Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis and blood vessel remodeling. Its expression is up-regulated in vascular smooth muscle cells by a number of conditions, including hypoxia. Hypoxia increases the transcriptional rate of VEGF via a 28-base pair enhancer located in the 5'-upstream region of the gene. The gas molecules nitric oxide (NO) and carbon monoxide (CO) are important vasodilating agents. We report here that these biological molecules can suppress the hypoxia-induced production of VEGF mRNA and protein in smooth muscle cells. In transient expression studies, both NO and CO inhibited the ability of the hypoxic enhancer we have previously identified to activate gene transcription. Furthermore, electrophoretic mobility shift assays indicated decreased binding of hypoxia-inducible factor 1 (HIF-1) to this enhancer by nuclear proteins isolated from CO-treated cells, although HIF-1 protein levels were unaffected by CO. Given that both CO and NO activate guanylyl cyclase to produce cGMP and that a cGMP analog (8-Br-cGMP) showed a similar suppressive effect on the hypoxic induction of the VEGF enhancer, we speculate that the suppression of VEGF by these two gas molecules occurs via a cyclic GMP-mediated pathway.

Low oxygen tension is a potent regulator of diverse biological processes, including erythropoiesis, angiogenesis, and vascular cell contractility. These effects are mediated by several proteins that are induced under hypoxic environments and modulate cell-cell interactions, cell proliferation, and differentiation. In the vasculature, hypoxia regulates the expression of genes encoding growth factors such as endothelin-1 (ET-1)\(^*\), platelet-derived growth factor-B (PDGF-B) and vascular endothelial growth factor (VEGF), as well as genes regulating the production of gas molecules such as nitric oxide (NO) and carbon monoxide (CO) (1–5). Whereas the expression of the endothelial nitric oxide synthase gene is suppressed by hypoxia, the expression of heme oxygenase-1 (HO-1), the enzyme catalyzing the production of CO, is up-regulated by hypoxia (5).

Mechanisms by which hypoxia alters gene expression include transcriptional and post-transcriptional regulation (4, 6, 7). Several hypoxia-responsive cis-acting elements have been identified (8, 9). We have reported the presence of a 28-bp enhancer located approximately 980 bp upstream of the VEGF transcription start site, which is necessary and sufficient to up-regulate transcription of the VEGF gene in response to hypoxia (10). This hypoxia response element contains a sequence homologous to (and now has been included into) the hypoxia-inducible factor 1 (HIF-1) consensus (11). HIF-1 is a basic helix-loop-helix transcription factor originally identified to mediate the transcriptional activation of the erythropoietin gene (8) leading to enhanced erythropoiesis under hypoxia. It was subsequently shown to regulate the expression of genes encoding glycolytic enzymes (12) and the gene for VEGF (10, 11) implicating it as an important regulator of the cellular responses to hypoxia. HIF-1 itself is also regulated by hypoxia at the posttranscriptional level (13–15), but intracellular events regulating its DNA binding activity and function are poorly understood.

Gas molecules are not only regulated by hypoxia but can also modify the effects of hypoxia on other genes. We reported that endothelial-derived NO inhibits both the basal and hypoxia-induced expression of ET-1 and PDGF-B genes in endothelial cells (16). Smooth muscle cell-derived CO was also shown to inhibit the hypoxic induction of these genes in a paracrine manner (17). In this report we investigated mechanisms by which NO and CO modulate hypoxic signal transduction leading to altered gene expression. We demonstrate that NO and CO inhibited the hypoxic induction of VEGF at the transcriptional level. This was due to decreased HIF-1 DNA binding activity, although HIF-1 protein levels actually increased. The uncoupling of HIF-1 production and HIF-1 function by NO and CO suggests the existence of additional control points for hypoxic signal transduction lying downstream of the putative oxygen sensor.

**EXPERIMENTAL PROCEDURES**

**Media and Cell Culture**—Bovine pulmonary artery endothelial cells (BPAEC), rat aortic smooth muscle cells (RASMC), and fetal smooth muscle cells (A7r5) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 0.1 mg/ml gentamicin sulfate. The cells were exposed to a normoxic environment consisting of 21% O\(_2\), 5% CO\(_2\), balance N\(_2\), or to a hypoxic mixture of 95% N\(_2\) and 5% CO\(_2\). The measured PO\(_2\) in the medium was 18–20 mm Hg under hypoxic conditions, as we reported previously (2).
Cells were cultured at 37 °C in humidified incubators.

Northern Blot and RNA Analysis—Total cellular RNA was isolated from cultured RASMC by guanidinium isothiocyanate extraction method. Total RNA (15 μg) was electrophoresed in 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes by blotting. The filters were hybridized with a CDNA probe specific for the rat VEGF gene (18). A mouse β-actin probe was used to normalize for RNA loaded. The CDNA fragments were labeled with [32P]-dCTP using a standard random-primed reaction to a specific activity of 1–2 × 10^6 cpm/μg. The membranes were hybridized for 2 h at 68 °C in QuikHyb solution (Stratagene, La Jolla, CA) with 2 × 10^6 cpm/ml of probe and washed twice in 2 × SSC (3 M NaCl, 0.3 M sodium citrate) containing 0.1% SDS at 60 °C for 30 min and then were exposed to film (X-Omat AR, Eastman Kodak Co.) with intensifying screens at −80 °C. For quantitation, we scanned autoradiographs with a laser densitometer (Ultrascan XL; LKB Instruments, Inc., Bromma, Sweden) running the Gel Scan XL software package (Amersham Pharmacia Biotech).

Transfection and Reporter Gene Assays—Transfections of BPAEC were carried out on cells at 50–80% confluence using Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's protocol. RASMC were transfected using DEAE-dextran method (19). Cell lysis was performed using the reporter gene lysis buffer from Promega (Madison, WI), and the activities of β-galactosidase and CAT were measured according to product diffusion method (20). Normalized CAT activity was the ratio of radioactivity (in counts/min) of labeled acetylated chloramphenicol to the optical density units from the cleavage product of α-nitrophenyl-β-D-galactopyranoside catalyzed by β-galactosidase.

Nuclear Protein Extraction and Electromobility Shift Assay—Nuclear proteins were isolated from BPAEC using the method described by Schreiber et al. (21), and the total protein was quantified with the Bio-Rad protein assay. Electromobility shift assay was performed according to the protocol described previously using as a probe a 35-bp DNA fragment (A-G) that contains the 28-bp hypoxic enhancer (10).

VEGF Peptide Quantification—RASMC were cultured in 100-mm plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as above. When the cells were approximately 80% confluent, the medium were changed to Dulbecco's modified Eagle's medium with 1% fetal bovine serum, and the cells were exposed to normoxic or hypoxic conditions for 48 h as above. The culture medium from these confluent cells were collected and concentrated 30-fold using Centricon-10 (Amicon, Inc., Danvers, MA). They were subsequently analyzed using a commercially available sandwich enzyme immunoassay for VEGF165 peptide (Quantikine®, R & D Systems, Minneapolis, MN). Values were expressed in picograms/ml, and the sensitivity of the assay was 5.0 pg/ml. The cells were trypsinized and counted in a Coulter counter, and the concentration of VEGF was reported in picograms/10^6 cells to normalize for cell number. To assess the effect of endogenously produced CO on the production of VEGF under hypoxia, the same experiment was done after addition of tin protoporphyrin IX (SnPP-9) or hemoglobin (Hb) to the medium at a final concentration of 100 and 50 μM, respectively, followed by hypoxia exposure for 48 h. Hb was purchased from Sigma and was prepared by treatment with excess sodium hydrosulfite (22). The experiment was performed five times in duplicate and the mean VEGF concentrations were compared using the Kruskal Wallis nonparametric analysis of variance test with a level of significance of p < 0.05.

Western Analyses—Nuclear proteins were extracted from BPAEC according to the method described previously (23). A total of 20 μg of nuclear proteins isolated from cells treated with different conditions was loaded on a 7% SDS-polyacrylamide gel. HIF-1α and HIF-1β were detected by the ECL Western kit from Amersham Pharmacia Biotech.

Reagents—SnPP-9 was purchased from Porphyrin Products, Inc. (Logan, UT). All other reagents used were obtained from Sigma unless otherwise indicated.

RESULTS

CO and NO Suppress the Hypoxic Induction of VEGF mRNA Expression—RASMC were exposed to hypoxia or normoxia for 3–48 h and, total cellular RNA was isolated for Northern analysis. After hybridization with the rat VEGF probe a 4-fold increase in VEGF mRNA was detected in cells exposed to hypoxia for 12 h, compared with the normoxic controls (Fig. 1A). Levels of VEGF mRNA started to increase at 6 h of hypoxia, reached a maximum at 12–24 h, and declined by 48 h. To examine the effect of NO on VEGF mRNA, cells were treated with the NO donor, S-nitrosglutathione (GSNO), for 12 h under hypoxic or normoxic conditions. GSNO (50 μM, lanes 3 and 4; 500 μM, lanes 5 and 6), and analyzed by Northern blot. C, VEGF mRNA was isolated from RASMC subjected to normoxia or hypoxia in the presence of Hb (50 μM, lanes 3 and 8), SnPP-9 (100 μM, lanes 4 and 9), methylene blue (10 μM, lanes 2 and 7); and dibutyryl (db) cGMp (1 mM, lanes 5 and 10), and Northern analysis was performed. The data presented in each panel are representative of three experiments.

To investigate the effects of hypoxia-induced CO on VEGF mRNA expression, RASMC were exposed to hypoxia or normoxia for 12 h as above and treated with the CO scavenger, Hb (50 μM), or the HO-1 inhibitor, SnPP-9 (100 μM), for the last 3 h of exposure. Fig. 1C demonstrates a 3-fold increase in VEGF mRNA after 12 h of hypoxia (lane 6) compared with normoxia (lane 1), which was induced approximately 2-fold further upon treatment with Hb or SnPP-9 (lanes 8 and 9, respectively). Treatment with the guananyl cyclase inhibitor, methylene blue (10 μM), also super-induced VEGF under hypoxic conditions (lane 7), whereas treatment with dibutyryl cGMp (1 mM, lane 10) dramatically suppressed the hypoxia induction of VEGF mRNA. Inhibiting HO-1 production with these agents did not increase VEGF mRNA levels under normoxia (lanes 2–4), since basal HO-1 activity is low under normoxic conditions. Dibutyryl cGMp, the second messenger molecule of NO and CO activity, further suppressed VEGF mRNA levels under both normoxia (lane 5) and hypoxia (lane 10). These data suggest that the hypoxic induction of VEGF mRNA is modulated by levels of endogenous NO or CO.
CO Regulates the Levels of Secreted VEGF Protein by Smooth Muscle Cells—To verify that the VEGF mRNA levels changed in parallel with levels of VEGF peptide secreted into the medium, we measured VEGF concentrations in the RASMC-conditioned medium. Fig. 2 shows the VEGF concentrations in the medium under various experimental conditions. Using an enzyme-linked immunosorbent assay, we were unable to detect any VEGF peptide in quiescent normoxic smooth muscle cells and detected only very low levels of protein in the conditioned medium of cells after 48 h of hypoxic exposure (mean = 0.2 pg/10⁶ cells). However, VEGF protein was consistently detected in the conditioned media after 48 h of hypoxic exposure in the presence of either SnPP-9 or Hb. The mean VEGF protein concentration after 48 h of hypoxic exposure in the presence of SnPP-9 or Hb was 0.61 pg/10⁶ cells. This was significantly higher than normoxia and 3-fold higher than hypoxia alone (p < 0.01). Although SnPP-9 is an inhibitor for both nitric oxide synthases and heme oxygenases and hemoglobin can scavenge both CO and NO, transcripts of NO synthase (inducible or constitutive) have not been detected in RASMC at any O₂ concentration, and inhibitors of NO synthesis had no effect on smooth muscle cell-derived cGMP levels (5). This suggests that in cultured RASMC, endogenous CO is the predominant negative regulator of VEGF production under hypoxic conditions.

CO and NO Suppress the Hypoxic Induction of VEGF through Attenuation of the Hypoxic Enhancer—Previous work has shown that hypoxia induces VEGF gene transcription through the activation of its enhancer (10, 25, 26), a 28-bp region that contains a HIF-1 consensus. To investigate whether the inhibitory effects of CO and NO occur at the transcriptional level, we tested the effect of these two gases on the expression of a reporter gene under the control of the VEGF hypoxic enhancer. Cultured BPAEC were transfected with reporter plasmid pV111/CATα, which contains the hypoxic enhancer upstream of the thymidine kinase promoter fused to the CAT gene (10), and GSNO was added to the medium at a concentration of 0.5 mM. After incubation for 24 h, cells were lysed and the reporter gene (CAT) activity was measured and normalized to β-galactosidase. We observed that in hypoxic cells, 0.5 mM GSNO reduced CAT activity by 50%. In normoxic cells the relative CAT activity was slightly increased by treatment with GSNO. The combined results from these effects are that the hypoxic induction was reduced from 18.4- to 4.6-fold by GSNO (Fig. 3A). A higher concentration of GSNO (5 mM) led to cell death and resulted in undetectable reporter gene activity (data not shown). In RASMC, GSNO inhibited the hypoxic induction in a dose-dependent manner. At 1 mM GSNO, the hypoxic induction was 8.7-fold (Fig. 3B, column 1), which is comparable with untreated hypoxic cells (data not shown). At 2 mM, the induction was reduced to 1.8-fold (column 2), and at 3 mM, the hypoxic induction was completely abolished (column 3).

Exogenous CO displayed a similar effect. RASMC were exposed to either hypoxia alone or hypoxia supplemented with 5% CO for 24 h after transfection. The CAT activity induced by hypoxia was reduced by 50% by the addition of exogenous CO (Fig. 3C). We observed a similar inhibition of reporter gene expression when BPAEC were used (data not shown).

The suppressive effect on the hypoxic enhancer was also demonstrated by endogenously produced CO. RASMC or fetal smooth muscle cells were transfected with the pV111/CATα reporter plasmid and treated with normoxia, hypoxia, or hypoxia plus hemin, the substrate of heme oxygenase and precursor of CO. Hemin in the medium is internalized into cells and metabolized to CO and bilirubin. As shown in Fig. 3D, in the presence of 5 and 50 µM hemin, the hypoxic induction of the VEGF enhancer was suppressed from 7.7-fold to 3.2- and 2.0-fold, respectively. Taken together, these findings suggest that endogenously produced or exogenously administered NO or CO serve to modulate the hypoxic responses of the VEGF enhancer, resulting in reduced VEGF production.

cGMP Levels Regulate VEGF Enhancer Function—Both NO and CO stimulate the activity of guanylyl cyclase (27, 28), which catalyzes the production of cGMP, an important second messenger for signal transduction. To determine whether the suppressant effect of CO and NO on the hypoxic induction of VEGF enhancer was through the action of cGMP, a cGMP analog, 8-Br-cGMP, was added to the culture medium to the concentration of 1 mM, and reporter gene activity was assayed using RASMC exposed to hypoxia or normoxia. In the presence of 8-Br-cGMP, the hypoxic induction of VEGF enhancer was suppressed from 8.9- to 4.9-fold (Fig. 5), paralleling the effect of dibutyryl cGMP on endogenous VEGF gene expression (Fig. 1C) and confirming the involvement of cGMP in this process.

DISCUSSION

VEGF plays an important role in the process of angiogenesis and vascular remodeling (29). Its expression has been shown to be regulated by various agents, such as transforming growth factor-β (30), estrogen (31), prostaglandin E₂ and F₃ (32), interleukin-1β (33), as well as hypoxia (25, 34, 35). Our previous study identified an enhancer in the human VEGF promoter region, which is necessary and sufficient to provide hypoxic induction when tested in a reporter system (10). The data in
this report demonstrate that induction of VEGF gene transcription via this hypoxic enhancer is subject to modulation by NO and CO, two gas molecules that are important in the regulation of blood vessel tone (36, 37).

NO has been reported previously to activate or suppress gene expression. For example, NO can activate gene transcription from AP-1-responsive promoters in mammalian cells (38), an effect mimicked by 8-Br-cGMP, indicating that NO may act by stimulating guanylyl cyclase production of cGMP. In contrast, NO suppresses the mRNA levels of macrophage-colony-stimulating factor induced by oxidized low density lipoprotein or tumor necrosis factor-α (39). This suppression was associated with decreased DNA-binding activity of the transcription factor NF-κB but was not mediated by cGMP. We have reported previously that NO can suppress the hypoxic induction of both ET-1 and PDGF-B gene expression (16) in vascular endothelial cells. Using the VEGF gene as a model, we once again demonstrated a suppressive effect of NO on the hypoxic induction of genes expressed by vascular cells. In this study, NO donors were able to suppress both the transcription of a reporter gene containing the VEGF hypoxic enhancer and the binding of the transcriptional activator HIF-1 to the enhancer. In contrast to the NO effect on the induction of macrophage-colony-stimulating factor, cGMP could mimic the suppressive effect of NO.

Earlier studies have shown that NO plays a role in microvascular permeability. An analog of L-arginine, Nω-nitro-L-arginine methyl ester, which can compete with L-arginine and suppress the activity of NO synthase, caused a rapid increase in microvascular permeability (40). Furthermore, the cytosolic second messenger cGMP has been shown to be the mediator between NO and the changes observed in vascular permeability (41). The mechanism by which cGMP regulates microvascular permeability is not clearly understood. It has been suggested that intracellular cGMP activates cGMP-dependent protein kinases that affect myosin phosphorylation. The modifications on the cytoskeletal filaments induce endothelial cell relaxation or contraction and change the size of interendothelial cell junctions, leading to the altered vascular permeability (41). In our study, we found that NO and cGMP suppressed the transcriptional rate of the VEGF gene. Since VEGF is a well known endothelial cell-specific vascular permeability factor (42–44), our results provide an alternative explanation for the
changed VEGF production and secretion from smooth muscle cells or endothelial cells can alter the permeability of vascular endothelium in a paracrine or an autocrine fashion, respectively. It should be noted that our results are not in contradiction to the possible pathway involving a cGMP-dependent protein kinase. In fact, protein phosphorylation events also occur following VEGF stimulation of endothelial cells. Thus, NO effects on an NO-dependent protein kinase and on VEGF expression could co-exist and be part of a complex system that regulates vascular permeability.

Given the similarities between NO and CO, one would expect that CO will have a similar physiological effect as NO. Indeed, previous studies by our group have shown that CO is produced in vascular smooth muscle cells and regulates cGMP levels in these cells (5). Furthermore, we and others have demonstrated the inhibitory effect of CO on the hypoxic induction of certain genes, including ET-1, PDGF (17), and VEGF (7). However, the molecular mechanisms of this regulation have not been reported previously. From the results reported here, we demonstrate that CO can regulate the transcriptional rate of a target gene and that for VEGF this effect occurs at the level of the hypoxic enhancer. Furthermore, we have shown that the binding of HIF-1 to the enhancer element was reduced by CO treatment and that this effect was “mimicked” by cGMP. This interference with the binding activity of HIF-1 suggests potential changes in phosphorylation or possibly NO/CO-mediated redox effects on HIF-1 dimerization and/or DNA binding. Wang and Semenza (45) have reported that phosphorylation of HIF-1 is required for DNA binding, and other reports (46, 47) have shown that HIF-1 protein is stabilized by a redox-dependent signaling pathway.

Our findings that CO inhibits HIF-1 DNA binding activity under hypoxia without altering the hypoxia-induced increases in HIF-1 protein may indicate that the molecular mechanisms of CO modulation of hypoxic signal transduction do not involve a putative heme protein O2 sensor at the apex of the cascade (48). They suggest the existence of additional control points, possibly intracellular protein modification events, that are downstream of O2 sensing and upstream of HIF-1α action. The results presented in this paper provide further evidence for the essential role of HIF-1 in the regulation of VEGF transcription in hypoxic cells. Posttranscriptional events also regulate the steady state levels of VEGF mRNA (25, 26). However, whether CO can effect these processes remains to be investigated.

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