Carbon Monoxide Controls the Proliferation of Hypoxic Vascular Smooth Muscle Cells*

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Excess vascular smooth muscle cell (VSMC) proliferation and contractility are key events in the pathophysiology of vascular disorders induced by hypoxia. We have recently reported that carbon monoxide (CO), produced by VSMC under conditions of hypoxia, can be a modulator of cGMP levels in both endothelial and smooth muscle cells. In this respect, some of the physiologic effects of CO in the vasculature parallel those of nitric oxide (NO), a well characterized regulator of vascular tone. We report here that under hypoxia, VSMC-derived CO is an important regulator of VSMC proliferation. Inhibiting CO formation or scavenging CO with hemoglobin increased VSMC proliferation in response to serum or to mitogens such as endothelin, whereas increasing CO production or exposing cells to exogenous CO lead to a markedly attenuated growth response. The effects of CO on VSMC proliferation correlated with changes in E2F-1 expression, the prototype member of a family of transcription factors that participate in the control of cell cycle progression. CO significantly suppressed E2F-1 expression, whereas, removal of CO from the cultures with hemoglobin lead to increased E2F-1 gene transcription, mRNA, and protein production as well as mRNA levels of c-myc, a target gene of E2F-1. Moreover, the actions of CO were mediated by the second messenger molecule, cGMP. Limiting VSMC growth by increasing the release of CO may represent a key event in the body’s compensatory responses to hypoxia.

Hypoxia has profound effects on blood vessel tone and cell growth. The cellular responses to hypoxia involve changes in DNA-protein interactions leading to altered gene expression (1, 2) and, ultimately, to cell proliferation and contractility. As more is learned about these responses to hypoxia, it has become evident that they involve complex cell-cell interactions mediated by both endothelial and smooth muscle cell-derived signals.

We have shown that under hypoxia, vascular smooth muscle cells (VSMC) up-regulate the expression of the heme oxygenase-1 (HO-1) gene, resulting in increased production of carbon monoxide (CO) (3). Cook et al. (4) using a sensitive gas chromatographic method, also reported HO-dependent CO production in the rat aorta, suggesting a physiological role for CO in VSMC relaxation. HO-1 is the inducible isoform of HO, which catalyzes the conversion of heme to biliverdin and CO (5, 6). The regulation of HO-1 by hypoxia hence affects both heme homeostasis in the body and CO levels in VSMC. CO has similar properties to the well known gas molecule, nitric oxide (NO). Both CO and NO activate guanylyl cyclase activity, thus raising intracellular cGMP levels. In addition to being a potent vasodilator, NO has been reported to inhibit the proliferation of tumor cells, (7) as well as cultured rat VSMC (8). Very little is known about the biology of CO. CO production is the activity of the constitutive enzyme HO-2 has been shown to be important in neuronal signal transduction (9) and may have endothelial-derived relaxing activity (10). We have reported that CO produced from the activity of HO-1 in hypoxic VSMC may play a physiologic role in the vasculature by regulating the production of the growth factors, endothelin-1 (ET-1), and platelet-derived growth factor-B (PDGF-B), thus indirectly regulating VSMC proliferation (11).

Hypoxia induces the expression of genes for growth factors and genes encoding glycolytic enzymes at least partially through the action of hypoxia-inducible factor-1 (1, 12–14). Hypoxia-inducible factor-1 is a basic-loop-helix-Per-Arnt/Sim heterodimeric transcription factor that is itself regulated by hypoxia (2). The mechanisms by which hypoxia regulates cell cycle-related processes, however, are poorly understood. Little is known about the downstream targets of growth control pathways that are activated by a hypoxic environment. The E2F family of transcription factors has been implicated in the regulation of genes essential for orderly cell cycle progression, such as c-myc, cyclins, and DNA polymerase-α (15, 16). E2F-1 was the first member of this family to be characterized and was shown to be critical for the G1/S phase transition (17, 18). E2F-5, on the other hand, the latest member of this family to be cloned (19–21) has a pattern of expression throughout the cell cycle that is distinct from that of E2F-1 and may play an active role in the G0/G1 transition and cell differentiation (19, 21). In this study, we examined the pattern of expression of these two genes in VSMC under conditions of hypoxia and correlated them with cell growth. Given that CO production is dramatically increased by hypoxia, whereas NO production is suppressed (22, 23), we investigated the effects of VSMC-derived CO on hypoxia-induced cell growth and monitored E2F gene expression as an indicator of cell cycle progression. We report that CO modifies the cellular responses to hypoxia by inhibiting VSMC proliferation and suppressing E2F-1 gene expression and protein production leading to decreased mRNA levels.
of c-myc, a target gene of E2F-1 action. In addition, CO inhibited E2F-1 expression and the corresponding mitogenic response of VSMC to growth factors such as ET-1 via a cGMP-dependent pathway. These actions implicate CO as a regulator of not only vascular tone, but also of VSMC growth.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of rat aortic VSMC were grown in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) with 10% newborn calf serum, were passed every 3 to 4 days as described previously (1, 3) and used between passages 5 and 10. When the cells reached 60–70% confluency, media were changed to Dulbecco's modified Eagle's medium with 0.2% newborn calf serum, and cultured for an additional 48 h prior to exposure to hypoxia. Various reagents were added immediately prior to hypoxia exposure. The hypoxic gas mixture (95% N2, 5% CO2) was preanalyzed and infused into airtight incubators with in-flow and out-flow valves (Bille-Urtonenbe, Del Mar, CA) at a flow rate of 3 liters/min for 15 min as previously to attain a PaO2 of 18–20 mm Hg (24). For the CO experiments, a preanalyzed gas mixture (5% CO, 5% CO2, room air) was infused at the same flow rate.

Cell Replication—Cell replication was assessed by counting cell number. After various incubation periods, cells were washed twice with ice-cold PBS, harvested, and centrifuged. Cell pellets were resuspended in ice-cold PBS and counted with a Coulter counter (Coulter Corp., Hialeah, FL). Growth rate was determined by plotting cell number over time in semilog scale and extrapolating the slope.

RNA Analysis—Total cellular RNA was prepared by guanidinium isothiocyanate extraction from VSMC exposed to normoxia or hypoxia in the presence or absence of reagents for various periods. Total RNA (15 μg/lane) was separated by electrophoresis on 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes by blotting. The filters were hybridized with cDNA probes specific for the following: rat c-myc (generous gift from Dr. Peter Brecher, Boston University Medical Center) and the rat E2F-1 and E2F-5 genes (19). The cDNA fragments were labeled with (α-32P)deCTP using a standard random-primed reaction to a specific activity of 1–2 × 109 cpm/μg. The membranes were hybridized for 2 h at 68 °C in QuikHyb solution (Stratagene, La Jolla, CA) with 2 × 106 cpm/ml of probe, and washed twice in 2 × SSC containing 0.1% SDS at 60 °C for 30 min, and were then exposed to film (X-Omat AR; Eastman Kodak, Rochester, NY) with excess reducing agent (26) to prevent oxidant-stress injury to the cells. After various incubation periods, cells were lysed, and solubilized with cold 65% ethanol. The cellular lysates were clarified by centrifugation at 8000 × g for 5 min, and the supernatants were added immediately prior to exposure to hypoxia. The hypoxic gas mixture (95% N2, 5% CO2) was preanalyzed and infused into airtight incubators with in-flow and out-flow valves (Bille-Urtonenbe, Del Mar, CA) at a flow rate of 3 liters/min for 15 min as previously to attain a PaO2 of 18–20 mm Hg (24). For the CO experiments, a preanalyzed gas mixture (5% CO, 5% CO2, room air) was infused at the same flow rate.

Nuclear Run-on Analysis—VSMC were serum-deprived for 48 h and then placed in 10% serum-containing medium in the presence or absence of 50 μM hemoglobin (Hb) prior to exposure to hypoxia for 12 h. Nuclei were isolated and in vitro transcription was performed as described previously (25). Hybridization to denatured probes (1 μg) slot-blotted on nitrocellulose filters was performed at 40 °C for 4 days in the presence of 50% formamide. cDNA for rat E2F-1, rat HO-1 (3), and β-actin were used as probes.

cGMP Accumulation in VSMC—After exposure to hypoxia or normoxia for 12 h, cGMP concentration was determined in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM), which was added to the plates for the last 20 min of incubation. Media were aspirated, cells washed with ice-cold phosphate-buffered saline and solubilized with cold 65% ethanol. The cellular lysates were clarified by centrifugation at 2000 × g for 15 min at 4 °C. The supernatants were evaporated at 60 °C in a vacuum oven and maintained at −80 °C until assay. cGMP concentrations were determined by radioimmunoassay (Amersham, Buckinghamshire, UK) using cGMP-specific antibodies. Western analysis was used to determine protein content as measured by a dye-binding assay (Bio-Rad) using bovine serum albumin as the standard.

Nuclear Extract Preparation and Western Analysis—VSMC were grown to 80% confluency and exposed to hypoxia or normoxia for 12 h in the presence or absence of Hb (50 μM) in fresh, serum-replete media. Cells were trypsinized, washed twice with PBS, and nuclear extracts were prepared as described previously (14). Briefly, cells were resuspended in 5 packed cell volumes of buffer A (10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl2, 10 mM KCl) with 2 mM dithiothreitol and proteasome inhibitors. Cell membranes were lysed using a Dounce homogenizer and after centrifugation, the nuclear pellet was lysed in buffer C (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM MgCl2). Nuclear proteins were isolated in the supernatant after centrifugation at 15,000 × g for 30 min, 30 μg of nuclear protein per sample were fractionated in 12% SDS-polyacrylamide gel electrophoresis, gel followed by transfer to nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat dry milk in PBS-T (0.05% Tween 20 in PBS) for 1 h. The blot was then incubated with antiserum monoclonal E2F-1 antibody (Transduction Laboratories, Lexington, KY) at a dilution of 1:100 for 16 h at 4 °C. After washing with PBS-T, the membrane was exposed to anti-mouse whole immunoglobulin horseradish peroxidase-conjugated antibody (Amersham) at a ratio of 1:2500 for 3 h at 4 °C. Immunoreactivity was detected using the standard enhanced chemiluminescence method (Amersham) according to the manufacturer's manual.

Reagents—Tin protoporphyrin IX (SnPP-9), zinc protoporphyrin IX (ZnPp-9), and cobalt protoporphyrin IX were purchased from Porphyrin Products, Inc. (Logan, UT). All other reagents used were obtained from Sigma, unless otherwise specified. Hb was prepared by treatment with excess reducing agent (26) to prevent oxidant-stress injury to the cells (27).

RESULTS

Hypoxia Enhances the Response of VSMC to the Mitogen ET-1—We exposed VSMC to hypoxia or normoxia for various periods of time and examined their growth in the two oxygen environments. Cultures were incubated in the presence of serum (10%) or ET-1 (10 nM) and cell number was compared with that of parallel cultures without serum or mitogens. ET-1 was used as the mitogen because its production is increased by hypoxia in endothelial cells (24) and was shown to stimulate proliferation of co-cultured VSMC (11). We found that hypoxia VSMC had a more pronounced proliferative response to ET-1 (Fig. 1) or to serum (not shown) than their normoxic counterparts. Similar findings have been reported for fibroblasts, which were shown to proliferate at higher rates in response to serum and growth factors under hypoxic conditions (28). In the absence of serum or exogenous growth factors, VSMC number did not increase significantly under hypoxia and increased only modestly under normoxia (Fig. 1). CO Decreases the Proliferative Response of VSMC to ET-1—We have reported that VSMC exposed to hypoxia express significantly higher levels of HO-1 compared with cells cultured under normoxic conditions, and that this up-regulation of HO-1 activity results in a dramatic increase in CO levels in the conditioned media (3). To examine if CO modulates the proliferative response of VSMC to exogenous mitogens, we exposed...
cells to hypoxia in the absence of serum (basal) or in serum-free media plus exogenous ET-1 and controlled the CO levels in the cultures through various treatments (Fig. 2). The presence of hemin, a potent inducer of HO activity and CO production, did not affect VSMC proliferation under either basal or ET-1-stimulated conditions. This is not unexpected because hypoxic VSMC already produce high levels of CO (3). The HO-1 inhibitors SnPP-9 and ZnPP-9 were used to decrease endogenous CO production and Hb was used to remove CO from the culture media. Although these agents did not alter the proliferation of VSMC under normoxia (not shown), hypoxic VSMC proliferated at significantly higher rates in their presence. Significantly, inhibition of HO or removal of CO with Hb resulted in increased VSMC proliferation even in the absence of growth factors (hatched bars). In fact, although maximal proliferation was observed in cells treated with ET-1 (solid bars), modulators of CO levels had an equal or more striking relative proliferative effect on VSMC under basal conditions. To confirm that it is the gaseous molecule CO that regulates VSMC growth, we exposed serum-replete VSMC to exogenous CO under normoxic conditions and demonstrated a significant suppression of proliferation compared with that of control cultures (Fig. 3). This anti-proliferative effect was negated by the presence of Hb, a potent scavenger of CO, while Hb by itself had no effect on VSMC growth under control conditions. We also treated normoxic cultures, grown in the presence of serum or ET-1, with hemin to stimulate CO production. VSMC proliferation in treated cultures was significantly reduced to 50% of the control numbers at time 0.

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**Fig. 2. Endogenous CO levels regulate VSMC proliferation under hypoxia.** VSMC number is determined 48 h after exposure to hypoxia in serum-deprived cells (basal, hatched bars) and in cells cultured in the presence of 10 nM ET-1 (solid bars). Cultures were treated with the HO-1 inhibitors SnPP-9 (100 μM) and ZnPP-9 (10 μM), Hb (50 μM), or the HO-1 inducer, hemin (Hem) (5 μM), to modulate the endogenous CO levels. The experiment was repeated three times in quadruplicate. Mean values ± S.E. (n = 4) are shown. **, p < 0.01; ***, p < 0.001 versus control numbers at time 0.

**Fig. 3. Exogenous CO decreases VSMC proliferation.** Serum-deprived VSMC cultures were exposed to 21% O₂ (control) or 5% CO environments in the presence or absence of Hb (50 μM) and cell number was determined at the times indicated. The experiment was repeated three times in quadruplicate. n = 4, **, p < 0.01; ***, p < 0.001 versus control.

**Fig. 4. Hypoxia regulates E2F-1 mRNA levels.** A, response to the growth factor, ET-1. Northern blot analysis of total RNA isolated from VSMC treated with ET-1 (10 nM) and exposed to hypoxia or normoxia for the time indicated. β-Actin mRNA is shown below and relative E2F-1 and E2F-5 mRNA levels normalized to actin are indicated in numbers below the bands. B, hypoxia suppresses E2F-1 mRNA in the absence of mitogens. Cells were cultured for the indicated time in the same oxygen environments as in A but without ET-1. Northern analysis was performed as above. In both A and B, the panels shown are representative of independent experiments performed at least twice.

12 h, peaked to about 4-fold by 24 h, and was again significantly elevated 3-fold above baseline levels at 48 h (lanes 5, 7, and 9, respectively). This effect was specific for E2F-1, as the mRNA levels of E2F-5, another member of the E2F family of cell cycle regulators as well as β-actin mRNA levels were unaffected by neither hypoxia nor ET-1. Hypoxia thus results in a more rapid and sustained elevation of VSMC E2F-1 levels upon exposure to ET-1, in good correlation with the enhanced mitogenic response of hypoxic VSMC to this growth factor (Fig. 1).

**Carbon Monoxide Regulates the Expression of Cell Cycle-specific Transcription Factor E2F-1**—To further characterize the effects of CO on VSMC proliferation, we examined the expression of the transcription factor E2F-1, a key regulator of cell cycle control (17, 18). Treatment of normoxic VSMC with ET-1 resulted in a roughly 4-fold rise in E2F-1 mRNA levels after 24 h with return to baseline levels by 48 h (Fig. 4A, lanes 6 and 8, respectively). In contrast, under hypoxia, E2F-1 mRNA increased 3-fold by
the below lanes 1, 2, 4 and parallel with the observed suppression of VSMC growth under VSMC cultured in the absence of serum or growth factors. In pressed E2F-1 mRNA levels (Fig. 5 and also suppresses the cell cycle-specific transcription tagonizes the growth response of VSMC to the mitogen ET-1 CO reversed this hypoxic effect (Fig. 5). The same blot was hybridized with the β-actin probe as described previously. Relative mRNA levels (normalized to β-actin mRNA) are indicated in numbers below the bands. Data are representative of two separate experiments. 

FIG. 5. Inhibitors of CO increase E2F-1 mRNA levels under hypoxia. A, effect of ET-1. Northern blot analysis of total RNA extracted from VSMC treated with ET-1 (10 nM) and exposed to normoxia or hypoxia for 12 h with no additions (lanes 1 and 2), in the presence of SnPP-9 (lanes 3 and 4), Hb (lanes 5 and 6), ZnPP-9 (lanes 7 and 8), or hemin (Hc) (lanes 9 and 10) in the same concentrations as in Fig. 2. E2F-5 mRNA levels are shown in the middle panel. The same blot was hybridized with the β-actin probe as described previously. Relative mRNA levels (normalized to β-actin mRNA) are indicated in numbers below the bands. Data are representative of two separate experiments. B, effect of CO in the absence of growth factors. E2F-1 mRNA levels are shown in response to the same agents as in A but in the absence of ET-1. The experiment was repeated twice.

Fig. 4B shows the effect of hypoxia on E2F-1 mRNA levels in VSMC cultured in the absence of serum or growth factors. In parallel with the observed suppression of VSMC growth under these conditions (see Fig. 1), E2F-1 mRNA levels were reduced by roughly 50% in a hypoxic environment (lanes 3, 5, and 7) compared with normoxia (lanes 1, 2, 4, and 6). Again, E2F-5 and β-actin mRNA levels were unaffected by these treatments. The effect of CO on E2F-1 mRNA levels is shown in Fig. 5. As above, when cells were treated with the mitogen ET-1, hypoxia significantly increased E2F-1 mRNA levels (Fig. 5A, lanes 1 and 2). However, E2F-1 mRNA was hyper-induced by hypoxia when CO levels were decreased by SnPP-9, ZnPP-9, or Hb (lanes 4, 6, and 8, respectively) but not when CO release was stimulated with hemin (lane 10). Again, this effect was specific for E2F-1 as mRNA levels of E2F-5 were unaffected by the presence or absence of CO (middle panel). Therefore, CO antagonizes the growth response of VSMC to the mitogen ET-1 (Fig. 2) and also suppresses the cell cycle-specific transcription factor E2F-1. In the absence of growth factors, hypoxia suppressed E2F-1 mRNA levels (Fig. 5B, lane 2) but inhibition of CO reversed this hypoxic effect (lanes 4, 6, and 8). Conversely, increasing CO with the addition of hemin did not prevent the suppression of E2F-1 by hypoxia (compare lanes 9 and 10). CO, therefore, modulates VSMC growth and cell-cycle progression (as assessed by E2F-1 expression) irrespective of exogenously added growth factors.

The effect of CO on the transcriptional rate of the E2F-1 gene was determined by nuclear run-on analysis (Fig. 6A). Cells were exposed to hypoxia in the presence or absence of the CO scavenger, Hb for 12 h. Transcriptional rates of HO-1, a gene dramatically regulated by endogenous levels of CO as we have previously reported (3), and β-actin were analyzed for comparison. Under hypoxia, removal of CO from the cultures with Hb markedly induced the transcriptional rate of both the E2F-1 and HO-1 genes (4- and 10-fold, respectively, as assessed by densitometry), whereas β-actin transcription was unaffected.

To determine if the changes in E2F-1 gene expression and mRNA levels result in changes of E2F-1 protein levels in VSMC, nuclear proteins were isolated and Western analysis performed using anti-E2F-1 antibody. This figure is representative of two independent experiments.

E2F-1 is known to regulate the transcription of other cell cycle-specific genes including c-myc (18). To examine whether the increased E2F-1 expression upon removal of CO from cell cultures results in increased expression of E2F-1 target genes, we monitored c-myc mRNA levels. In parallel with enhanced VSMC proliferation and E2F-1 mRNA expression, we found a 2-fold increase in c-myc mRNA levels when cells were exposed to hypoxia for 12 h and CO was removed from the cultures with Hb (results not shown). Combined with the above findings, these results point to CO as the modulator of genes encoding growth factors and transcription factors critical to cell cycle progression and cell proliferation.

cGMP Mediates the Suppression of E2F-1 by CO—Exogenous CO is known to activate guanylyl cyclase and elevate cGMP levels (29, 30). We have previously reported that the CO produced by VSMC under hypoxia increases cGMP content in both VSMC (3) as well as adjacent endothelial cells (11). As previously, we found a significant 9-fold rise in cGMP levels in VSMC exposed to hypoxia for 12 h (23 pmol/mg of cell protein) compared with normoxic controls (2.5 pmol/mg of cell protein)
Proliferation of vascular cells plays an essential role in the pathogenesis of cardiovascular diseases such as atherosclerosis, intimal hyperplasia, and pulmonary hypertension. Hypoxia, often an underlying factor in these diseases, is a strong stimulus for vascular cell proliferation. Hypoxia has been shown to increase the expression of the growth factors ET-1 (24) and PDGF-B (25) while decreasing the production of NO (22, 23), an inhibitor of VSMC growth, thus predisposing to excess smooth muscle cell proliferation in the vasculature.

We report here that cultured VSMC exposed to hypoxic conditions proliferated at significantly increased rates in response to the mitogen ET-1 compared with normoxic cells. These findings are in agreement with a previous study showing an increased proliferative response of fibroblasts to serum and growth factors under low oxygen conditions (28). In addition, we report that hypoxia specifically increased mRNA and protein levels of E2F-1, a transcription factor involved in the orderly progression of cells from the G1 to the S phase but it did not affect the expression of E2F-5, another member of the E2F family, which presumably controls a checkpoint in earlier phases of the cell cycle. In the absence of growth factors, VSMC proliferation rate remained low under hypoxia and similarly, E2F-1 mRNA levels remained suppressed with continued hypoxia as cells were growth arrested. Although the cascade of events mediating the hypoxic response has yet to be characterized, our results indicate that E2F-1 plays a critical role in this pathway.

VSMC were recently shown to produce CO under conditions of hypoxia (3) which inhibited the production of ET-1 and PDGF-B in adjacent endothelial cells in a paracrine manner (11). We hypothesized that VSMC-derived CO may also have endothelial-independent effects on VSMC growth and in the current study designed experiments to test this hypothesis. We approached this question at multiple levels by modulating endogenous CO production or by administering exogenous CO as well as examining potential intracellular signaling molecules that could mediate the actions of CO.

VSMC were treated with ZnPP-9 or SnPP-9 to inhibit CO production or with Hb to scavenge CO from the media. When CO was removed from the cultures with these treatments under hypoxic conditions, VSMC demonstrated even a more pronounced growth response to the mitogen ET-1. These agents had no effect on VSMC growth under normoxic conditions when CO levels are low to absent (3). Since metalloporphyrins are not only strong inhibitors of HO activity but have also been re-
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We propose that CO, by antagonizing the proliferative effects of hypoxia (11), may represent the body’s response to growth factors (35). Indeed, our studies suggest that E2F-1 may be a downstream target of CO on cell cycle control pathways.

CO shares some of the properties of NO. Both are gas molecules normally produced in the body and are capable of activating guanylyl cyclase to increase cGMP levels (8). However, under hypoxia, NO synthesis is suppressed in endothelial cells resulting in depressed cGMP levels in both endothelial cells and adjacent VSMC (22). Furthermore, transcripts of nitric-oxide synthase were not detectable in VSMC in the absence of cytokine stimulation (3, 36) and treatment of VSMC with nitric-oxide synthase inhibitors did not eliminate cGMP accumulation under either normoxia or hypoxia (11) (see also Fig. 7). In this study we showed that it is the endogenously-derived CO in hypoxic VSMC that increases cGMP levels and demonstrated that the expression of E2F-1 is inversely related to cGMP levels. We therefore propose that it is the VSMC-derived CO and not NO, that regulates E2F-1 gene expression and VSMC growth via a cGMP-dependent pathway in response to hypoxia.

The increased production of ET-1 and PDGF-B by hypoxic endothelial cells combined with the suppression of endothelial nitric-oxide synthase would be expected to accelerate VSMC growth. On the other hand, a counter-proliferative system is also in effect under hypoxia. Using a co-culture system of endothelial cells and VSMC, we reported that VSMC-derived CO suppressed the hypoxia increases in ET-1 and PDGF-B production by endothelial cells in a paracrine manner (11). In this report, we showed that CO has additional effects on VSMC proliferation that are endothelial cell-independent. Therefore, CO has both direct and indirect anti-proliferative effects on VSMC growth. In this manner, CO may represent the body’s adaptive responses to hypoxia with both vasoactive and anti-proliferative effects both of which are partly mediated by cGMP. We propose that CO, by antagonizing the proliferative effects of hypoxia on VSMC growth, may limit the cellular hyperplasia within the blood vessel wall, thus potentially decreasing the severity of cardiovascular disorders such as atherosclerosis and pulmonary hypertension.

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