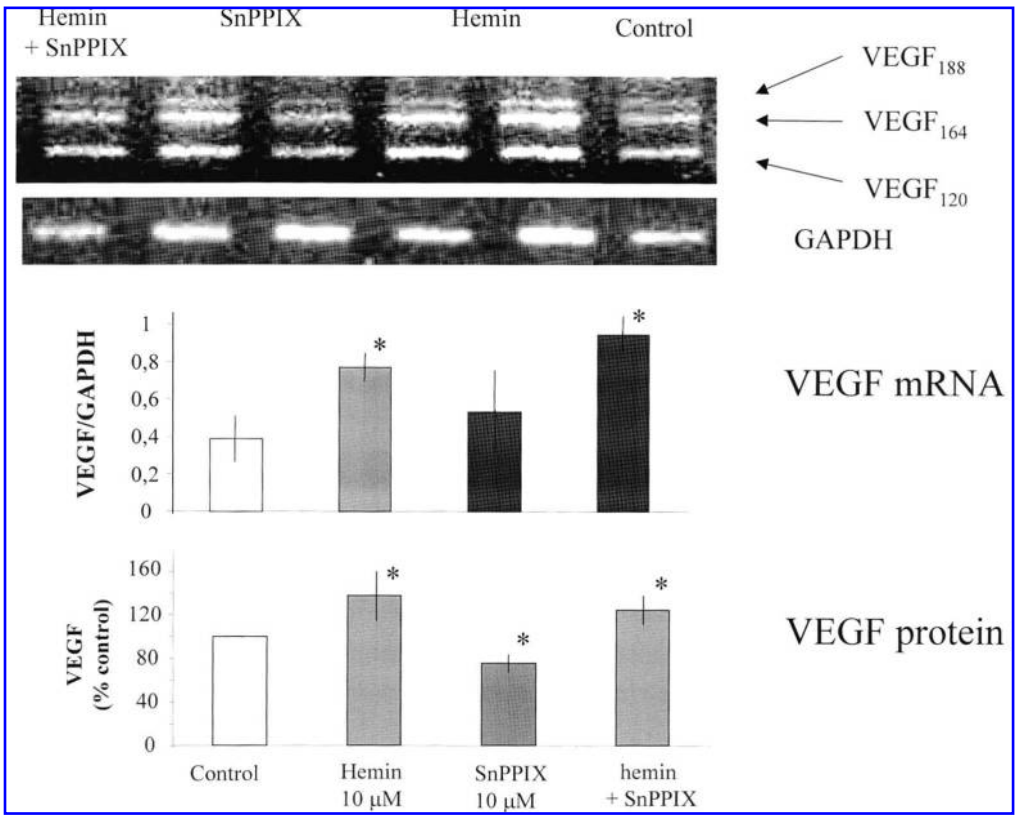

FIG. 5. Regulation of VEGF mRNA expression by SnPPIX and hemin. Rat VSMC cells were treated for 12 h. RNA was isolated as described in Materials and Methods, and VEGF and GAPDH mRNA expression was measured by ELISA mRNA assay or used for RT-PCR. Note that both hemin and SnPPIX enhanced VEGF mRNA expression (**A** and **B**). However, VEGF protein synthesis, determined by ELISA, was increased only after hemin treatment (**C**). The data are the means of two independent experiments (protein measurement) and are the representative results of one of three independent measurements (VEGF mRNA ELISA). Results are expressed as means ± SD (n = 6). *p < 0.05 vs. control.

SnPPIX, but not NOS inhibitors, prevents VEGF synthesis during hypoxia

Under hypoxic conditions, rat VSMC generated up to eight times more VEGF than cells exposed to normoxia (Fig. 6). As in normal conditions, hypoxia-induced VEGF protein synthesis was significantly inhibited by SnPPIX. In contrast, NOS inhibitors (L-NAME and ITU) were without any effect (Fig. 6).

Overexpression of HO-1 results in enhanced VEGF synthesis

Overexpression of the HO-1 gene following transfection to rat of human VSMC resulted in the enhanced expression of VEGF mRNA (Fig. 7A). Co-transfection of rat VSMC with an HO-1 expression plasmid together with a VEGF-luciferase reporter plasmid resulted in the augmentation of VEGF promoter activity, as determined by increased luciferase production (Fig. 7B). Similar results were obtained in NIH 3T3 cells (data not shown). Thus, the stimulatory effect of HO-1 on the VEGF expression occurs, at least in part, at the promoter level. As a consequence of enhanced VEGF gene transcription, the HO-1-transfected VSMC, either human or rat, generated up to four times more VEGF than cells transfected with control plasmid (Fig. 7C). The effect was dependent on the amount of the introduced pcHO-1 plasmid (Fig. 7C).

Effect of HO products on VEGF synthesis in VSMC

To investigate which products of HO activity are involved in the induction of VEGF synthesis, we tested the effects of biliverdin, bilirubin, iron, and CO. At physiological concentrations (0.5–10 μM), neither biliverdin nor bilirubin has any effect on VEGF synthesis by VSMC (data not shown). Ferric and ferrous ions decreased both basal and cytokine-induced VEGF synthesis (Fig. 8A and C), whereas the iron chelator deferoxamine significantly promoted basal, cytokine- (Fig. 8B and C), and hemin-induced (data not shown) VEGF synthesis. Thus, we hypothesized that CO could be involved in the induction of VEGF synthesis. To test this hypothesis, we exposed cells for 24 h in normoxic conditions in the presence of 1% CO. This treatment resulted in a significantly ($p < 0.001$) higher VEGF content in the media compared with normoxic conditions (Fig. 9A). Although some toxicity has been observed in the presence of this concentration of CO (Fig. 9B), the VEGF generation was enhanced up to 20 times (Fig. 9A). In other sets of experiments, we have treated either rat or human VSMC with hemin and/or oxy-Hb. In the presence of oxy-Hb, the VEGF production was significantly decreased (Fig. 9C).

To check whether the toxicity may be the major determinant of enhanced VEGF release of VEGF after CO or hemin, we treated VSMC with toxic amounts of hemin (100 μM) or dimethyl sulfoxide (DMSO) (1%). In all these cases, VEGF synthesis was potently decreased, whereas LDH release was enhanced (data not shown).

DISCUSSION

In the present study, we demonstrated that HO, the enzymes generating CO, iron, and biliverdin from the substrate heme, influence VEGF synthesis in both normoxic and hypoxic conditions. Increased HO-1 expression resulted in an augmented VEGF synthesis, whereas inhibition of HO activity led to a decrease in VEGF production.

As HO-1 and iNOS are strongly up-regulated under hypoxic conditions (13, 30), we aimed to elucidate the contribu-

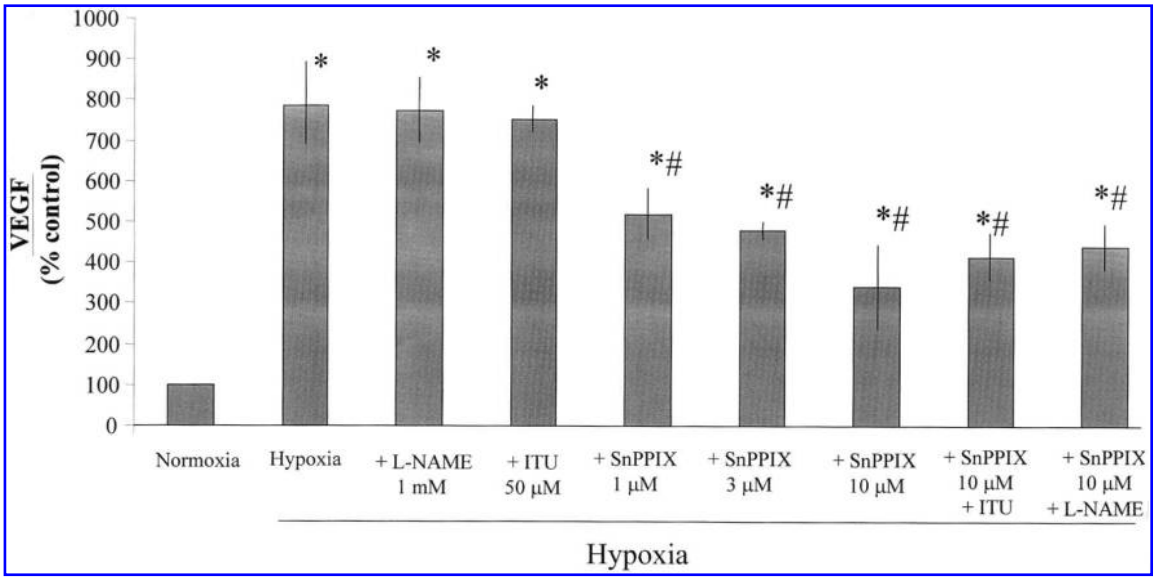

FIG. 6. Effect of NOS and HO pathways on VEGF synthesis during hypoxia. Rat VSMC were exposed to hypoxia (pO_2 = 2 mm Hg) for 24 h in the presence or absence of NOS inhibitors (L-NAME or ITU), SnPPIX, or both (L-NAME + SnPPIX or ITU + SnPPIX). Control groups are represented by cells exposed to normoxic conditions. Results are expressed as means ± SD (n = 3–5). *$p < 0.001$ vs. normoxic control; #$p < 0.01$ vs. hypoxia.

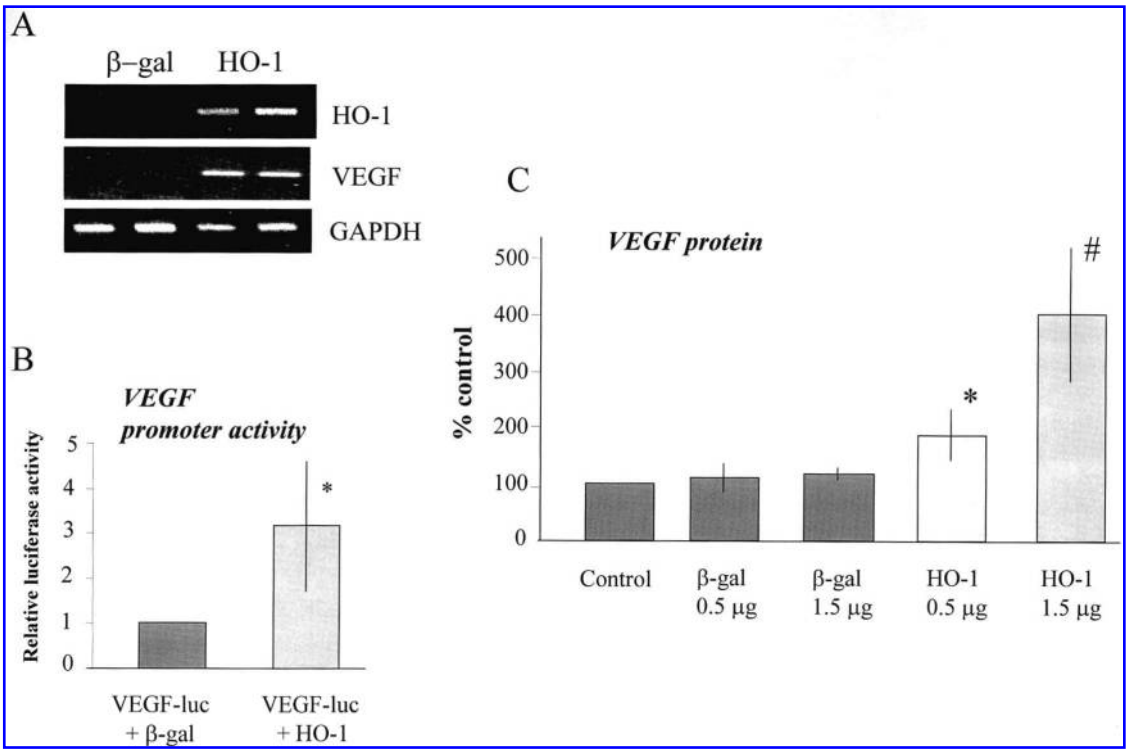

FIG. 7. Effect of HO-1 transfection on VEGF expression. (**A**) Rat VSMC were transfected with 0.5 μg of pcDNA-HO-1 plasmid or control (pSVβ-gal or pCMVβ-gal plasmid) according to methods described previously (22). RNA was isolated after 72 h and used for RT-PCR. A representative picture of one of three similar RT-PCR is shown in part A. For determination of promoter activity (**B**), rat VSMC were co-transfected with VEGF-luc plasmid containing luciferase gene driven by human VEGF promoter and by pcHO-1 or pSVβ-gal plasmid. The luciferase production was determined in the cellular extract 72 h after transfection. Results are expressed as means ± SD ($n = 7$). *$p < 0.05$ vs. cells transfected with VEGF-luc plasmid and β-gal control vector. (**C**) Human VSMC were transfected with different doses of control, pSVβ-gal plasmid, or pcHO-1 plasmid. VEGF production was measured in culture media collected 72 h after transfection. Results are expressed as means ± SD ($n = 4$–8). *$p <$ 0.05, #$p < 0.01$ vs. control cells or cells transfected with control plasmid.

tion of these metabolic pathways in hypoxia-mediated VEGF synthesis. The present data indicate that iNOS does not play a major role in hypoxia-mediated induction of VEGF, but inhibition of NO generation in normoxic conditions decreased VEGF synthesis. In contrast, inhibition of HO activity by SnPPIX resulted in a profound reduction of hypoxia-induced VEGF synthesis and abolished VEGF production in normoxic conditions.

We investigated the possible role of HO-1-derived catabolites. Neither biliverdin nor bilirubin influenced basal VEGF production, whereas iron attenuated the generation of VEGF. This is in agreement with previous reports showing that iron inhibits hypoxia-inducible factor-1α (HIF-1α) activity (33). Furthermore, the iron chelator deferoxamine is a potent activator of VEGF synthesis (2, 17, this study). Therefore, we hypothesized that increased CO as a result of HO-1 activation stimulates VEGF synthesis. In accordance with this view, cells exposed for 24 h to an atmosphere containing 1% CO generated 20 times more VEGF than VSMC grown in normoxic conditions. Thus, CO appears to be a crucial regulator of VEGF synthesis.

Our results show that oxy-Hb, a scavenger of CO, partially abolished basal or hemin-induced VEGF synthesis. It is unlikely that hemoglobin enters cells and, therefore, can only scavenge CO from outside. Thus, hemoglobin may only partially prevent CO activity. In addition, as hemoglobin can ultimately induce HO-1 expression (32), the inhibitory effect of this heme-dependent protein cannot be total.

Our study is in agreement with recent observations of Marti and Risau (31), who demonstrated that exposure of mice to 0.1% CO for 6 h resulted in the induction of VEGF expression in numerous organs, and the extent of this induction was similar to that obtained in mice kept under 6% oxygen. Thus, it cannot be excluded that hypoxia originated from CO exposure enhances VEGF expression. Interestingly, other investigators reported an apparent enhancement of VEGF synthesis in kidney of animals exposed to 1% CO (26).

Our results appear to be conflicting with other studies (19, 29, 39) describing the influence of CO on hypoxia-induced HIF-1α activation (19, 39) and VEGF expression (29). In a report showing the inhibitory effect of CO on the activity of HIF-1α, HepG2 cells were cultured in hypoxic conditions and were further treated with very high concentrations of CO (up to 80%); no data concerning cell viability were reported in those studies (19). In our experiments performed under conditions using a much lower concentration of CO (1%),

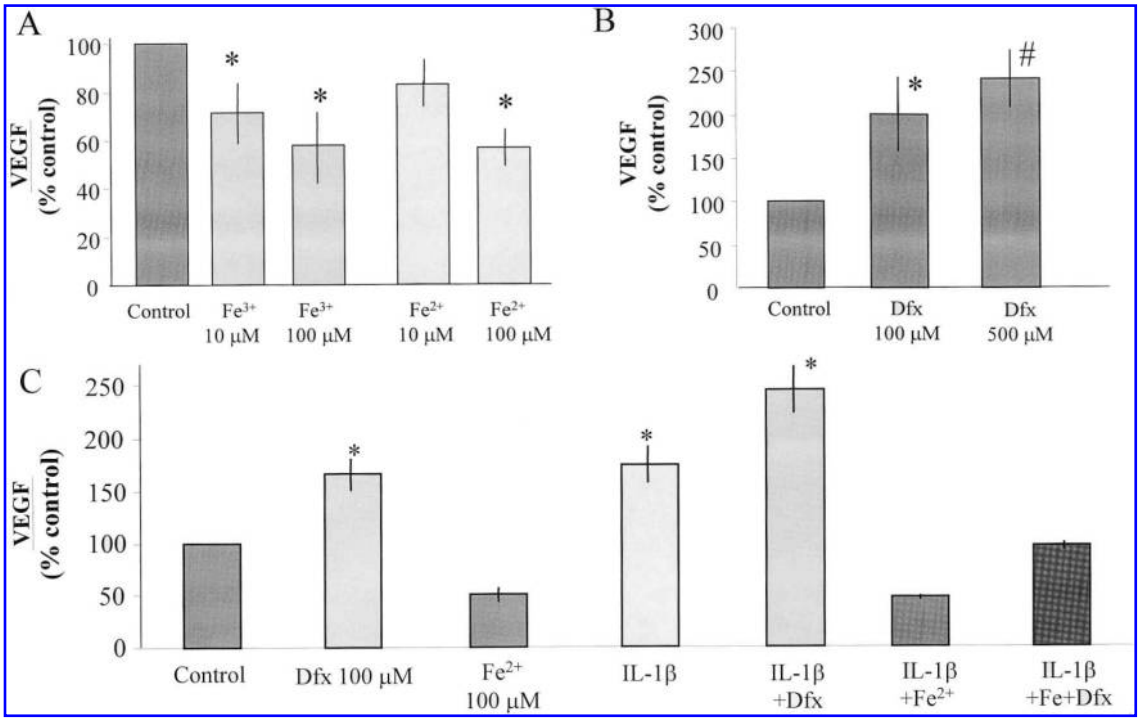

FIG. 8. Effect of iron and the iron chelator deferoxamine (Dfx) on VEGF synthesis. Rat VSMC were treated with ferric or ferrous sulfate (**A**) or with deferoxamine alone (**B**) or in combination with IL-1β (**C**). Iron ions and deferoxamine influenced both basal (A and B) and cytokine-induced (C) VEGF synthesis. Results are expressed as means ± SD (n = 4–8). *$p < 0.05$ vs. control; #$p < 0.001$ vs. control.

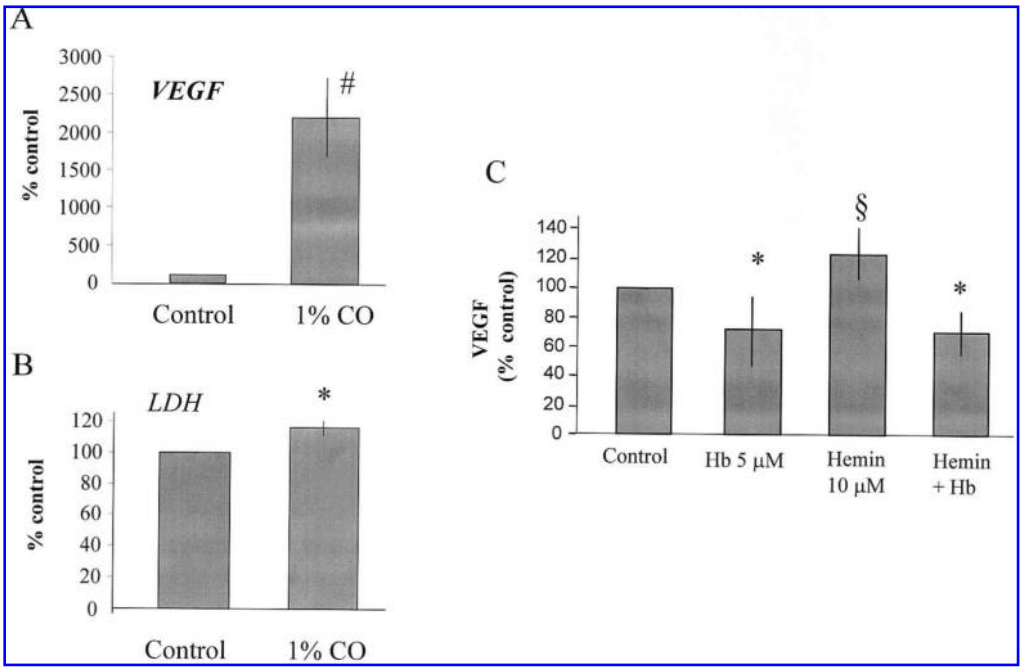

FIG. 9. Effect of CO on VEGF synthesis in VSMC. Rat VSMC were treated for 24 h with a gas mixture of 1% CO/5% CO_2/21% O_2 or in normoxic conditions (air/5% CO_2). Although 1% CO induced some cytotoxicity (**B**), this treatment very potently enhanced VEGF synthesis in rat VSMC (**A**). Human VSMC were treated with hemin and/or oxy-Hb (Hb), and VEGF concentration in the media was determined after 24 h. Oxy-Hb slightly diminished VEGF synthesis (**C**). A similar effect of oxy-Hb, which scavenges CO, was observed in rat VSMC culture. Results are expressed as means ± SD (n = 8–10). *$p < 0.05$ vs. control; #$p < 0.001$ vs. control; §$p < 0.05$ vs. control or oxy-Hb.

cells displayed some degree of distress, although VEGF synthesis was very potently increased. Thus, one can speculate that the effect on VEGF synthesis of very high concentrations of CO (10–80%) applied during hypoxia might be different from lower (1%) concentrations of this gas.

We observed that SnPPIX consistently, and in a concentration-dependent manner, diminished VEGF synthesis in both normoxic and hypoxic conditions. SnPPIX is a very potent inhibitor of HO activity, and recently it has been shown to decrease very strongly CO production in VSMC (35). Our study shows that low concentrations of SnPPIX (10 μM) inhibit VEGF protein synthesis, as the amount of this growth factor was diminished both in culture media and in the cell cytosol. In contrast to our data, the study of Liu and co-workers (29) reported an induction of VEGF mRNA expression by 100 μM SnPPIX in hypoxia. In our experiments, SnPPIX enhanced VEGF mRNA expression, but the synthesis of VEGF protein was always decreased. This may indicate that the effect of SnPPIX on VEGF mRNA expression is unrelated to HO-1. As SnPPIX always potently diminished VEGF production, we suggest that SnPPIX influences VEGF synthesis at the post-transcriptional level. We hypothesize that this effect is specific for VEGF, as the generation of uPA (urokinase plasminogen activator) and IL-8 (interleukin-8) was not impaired by SnPPIX (Jozkowicz and Dulak, unpublished observations).

In another study, Eyssen-Hernandez and colleagues reported that hemin did not induce VEGF mRNA expression in cardiac myocytes (11). In view of our data indicating a moderate increase in VEGF after hemin treatment, we speculate that iron released from heme might have attenuated the stimulatory effect of HO-derived CO on VEGF synthesis. In a recent elegant study, Suttner and Dennery (40) demonstrated that high activity of HO-1 results in the release of a large amount of iron, which in certain circumstances can lead to increased cytotoxicity. Accordingly, we found that deferoxamine up-regulated basal VEGF production and enhanced hemin- or IL-1-induced VEGF synthesis. Thus, it is possible that the total amount of VEGF generated due to increased HO activity is dependent on the ratio of CO and free iron. Liu and co-workers also showed an inhibition of VEGF mRNA expression when hemin was applied to cells during hypoxia (29). Thus, in hypoxic conditions the effect of hemin might be different from that elicited in normoxia.

The novel finding of our study is that activation of the HO-1 gene(s) up-regulates VEGF synthesis in both normoxia and hypoxia. In nonstimulated cells, the VEGF synthesis may be dependent on the activity of both HO-1 and HO-2, and inhibition of basal VEGF production by SnPPIX or ZnDPPIX supports this assumption. After stimulation with cytokines in normoxic conditions, CO may cooperate with NO in VEGF regulation. We postulate a major contribution of CO, as upon inhibition of HO activity by SnPPIX, which potently decreases CO production in VSMC (35), VEGF synthesis was totally abolished and at the same time NO synthesis significantly increased. We exclude the possibility that higher amounts of NO, generated in the presence of SnPPIX, inhibit VEGF synthesis, as VEGF was enhanced by both low (1–5 μM) and high (50 μM) NO concentrations, but only when HO activity was not inhibited (8; unpublished data).

As shown by our data, the increase in VEGF expression by HO activity appears to be modulated at the transcriptional level. In fact, when HO-1 expression was stimulated by gene transfer, we observed a significant induction of VEGF promoter activity and protein synthesis. Similarly, cytokine-induced VEGF synthesis (which is accompanied by HO-1 induction) is associated with augmented VEGF expression (8). It is then not surprising that hemin treatment resulted in up-regulation of VEGF mRNA synthesis, as evidenced by mRNA ELISA quantitative assay. We also observed that cobalt protoporphyrin, which induces HO-1 expression (27), enhanced VEGF promoter activity and VEGF mRNA synthesis in VSMC (unpublished observations). In addition, overexpression of eNOS, which results in induction of VEGF promoter activity and VEGF synthesis (8, 21, 22), is also accompanied by induction of HO-1 expression (unpublished observations).

The molecular mechanism(s) underlying the observed up-regulation of VEGF synthesis by the HO/CO system remains to be clarified. It is possible that HIF-1α transcription factor is playing a role in this effect. The stability of HIF-1α protein, the major factor involved in the regulation of VEGF synthesis under hypoxia, is dependent on iron availability. Iron is essential for the degradation of HIF-1α by proteasomes (33). Thus, iron can be regarded as the inhibitor of VEGF synthesis in the system in which HO activity is enhanced. The concomitant release of an inducer (CO) and a blocker (iron) of VEGF synthesis may explain why the increase of this growth factor after hemin treatment is not as high as the one obtained after increase in HO activity by stimuli other than heme.

Our data may have relevance to other mechanisms governing regulation of VEGF synthesis. It has been demonstrated that modified low-density lipoproteins (LDL) enhance VEGF synthesis in macrophages (20, 38) and VSMC (9). Interestingly, oxidized LDL also induce HO-1 expression, which is abundantly present in the atherosclerotic plaque (42). In vessels of hypercholesterolemic rabbits, we have recently observed that expression of VEGF is co-localized with HO-1 protein (Dulak *et al,* in preparation). In a recent article, Inoue and colleagues demonstrated that oxidized LDL-mediated induction of VEGF synthesis is associated with increased activity of peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands (20). These transcription factors seem to play a role in up-regulation of the HO-1 gene (25); accordingly, PPAR-γ-mediated induction of VEGF in human microvascular endothelial cells is inhibited by SnPPIX and hemoglobin, but not by CuPPIX, indicating an involvement of HO-1 in this up-regulation (23).

In conclusion, our data reveal the existence of novel pathways in the modulation of VEGF synthesis by NO and CO. We suggest that the reciprocal relationship between this potent angiogenic factor and endogenously generated gaseous molecules may open a new field for therapeutic interventions.

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ABBREVIATIONS

CO, carbon monoxide; CuPPIX, copper protoporphyrin IX; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF-1α, hypoxia-inducible factor-1α; HO, heme oxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; ITU, isothiourea; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; L-NAME, L-methyl ester L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; oxy-Hb, oxyhemoglobin; PBS, phosphate-buffered saline; PPAR-γ, peroxisome proliferator-activated receptor-γ; RT-PCR, reverse transcription–polymerase chain reaction; SnPPIX, tin protoporphyrin IX; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells; ZnDPPIX, zinc (II) deutero protoporphyrin IX-2,4-bisethyleneglycol.

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