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Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP

(hypoxia/vessel tone/gene regulation)

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ABSTRACT **Carbon monoxide (CO) is a product of the enzyme heme oxygenase (HO; EC 1.14.99.3). In vascular smooth muscle cells, exogenously administered CO increases cyclic guanosine 3′,5′-monophosphate (cGMP), which is an important regulator of vessel tone. We report here that smooth muscle cells produce CO via HO and that it regulates cGMP levels in these cells. Hypoxia, which has profound effects on vessel tone, significantly increased the transcriptional rate of the HO-1 gene resulting in corresponding increases of its mRNA and HO enzymatic activity. In addition, under the same conditions, rat aortic and pulmonary artery smooth muscle cells accumulated high levels of cGMP following a similar time course to that of HO-1 production. The increased accumulation of cGMP in smooth muscle cells required the enzymatic activity of HO, since it was abolished by a specific HO inhibitor, tin protoporphyrin. In contrast, N^{ω}-nitro-L-arginine, a potent inhibitor of nitric oxide (NO) synthesis, had no effect on cGMP produced by smooth muscle cells, indicating that NO is not responsible for the activation of guanylyl cyclase in this setting. Furthermore, conditioned medium from hypoxic smooth muscle cells stimulated cGMP production in recipient cells and this stimulation was completely inhibited by tin protoporphyrin or hemoglobin, an inhibitor of CO production and a scavenger of CO, respectively. This report shows that HO-1 is expressed by vascular smooth muscle cells and that its product, CO, may regulate vascular tone under physiologic and pathophysiologic (such as hypoxic) conditions.**

Regulation of blood vessel tone is critical to maintaining adequate tissue oxygenation and perfusion. This phenomenon involves a delicate balance between vasodilators and vasoconstrictors. Hypoxia, for example, has profound effects on blood vessel tone, principally through the release or inhibition of vasoactive mediators from endothelial cells (1, 2). One endothelium-derived mediator is nitric oxide (NO), a potent vasodilator that helps maintain normal vascular tone by stimulating guanylyl cyclase in smooth muscle cells (SMCs) and elevating cGMP levels. Endothelial NO was shown to be suppressed by a hypoxic state resulting in low cGMP levels (3). NO is normally produced by the body and serves as an important chemical messenger not only in the regulation of vessel tone, but also in neuronal transmission. Like NO, carbon monoxide (CO) is an endogenously produced gas molecule that activates guanylyl cyclase (4). Although a role for CO has been suggested in neuronal signal transduction (5), it is not known whether CO plays a physiologic role in the vasculature.

There are at least two endogenous sources of CO production, one of which is from the oxidation of organic molecules, but the predominant source is from the degradation of heme (6). Heme is metabolized to biliverdin and CO by heme oxygenase (HO; EC 1.14.99.3) (7). Two isoforms of HO have been characterized: HO-1 and HO-2 (8). HO-1 is inducible by a variety of agents (9, 10), whereas HO-2 is not inducible (11). The CO generated from the activity of HO could therefore, serve to elevate cGMP levels in the same or neighboring cells and exert its effects in an autocrine or paracrine manner. We designed the present study to determine whether CO could be produced by vascular SMCs and to investigate whether it could have a physiological role in the regulation of cGMP levels in these cells in response to a hypoxic stimulus.

MATERIALS AND METHODS

Cell Culture. Primary cultures of rat aortic SMCs (rSMCs) and rat pulmonary artery SMCs were harvested from male Sprague–Dawley rats (200–250 g) (12). An antibody to SMC α-actin was used to assess the purity of the culture, demonstrating that 99% of the cells were of smooth muscle origin. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO) and gentamicin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator and were used between passages 5 and 10. rSMCs at confluence were exposed to low O_2 tensions in airtight chambers flushed with preanalyzed gas mixtures and kept at 37°C. Hypoxic conditions were established with airtight incubators (Billups-Rothenberg, Del Mar, CA) as described (2). When 1% or 3% O_2 was infused, the lowest P_{O_2} reached in the medium was 30 torr and 45 torr, respectively (1 torr = 133 Pa).

Amplification of SMC HO-1 and HO-2 cDNA Fragments. HO-1 and HO-2 cDNA fragments were amplified from rSMC RNA by the reverse transcription–polymerase chain reaction (PCR) (13). Primers were designed according to the published sequence of human monocyte HO-1 (14) and rat testis HO-2 (15). The forward (5′-GAATTCAGCATGCCCCAGGATTTG-3′) and reverse (5′-TCTAGACTAGCTGGATGTTGAGCAGGA-3′) primers for HO-1 were used to amplify a 615-bp fragment, and the forward (5′-GAATTCGGGACCAAGGAAGCACAT-3′) and reverse (5′-TCTAGACTATGTAGTACCAGGCCAAGA-3′) primers for HO-2 were used to amplify an 828-bp fragment. The PCR fragments were then subcloned and sequenced by the dideoxy chain-termination method (16) to confirm their identity.

RNA Analysis. Total cellular RNA was prepared by guanidinium isothiocyanate extraction from rSMCs exposed to various O_2 tensions for various periods. Total RNA (15 μg per lane) was electrophoresed in 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes by blotting. As probes, we used the 615-bp DNA fragment of the rat HO-1 gene and the 828-bp DNA fragment of the rat HO-2

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Abbreviations: HO, heme oxygenase; SMC, smooth muscle cell; rSMC, rat aortic SMC; SnPP-9, tin protoporphyrin IX.

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gene. The cDNA fragments were labeled with [α-^{32}P]dCTP in a standard random-primed reaction to a specific activity of 1–2 $\times$ 10^9 cpm/μg. The membranes were hybridized with probe at 2 $\times$ 10^6 cpm/ml in QuikHyb solution (Stratagene) at 68°C for 2 hr and washed twice in 2$\times$ standard saline citrate (SSC)/0.1% SDS at room temperature for 15 min and once in 0.1$\times$ SSC/0.1% SDS at 60°C for 30 min. The membranes were then exposed to film (X-Omat AR; Eastman Kodak) with intensifying screens at −80°C. The membranes were subsequently stripped and rehybridized with a ^{32}P-labeled, 800-bp *Pst* I fragment of the mouse β-actin gene. For quantitation, we scanned autoradiographs with a laser densitometer (Ultroscan XL; LKB) running the GEL SCAN XL software package (Pharmacia LKB).

Nuclear Run-On Analysis. Confluent cultures of rSMCs were exposed to hypoxia for 12 hr. Nuclei then were isolated and *in vitro* transcription was performed (3, 17). 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. cDNAs for rat HO-1, rat HO-2, and β-actin were used as probes.

HO Enzyme Activity. HO activity in rSMC microsomes was measured by bilirubin generation (18, 19). rSMCs at confluence in 15-cm-diameter dishes were exposed to hypoxia for 6, 12, 24, and 48 hr in the presence or absence of a specific inhibitor of HO, tin protoporphyrin IX (SnPP-9) (20). SnPP-9 was added (10 μM, 100 μM, or 1 mM) 1 hr before the end of the exposure period. At the end of each incubation, a microsomal fraction was prepared (19). The supernatant was added to a reaction mixture (1.2 ml) containing rat liver cytosol (2 mg), NADP (0.8 mM), glucose-6-phosphate (1 mM), and glucose-6-phosphate dehydrogenase (0.2 unit). Finally, 20 μl of 2.5 mM hemin was added as the substrate. The mixtures were aerobically incubated for 10 min at 37°C in the dark. The reaction was stopped by placement on ice, and the amount of bilirubin formed was measured with a double-beam spectrophotometer as ΔOD 464 − 530 nm (extinction coefficient, 40 $mM^{-1} \cdot cm^{-1}$ for bilirubin). HO activity was expressed as pmol of bilirubin formed per mg of cell protein in 60 min. The protein content was determined by a dye-binding assay (Bio-Rad).

cGMP Accumulation in Hypoxia-Exposed rSMCs. To determine the relationship between cGMP concentration and the production of HO-1 by hypoxia, rSMCs at confluence were exposed to hypoxia for 6, 12, 24, and 48 hr and cGMP concentrations were determined at each time point, in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM), which was added to the plate 20 min before cell extracts were obtained. The effect of SnPP-9 on hypoxia-induced cGMP accumulation was evaluated by exposing cells to hypoxia for 6 hr and adding SnPP-9 (10 μM, 100 μM, or 1 mM) to plates 1 hr before the end of the exposure period. To eliminate the role of NO on hypoxia-induced cGMP accumulation, rSMCs were incubated in L-arginine-free medium for 24 hr and then exposed to 0% O_2 for 6 hr. The NO synthase inhibitor N^{ω}-nitro-L-arginine (2.5 mM) was added to plates 1 hr before the end of the exposure period, and cGMP concentration was determined in the presence of isobutylmethylxanthine. In the conditioned-medium experiments, donor rSMCs were exposed to 0% or 21% O_2 for 6 hr and treated with hemoglobin (Hb, 50 μM) or SnPP-9 (1 mM) for the last hour. The media were then transferred to the recipient rSMCs pretreated with SnPP-9. After 10 min, isobutylmethylxanthine was added, and after an additional 20-min incubation, cGMP concentrations in recipient cells were determined.

Measurements of cGMP Concentrations. cGMP was extracted from the cells by rapid aspiration of medium, washes with ice-cold phosphate-buffered saline, and the addition of ice-cold ethanol to 65% (vol/vol). The cells were harvested and centrifuged at 2000 $\times$ *g* for 5 min at 4°C. Supernatants were transferred to fresh tubes, evaporated at 60°C in a vacuum oven until completely dry, and kept at −80°C till assay. The cGMP concentration of cell extracts was determined by radioimmunoassay (Amersham). cGMP concentration was normalized to protein content as determined by a dye-binding assay (Bio-Rad) using bovine serum albumin as the standard.

Measurement of Carboxyhemoglobin (HbCO) in Conditioned Medium. To examine whether rSMCs released CO into the medium Hb (50 μM) was added to the cells for the last hour of incubation, and HbCO was measured spectrophotometrically.

Reagents. Glucose-6-phosphate dehydrogenase was purchased from Boehringer Mannheim, and SnPP-9 was purchased from Porphyrin Products (Logan, UT). All other reagents utilized were obtained from Sigma unless otherwise specified. Pure HbO_2 was prepared by treatment with excess reducing agent (21).

Data Analysis. Significant difference were determined by one-way ANOVA, and $P < 0.05$ was considered statistically significant.

RESULTS

Hypoxia Increases Rat HO-1 mRNA Levels in rSMCs. rSMCs and rat pulmonary artery SMCs were exposed to 0% O_2 (Po_2 = 18–20 torr) or to an ambient environment (Po_2 = 130 torr) for various periods. Northern blot analysis of rSMC RNA after hybridization with the rat HO-1 cDNA probe showed a 6-fold increase in HO-1 mRNA levels in cells exposed to hypoxia for 12 hr, compared with controls in a 21% O_2 environment. Reexposure of cells to 21% O_2, after a hypoxic exposure, resulted in a return of HO-1 mRNA to near basal levels by 24 hr (Fig. 1*A*). The time course of hypoxia-induced HO-1 gene expression showed that HO-1 mRNA started to increase within 2 hr, reached a maximum by 15 hr of hypoxia, and declined to basal levels during the subsequent 24–48 hr (Fig. 1*B*). To better define the range of O_2 tension in which the HO-1 gene is regulated, we compared the effects of 3% or 1% O_2 with those of 0% O_2 environments on HO-1 mRNA. Fig. 1*C* demonstrates that 0% O_2, the lowest O_2 tension, induced mRNA more markedly than 1% O_2, which was, in turn, more effective than 3% O_2. On the other hand, HO-2 mRNA levels were lower than those of HO-1, and were constant in all environments, not regulated by a hypoxic state (data not shown).

Hypoxia Regulates the Rate of HO-1 Gene Transcription. Nuclear run-on analysis was carried out to determine the transcriptional rate of the HO-1 gene under hypoxic and normoxic conditions. Transcriptional rates of two other genes expressed by rSMCs, HO-2 and β-actin, were analyzed for comparison. Hypoxia stimulated the transcriptional rate of the HO-1 gene markedly above the rate observed at 21% O_2 (Fig. 2). In contrast, the transcriptional rates of the HO-2 and β-actin genes were not increased by hypoxia. The half-life of the HO-1 transcript was found to be 3 hr in both hypoxic and normoxic conditions (data not shown). The hypoxia-induced increase in HO-1 mRNA in rSMCs is therefore predominantly due to increased gene transcription as opposed to enhanced stability of the mRNA transcript.

Hypoxia Increases HO Activity. To examine whether an increase in HO-1 enzyme activity accompanies hypoxia-induced changes in gene expression, HO activity was measured by bilirubin generation in rSMC microsomes. Hypoxia exposure increased HO enzymatic activity in rSMCs to a peak of 6.0-fold above control levels (713.72 ± 23.74 pmol of bilirubin formed per mg of cell protein in 60 min) by 12 hr and declined subsequently (Fig. 3*A*). These changes paralleled those in mRNA expression for HO-1. SnPP-9 inhibited the HO activity in a dose-dependent manner under both conditions (Fig. 3*B*).

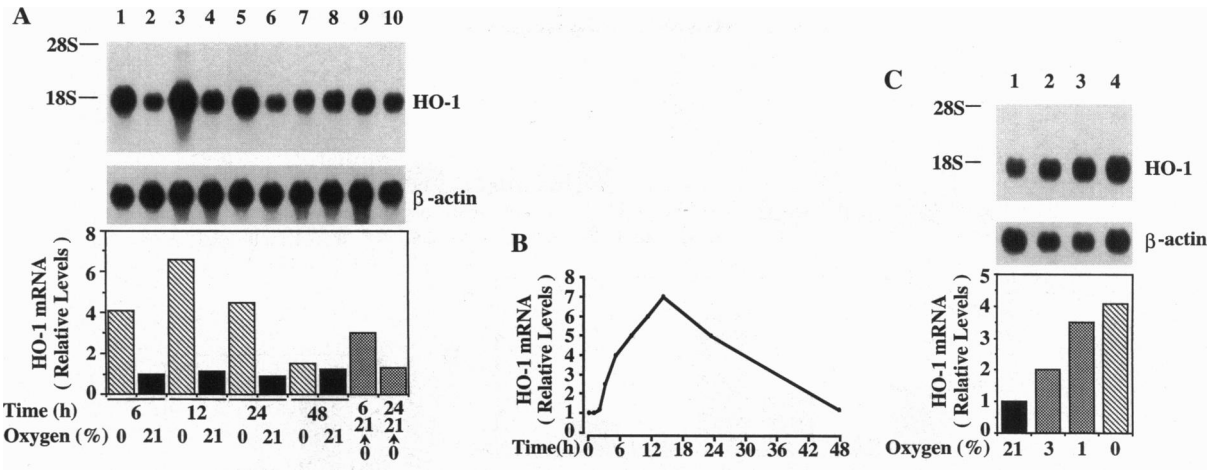

FIG. 1. HO-1 mRNA induction by hypoxia. (*A*) (*Top*) Total RNA was extracted from rSMCs exposed to 0% O_2 for 6 hr (lane 1), 12 hr (lane 3), 24 hr (lane 5), 48 hr (lane 7), or to 0% O_2 for 24 hr followed by 21% O_2 for 5 hr (lane 9) or 24 hr (lane 10), and Northern blot analysis was performed. Lanes 2, 4, 6, and 8 represent signal from rSMCs at each indicated period under 21% O_2. Positions of 28S and 18S rRNAs are indicated as size markers. (*Middle*) Same blot hybridized to the β-actin probe. (*Bottom*) Relative mRNA levels (normalized to β-actin mRNA) derived from laser densitometric analysis of amounts of HO-1 transcripts expressed under hypoxia compared with RNA from cells exposed to 21% for 6 hr. (*B*) Time course of HO-1 mRNA induction by hypoxia. The HO-1 mRNA levels (normalized to β-actin mRNA) at 0% O_2 were divided by normalized HO-1 mRNA levels in rSMCs at 21% O_2 and are presented for 1, 2, 3, 6, 9, 12, 15, 24, and 48 hr. (*C*) HO-1 mRNA levels are regulated at a narrow range of O_2 tension. (*Top*) Total RNA was extracted from rSMCs exposed to 0%, 1%, or 3% O_2 for 6 hr. (*Middle*) Same blot hybridized to the β-actin probe. (*Bottom*) Relative levels presented as in *B*. Data shown are representative of six independent experiments.

Hypoxia Regulates cGMP Concentration in rSMCs Through HO. We then examined whether the elevated HO activity as a result of hypoxia could increase cGMP in rSMCs. Hypoxia significantly increased cGMP levels above those at 21% O_2 concentration at all four time points, with a maximum of 9.1 fold (22.88 ± 0.26 pmol per mg of cell protein) at 12 hr (Fig. 4*A*). The time course of the increase in cGMP was essentially similar to that of HO-1 production. To confirm that it is the increased HO activity which regulates cGMP production in rSMCs under hypoxic conditions, we added SnPP-9 to the cultures. In addition, to eliminate NO as the molecule mediating this increase in cGMP concentration, we incubated cells with N^{ω}-nitro-L-arginine (2.5 mM). The inhibitor of HO-1, SnPP-9, lowered rSMC cGMP concentrations under both conditions in a dose-dependent manner, whereas N^{ω}-nitro-L-arginine, the inhibitor of NO synthesis, had no effect on cGMP levels (Fig. 4*B*). In addition, we were unable to detect transcripts for NO synthase in rSMCs under any O_2 environment (data not shown). A transferable HO-derived factor in the medium of hypoxic rSMCs was found to stimulate cGMP production in recipient SMCs (Fig. 4*C*). In this experiment, endogenous HO-1 activity in the recipient cells was blocked with SnPP-9 pretreatment. Conditioned medium from hypoxic rSMCs stimulated a 2-fold rise in cGMP in recipient cells compared with medium from normoxic donor cells (Fig. 4*C*, bars 4 and 3, respectively). Medium obtained from donor cells pretreated with SnPP-9, however, failed to raise cGMP concentrations in recipient rSMCs (bar 5), and similarly, when Hb, a scavenger of CO, was added to the donor medium, the rise in cGMP content of the recipient cells was inhibited (bars 6 and 7). These findings indicate that CO, the biological product of HO-1 activity, is responsible for the rise in cGMP.

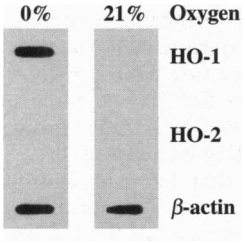

FIG. 2. Hypoxia increases HO-1 gene transcription. rSMCs were exposed to 0% or 21% O_2 for 12 hr. Nuclei were isolated and transcription was allowed to resume *in vitro* in the presence of [α-^{32}P]UTP. Equal amounts of ^{32}P-labeled, *in vitro* transcribed RNA were hybridized to denatured cDNA for the HO-1, HO-2, and β-actin genes, previously immobilized on nitrocellulose filters. Shown is a representative autoradiogram of two independent experiments.

Hypoxia Increases CO Release by rSMCs. To confirm that rSMCs exposed to hypoxia release more CO (via HO) than rSMCs in normoxia, we quantified the relative amounts of CO produced by adding Hb to the medium and measuring HbCO levels. Hypoxic rSMCs generated 6-fold greater CO than cells incubated in 21% O_2 environments, and the CO produced could be decreased by inhibiting HO-1 activity with SnPP-9 in a dose-dependent manner (Fig. 4*D*). Hypoxia, therefore, increases HO-1 gene expression, leading to increased enzymatic

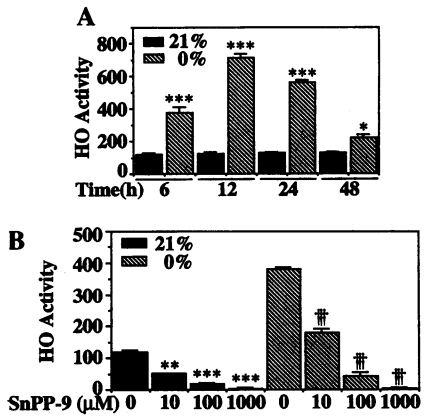

FIG. 3. (*A*) Hypoxia increases HO enzyme activity. Bars represent the HO activity (pmol of bilirubin formed per mg of cell protein in 60 min) (mean ± SEM) of four experiments performed in duplicate. *, $P < 0.05$; ***, $P < 0.001$ versus rSMCs under 21% O_2 at each time point. (*B*) SnPP-9 decreases hypoxia-induced HO-1 activity. rSMCs were exposed to 0% or 21% O_2 for 6 hr and SnPP-9 was added for the last hour. Data represent mean HO activity of four experiments performed in duplicate. **, $P < 0.01$; ***, $P < 0.001$ versus untreated rSMCs under 21% O_2; †††, $P < 0.001$ versus untreated rSMC exposed to 0% O_2.

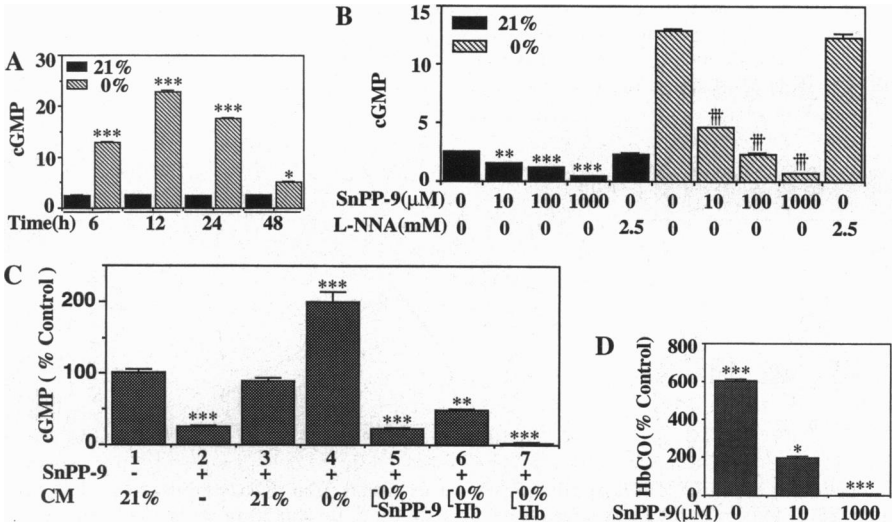

FIG. 4. (*A*) Hypoxia increases cGMP in rSMCs. cGMP concentrations (pmol per mg of cell protein) (mean ± SEM) are shown for four experiments performed in duplicate. *, $P < 0.05$; ***, $P < 0.001$ versus 21% O_2 control at each indicated time. (*B*) Effect of SnPP-9 or N^{ω}-nitro-L-arginine (L-NNA) on hypoxia-induced cGMP concentrations. rSMCs were exposed to 0% or 21% O_2 for 6 hr in the presence or absence of SnPP-9 or L-NNA. Data represent the cGMP concentrations (pmol per mg of cell protein) (mean ± SEM) of four experiments performed in duplicate. **, $P < 0.01$; ***, $P < 0.001$ versus untreated rSMCs under 21% O_2; †††, $P < 0.001$ versus untreated rSMCs exposed to 0% O_2. (*C*) Conditioned medium from rSMCs exposed to 0% O_2 increases cGMP concentration in recipient rSMCs pretreated with SnPP-9. Bar 1, control cGMP levels in untreated rSMCs incubated with conditioned medium (CM) from 21% O_2-exposed donor rSMCs; bar 2, cGMP levels in SnPP-9-treated rSMCs with medium unchanged in 21% O_2; bars 3 and 4, cGMP levels in SnPP-9-treated rSMCs incubated with medium from 21% O_2-exposed donor rSMCs (bar 3) or 0% O_2-exposed donor rSMCs (bar 4); bar 5, cGMP levels in SnPP-9-treated rSMCs incubated with medium from 0% O_2-exposed donor rSMCs that were also pretreated with SnPP-9; bars 6 and 7, cGMP levels in SnPP-9-treated rSMCs incubated with conditioned medium from hypoxic donor cells in the presence of 1 μM and 50 μM Hb respectively, for the last 30 min. Data are presented as percentages of cGMP levels in untreated recipient rSMCs (control) of three experiments in duplicate. **, $P < 0.01$; ***, $P < 0.001$ versus control. (*D*) Measurement of HbCO in conditioned medium of rSMCs under hypoxia for 24 hr and conditioned media obtained from the same hypoxic conditions but in the presence of 10 μM and 1 mM SnPP-9. Values are represented as percentages of control HbCO levels in conditioned medium of rSMCs exposed to 21% O_2 for 24 hr. *, $P < 0.05$; ***, $P < 0.001$ versus control ($n = 4$).

activity and to the production of CO which, in turn, stimulates the generation of cGMP.

Endogenous CO Levels Regulate HO-1 mRNA. It has previously been reported that exogenous administration of CO inhibits the hypoxic increases in gene expression (22, 23). We have also found that exogenous CO suppresses the hypoxic induction of HO-1 mRNA (data not shown). Since the response of HO-1 to hypoxia is biphasic, with a rapid rise and subsequent fall to baseline by 48 hr (see Fig. 1*B*), we hypothesized that endogenous CO accumulation may be responsible for the late suppression of HO-1 at 48 hr. We thus inhibited CO production with SnPP-9 and found that although SnPP-9 had no effects on the basal level of HO-1 mRNA (data not shown), in its presence, HO-1 transcript levels remained elevated at 48 hr of hypoxia (Fig. 5) and failed to return to baseline. These findings support the hypothesis of feedback suppression of HO-1 by endogenous accumulation of CO.

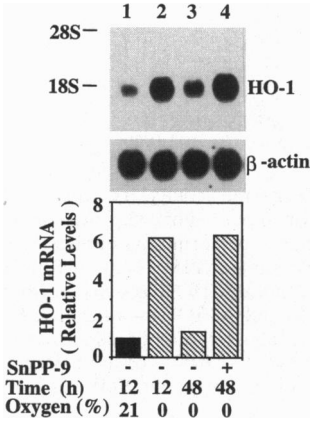

FIG. 5. HO-1 mRNA remains elevated with SnPP-9. (*Top*) Total RNA was extracted from rSMCs exposed to 21% O_2 for 12 hr (lane 1), 0% O_2 for 12 hr (lane 2), or 0% O_2 for 48 hr (lane 3) in the absence of SnPP-9 or to 0% O_2 for 48 hr in the presence of SnPP-9 (lane 4), and Northern blot analysis was performed. (*Middle* and *Bottom*) β-Actin and relative mRNA levels as described in Fig. 1*B*.

DISCUSSION

cGMP is a messenger molecule that mediates SMC relaxation in the vasculature and neuronal transmission in the brain. It is formed from the action of guanylyl cyclase, which in turn is activated by NO. Verma *et al.* (5) reported that CO may also be a physiologic regulator of cGMP and function as a neurotransmitter in the brain, and potentially as a dilator of vascular smooth muscle outside the brain. Direct stimulation of guanylyl cyclase activity in SMCs by exogenous CO has been proposed (4, 24), but the role of CO that is generated outside the brain is not known. CO is formed principally from the breakdown of heme to biliverdin by the enzyme HO. According to Verma *et al.* (5), HO-2 colocalizes to a significant degree with guanylyl cyclase throughout the rat brain, and HO-2 mRNA is localized in SMCs in blood vessels. In agreement with Verma *et al.*, we report here that rSMCs express HO-2 mRNA constitutively at low levels. However, we found that in addition to expressing HO-2, rSMCs express HO-1 at higher baseline levels and furthermore, upregulate its production dramatically in response to hypoxia, resulting in high cGMP accumulation. In contrast, HO-2 mRNA expression remains unchanged with hypoxia. These finding suggest that CO produced by the activity of HO-1 serves to increase cGMP levels in rSMCs by activating guanylyl cyclase.

We report here that hypoxia significantly increases the transcriptional rate of the gene encoding HO-1, resulting in correspondingly increased mRNA levels and HO-1 enzymatic activity. HO-1 activity was assayed by amounts of bilirubin produced as well as by CO levels and cGMP content. Significantly higher CO levels were released into the medium of hypoxic cells, and similarly, significantly higher bilirubin and intracellular cGMP levels were measured in hypoxic SMCs than in SMCs in an 21% O_2 environment. To test whether the cGMP increases were specifically due to the activity of HO-1,

we used a potent selective HO inhibitor, SnPP-9. We found that SnPP-9 lowered cGMP concentrations dose-dependently in both oxygen conditions. Furthermore, the conditioned-medium experiments showed that a "factor" released in the medium of hypoxic cells can increase cGMP levels in normoxic rSMCs. This was demonstrated in experiments whereby medium from donor rSMCs restored cGMP contents in recipient cells pretreated with SnPP-9, whereas SnPP-9- and Hb-pretreated donor rSMCs failed to increase cGMP. We also eliminated NO as the molecule responsible for the increases in cGMP by showing that N^{ω}-nitro-L-arginine, a potent inhibitor of NO synthesis, had no effect on cGMP production by rSMCs. In addition, we were unable to detect transcripts for NO synthase in rSMCs under any O_2 environment. Although we cannot rule out another Hb-inhibited substance, these observations suggest that the factor in the transferred medium from donor rSMCs responsible for increasing cGMP levels in recipient cells is CO, a product of HO-1 activity, inhibited by SnPP-9 and Hb.

Hypoxia has been reported to induce the expression of several mammalian genes, including those encoding platelet-derived growth factor B (PDGF-B) (1), endothelin (ET-1) (2), and vascular endothelial growth factor (VEGF) (22, 25). These hypoxia-induced genes may mediate some of the hypoxia-related vascular disorders, such as excessive proliferation and constriction of smooth muscle cells, as well as increased permeability of endothelial cells. Excluding VEGF, the other factors regulated by hypoxia are produced by endothelial cells and thus control SMC function in a paracrine manner. The findings in this report suggest that hypoxia can regulate SMC function directly by stimulating HO-1 gene expression in these cells causing a rise in SMC cGMP. To our knowledge, it is the first report to demonstrate that SMCs express HO-1 and furthermore, that the expression of this gene is regulated by hypoxia. Although endothelial cell-derived NO is the molecule classically described to control cGMP content in the vasculature, it is the SMC-derived CO, and not NO, that increases cGMP in response to hypoxia. Of interest, exogenous CO has been reported to suppress the hypoxic increases of the PDGF-B, ET-1, and VEGF genes (22, 23). Exogenous administration of CO also suppressed the induction of the HO-1 gene by hypoxia. Furthermore, our time-course studies indicate that the induction of HO-1 by hypoxia is suppressed at 48 hr (Fig. 1*B*). However, when the production of CO in rSMCs is inhibited by SnPP-9, HO-1 mRNA levels remain elevated beyond 48 hr (Fig. 5). Taken together, these findings suggest that the hypoxia-induced HO-1 gene transcription may be inhibited by the CO generated from the activity of HO-1 itself, thus resulting in a negative feedback mechanism. We speculate that suppression of vasoactive mediators *in vivo* by the local production of CO in response to hypoxia may serve to regulate SMC function and vascular tone.

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