CO modulates pulmonary vascular response to acute hypoxia: relation to endothelin

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Zhang, Fan, Jun Ichi Kaide, LiMing Yang, Houli Jiang, Shuo Quan, Rowena Kemp, Weiying Gong, Michael Balazy, Nader G. Abraham, and Alberto Nasjletti. CO modulates the pulmonary vascular response to acute hypoxia: relation to endothelin. Am J Physiol Heart Circ Physiol 286: H137–H144, 2004.—Pulmonary intralobar arteries express heme oxygenase (HO)-1 and -2 and release carbon monoxide (CO) during incubation in Krebs buffer. Acute hypoxia elicits isometric tension development (0.77 ± 0.06 mN/mm) in pulmonary vascular rings treated with 15 μmol/l chromium meso-porphyrin (CrMP), an inhibitor of HO-dependent CO synthesis, but has no effect in untreated vessels. Acute hypoxia also induces contraction of pulmonary vessels taken from rats injected with HO-2 antisense oligodeoxynucleotides (ODN), which decrease pulmonary HO-2 vascular expression and CO release. Hypoxia-induced contraction of vessels treated with CrMP is attenuated (P < 0.05) by endothelium removal, by CO (1–100 μmol/l) in the bathing buffer, and by endothelin-1 (ET-1) receptor blockade with L-754142 (10 μmol/l). CrMP increases ET-1 levels in pulmonary intralobar arteries, particularly during incubation in hypooxygenated media. CrMP also causes a leftward shift in the concentration-response curve to ET-1, which is offset by exogenous CO. In anesthetized rats, pretreatment with CrMP (40 μmol/kg iv) intensifies the elevation of pulmonary arterial pressure elicited by breathing a hypoxic gas mixture. However, acute hypoxia does not elicit augmentation of pulmonary arterial pressure in rats pretreated concurrently with CrMP and the ET-1 receptor antagonist L-745142 (15 mg/kg iv). These data suggest that a product of HO activity, most likely CO, inhibits hypoxia-induced pulmonary vasoconstriction by reducing ET-1 vascular levels and sensitivity.

hypoxic pulmonary vasoconstriction; heme oxygenase

ARTERIAL VESSELS generate carbon monoxide (CO) along with biliverdin via metabolism of heme by heme oxygenase (HO) isoforms -1 and -2 (36). Arterial vessels express HO-2 constitutively, whereas HO-1 is primarily expressed as the result of exposure to inducing factors or conditions (24, 30). Products of heme metabolism by HO possess biological activities that influence vascular functions. Biliverdin and its metabolic product bilirubin are antioxidants capable of preventing oxidant-induced microvascular leukocyte adhesion (6). CO stimulates soluble guanylate cyclase (19) and Ca2+-activated K+ (KCa) channels (10, 30) in vascular smooth muscle, interferes with the expression and release of endothelin-1 (ET-1) in endothelial cells (18), and inhibits endothelial nitric oxide synthase (27). CO of vascular origin attenuates vasoconstrictor responsiveness to myogenic stimuli in rat gracilis muscle arterioles (37) and to constrictor agonists in the rat renal interlobar arteries (10), the rat tail artery (30), and aorta (3). CO of vascular origin was also implicated in the mediation of dilatory responses to acetylcholine in porcine pulmonary arteries (35), to heme-1-lysinate in small rat mesenteric arteries (22), and to acute hypoxia in cerebral arteries of piglets (15).

Hypoxia causes constriction of pulmonary arterial vessels in vitro and in vivo (9, 14, 23, 38). The pulmonary vasoconstriction elicited by hypoxic conditions is attenuated by blockade of ET-1 subtype A (ET-A) receptors, suggesting contribution of ET-1 to the mechanism underlying the constrictor response (9, 23). That HO-derived CO downregulates ET-1 expression and release in endothelial cells (18) raises the possibility that CO inhibits the ET-1-dependent component of the constrictor response to hypoxia in pulmonary vessels. However, the result of studies examining modulation of hypoxia-induced pulmonary vasoconstriction by CO is less than conclusive. Although exogenous CO dilates pulmonary vessels preconstricted with an agonist of thromboxane receptors (21), exogenous CO does not consistently elicit pulmonary vasodilation during hypoxic conditions (1, 28). While experimental interventions that enhance lung HO-1 expression prevent the pulmonary hypertension produced by chronic hypoxia in rats (4), the elevation of pulmonary arterial pressure produced by chronic hypoxia was similar in HO-1 null mice and the corresponding wild-type controls (33). In addition, the inhibitors of HO affect hypoxia-induced pulmonary vasoconstriction inconsistently, having little or no effect under basal conditions, while intensifying the response when nitric oxide synthesis is decreased (2).

The present study was designed to test the hypothesis that CO produced by pulmonary arterial vessels attenuates hypoxia-induced pulmonary vasoconstriction by interfering with the vascular formation or actions of ET-1. We conducted experiments in the small pulmonary arteries of rats for the following reasons: 1) to quantify the generation of CO and determine whether it is affected by acute hypoxia, 2) to examine the effect of interventions that decrease HO activity or expression on the vascular response to acute hypoxia, and 3) to determine the effect of HO inhibition on constrictor responsiveness to ET-1 and vascular ET-1 levels.

MATERIALS AND METHODS

Chemicals. Chromium meso-porphyrin (CrMP) was purchased from Porphyrin Products (Logan, UT), and stock solutions were prepared in 50 mmol/l Na2CO3, CO was purchased from Tech Air (White Plains, NY), and CO-saturated solution (1 mmol/l) was prepared immediately before use (37). ET-1 was purchased from Bachem Bioscience (King of Prussia, PA). L-754142 was a gift from Merck Research Laboratories (Rahway, NJ).

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Antisense (AS) oligodeoxynucleotides (ODN) complementary to rat HO-1 mRNA (HO-1 AS-ODN) and rat HO-2 mRNA (HO-2 AS-ODN) and the corresponding scrambled (S) ODN (HO-1 S-ODN and HO-2 S-ODN) were synthesized by Genosys Biotechnologies (Woodland, TX); each ODN was phosphorothioated on the first three bases of the 3’ end and was purified by high-pressure liquid chromatography. The sequence of HO-1 AS-ODN is 5’-GGGCGTCCATCGCGGACTG-3’ and target bases +10 to −9 of HO-1 mRNA; the sequence of HO-2 AS-ODN is 5’-TCTGAAGACATGTTGCTGA-3’ and targets bases +11 to −9 of HO-2 mRNA. The sequence of HO-1 S-ODN is 5’-TCCAGGGGCTACAGCCTGG-3’, and the sequence of HO-2 S-ODN is 5’-GATCTGACCTCAAGTGATTG-3’. The effectiveness of HO-1 AS-ODN and HO-2 AS-ODN to reduce tissue expression of HO-1 and HO-2, respectively, was documented previously (5, 10).

Animals. Experiments were conducted on male Sprague-Dawley rats (200–300 g; Charles River, Wilmington, MA) according to protocols approved by the Institutional Animal Care and Use Committee. In the experiments using isolated pulmonary vessels, the animals were anesthetized (60 mg/kg ip pentobarbital sodium), heparin (1,000 U/kg) was injected intravenously, and the lungs were removed. Immediately thereafter, the lungs were placed on a dish filled with ice-cold Krebs buffer composed of (in mmol/l): 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25.0 NaHCO₃, and 11.1 dextrose, and pulmonary intralobar arteries (100–200 µm of internal diameter) were dissected out for use in vascular contractility studies and for assessment of HO isoform expression, CO production, and ET-1 levels. In some experiments, the animals were treated 24 h before experimentation with HO-1 AS-ODN, HO-2 AS-ODN, HO-1 S-ODN, or HO-2 S-ODN. The ODN were encapsulated in liposomes (1:1 molar ratio nucleotide/lipid) prepared using premixed 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane and L-(oleoyloxy)-3-(trimethylammonio)propane and target bases GATTG-3’/H11032 and GATCTGACTTCAAGT-3’/H11032. The effectiveness of HO-1 AS-ODN and HO-2 AS-ODN to reduce tissue expression of HO-1 and HO-2, respectively, was documented previously (5, 10).

Assessment of vascular ET-1 levels. Segments of pulmonary intralobar arteries were placed inside glass vials containing Krebs buffer (1 ml) gassed with 95% O₂-5% CO₂ or 95% N₂-5% CO₂, the vials were capped, and the samples were incubated for 60 min at 37°C. When so noted, the incubations were conducted in buffer containing CrMP (15 µmol/l). At the conclusion of the incubation, the tissues were homogenized in 1 ml/l HCl containing 1% acetic acid, 1% trifluoroacetic acid, and 1% NaCl, and the resulting sample was processed (12) and assayed for ET-1 by radioimmunoassay using a commercially available kit (Peninsula Laboratories; San Carlos, CA).

Effect of hypoxia on isometric tension development in pulmonary intralobar arteries. Pulmonary arterial vessels with an internal diameter averaging 153 ± 12 µm were cut into ring segments 2 mm in length. The vascular rings were mounted on 25 µm stainless steel wires in the chambers of a multiwell myograph (J.P. Trading; Aarhus, Denmark) for measurement of isometric tension (38). The vessels were bathed in Krebs buffer maintained at 37°C and gassed with 95% O₂-5% CO₂, unless indicated otherwise. After an equilibration period (30 min), the vessels were stretched radially so that the internal circumference was equivalent to 90% of the vessel’s normal size if relaxed under a transmural pressure of 17.5 mmHg (20), which approximates the mean pressure measured in the pulmonary artery of rats under normoxic conditions. Isometric tension was monitored continuously and was expressed as milliNewtons per millimeter of vessel length.

Experiments were initiated by exposing the vessels to Krebs buffer modified by increasing the concentration of KCl to 80 mmol/l (by equimolar exchange with NaCl) to ascertain reproducibility of contractile responses. Subsequently, the vessels were washed with unmodified Krebs buffer and allowed to rest for 30 min before hypoxic conditions were established by gassing the bathing buffer with 95% N₂-5% CO₂ and assessment of ensuing changes in isometric tension. The effect of hypoxia on isometric tension was investigated in pulmonary intralobar arteries taken from rats treated 24 h before experimentation with HO-1 AS-ODN, HO-2 AS-ODN, or the corresponding HO-1 S-ODN or HO-2 S-ODN. The effect of hypoxia also was examined in endothelium-intact and endothelium-denuded (38) pulmonary vessels from untreated normal rats, bathed in buffer containing and not containing the HO inhibitor CrMP (15 µmol/l). In these experiments, the effectiveness of the denudation procedure was ascertained by documenting that acetylcholine (10 µmol/l)-induced relaxation of denuded pulmonary vessels precontracted with PGF₂α (10 µmol/l) was minimal (<10% of PGF₂α-induced tone) relative to the response in endothelium-intact vessels (>50% of PGF₂α-induced tone). Additional studies (32), conducted in preparations bathed in buffer containing CrMP (15 µmol/l), compared the vascular response to hypoxia in vessels exposed and not exposed to exogenous CO (1–100 µmol/l), biliverdin (10 µmol/l), or to L-754142 (10 µmol/l), a nonselective antagonist of ET-1 receptors. The pulmonary vascular response to acute hypoxia in preparations treated and not treated with CrMP (15 µmol/l) was also examined after inclusion of 1.0 µmol/l PGE₂ into the buffer, which elicited a modest elevation of isometric tension (from 0.36 ± 0.09 to 0.44 ± 0.09, n = 12; P < 0.05), believed to be a priming factor for expression of hypoxia-induced contraction of pulmonary arterial smooth muscle ex vivo (14).
RESULTS

HO expression and CO production in pulmonary arterial vessels. Proteins with the molecular mass of HO-1 and HO-2 were identified by immunoblotting in homogenates of pulmonary intralobar arteries (Fig. 1). The expression of HO-1 protein in pulmonary vessels was less intense (P < 0.05) in rats treated with HO-1 AS-ODN than in rats treated with HO-1 S-ODN (Fig. 1). The expression of HO-2 protein in vessels of rats treated with HO-2 AS-ODN was less intense (P < 0.05) than in vessels of rats treated with HO-2 S-ODN (Fig. 1).

Pulmonary intralobar arteries released CO into the headspace during incubation in Krebs buffer for 60 min (Fig. 2). CO release was virtually identical in vessels taken from rats treated with HO-1 S-ODN and HO-1 AS-ODN but was decreased (P < 0.05) in vessels from animals treated with HO-2 AS-ODN relative to the release from vessels of rats treated with HO-2 S-ODN. CO release from vessels bathed in media containing the HO inhibitor CrMP also was diminished (P < 0.05). CO release from vessels bathed in media gassed with 95% O2-5% CO2 (280 ± 44 pmol/l) was comparable to the release from vessels bathed in media gassed with 95% N2-5% CO2 (287 ± 70 pmol/l).

Effect of interventions that decrease HO expression and/or CO production on the pulmonary vascular response to acute hypoxia. KCl at 80 mmol/l produced comparable increase of isometric tension in rings of pulmonary intralobar artery taken from rats treated with HO-1 S-AS-ODN, HO-1 S-ODN, or HO-2 S-ODN.

Figure 1. Assessment of heme oxygenase (HO)-1 and HO-2 expression by immunoblotting in homogenates of rat pulmonary intralobar artery taken from rats treated with HO-1 scrambled-oligodeoxynucleotide (S-ODN), HO-1 antisense (AS)-ODN, HO-2 S-ODN, and HO-2 AS-ODN. The bar graph depicts the density of HO-1 and HO-2 bands in relation to that of β-actin. Results are means ± SE. n, Number of experiments. *P < 0.05.

Figure 2. Release of carbon monoxide (CO) into the headspace gas during incubation of rat pulmonary intralobar arteries in media gassed with 95% O2-5% CO2. The arteries were obtained from untreated rats and from rats treated with HO-1 S-ODN, HO-1 AS-ODN, HO-2 S-ODN, or HO-2 AS-ODN. Pulmonary intralobar arteries from untreated rats were incubated in the absence and presence of 15 mmol/l chromium mesoporphyrin (CrMP). Results are means ± SE. n, Number of experiments. *P < 0.05.
The inclusion of ET-1 and hypoxia-induced pulmonary vascular contraction.

The tension development induced by acute hypoxia in rings of pulmonary intralobar arteries exposed to CrMP was greatly attenuated by the addition of the ET-1 receptor antagonist L-754142 (10 μmol/l) to the bathing buffer (Fig. 6).

Figure 7 illustrates the effect of ET-1 on tension development in rings of pulmonary intralobar arteries bathed in buffer not containing and containing the HO inhibitor CrMP (15 μmol/l), alone and with CO (1 μmol/l). ET-1 elicited concentration-dependent tension development in all the groups. Exposure of the vessels to CrMP caused a leftward shift in the concentration-response curve to the constrictor peptide, decreasing the EC50 but not the Rmax. The sensitization caused by ex vivo treatment with CrMP was offset by exogenous CO, which caused a rightward displacement of the concentration-response curve to ET-1 and increased the EC50 without altering the Rmax.

Figure 8 depicts data on ET-1 levels in pulmonary intralobar arteries incubated for 60 min in Krebs buffer gassed with 95% O2-5% CO2 or with 95% N2-5% CO2, both in the presence and absence of CrMP. ET-1 levels were comparable in vessels incubated during hypoxic and nonhypoxic conditions in the presence of CrMP and CrMP and biliverdin (10 μmol/l). Results are means ± SE. Number of experiments. *P < 0.05, relative to data obtained during nonhypoxic conditions at time 0.
absence of CrMP. CrMP increased \( P < 0.05 \) the vascular level of ET-1 both in nonhypoxic and hypoxic preparations. ET-1 levels were higher \( P < 0.05 \) in hypoxic vessel treated with CrMP than in nonhypoxic preparations treated with the HO inhibitor. Also shown in Fig. 8, pulmonary intralobar arteries taken from rats treated with HO-2 S-ODN displayed similar levels of ET-1 when incubated under hypoxic and nonhypoxic conditions. In contrast, pulmonary vessels taken from rats treated with HO-2 AS-ODN displayed higher \( P < 0.05 \) levels of ET-1 when incubated under hypoxic than nonhypoxic conditions. Relative to data in pulmonary arteries from rats treated with HO-2 S-ODN, pulmonary arteries from rats treated with HO-2 AS-ODN had higher \( P < 0.05 \) levels of ET-1 both during incubation under nonhypoxic and hypoxic conditions.

**DISCUSSION**

This study shows that rat pulmonary intralobar arteries express both HO-1 and HO-2 proteins, which is consistent with previous observations (17, 35) in pulmonary arteries of other animal species, and that the expression of HO-1 and HO-2 is depressed in vessels taken from rats treated with HO-1 AS-ODN and HO-2 AS-ODN, respectively. The study also demonstrates that rat pulmonary intralobar arteries incubated in Krebs buffer release CO into the headspace gas. CO release from these vessels is largely HO dependent because it was decreased to \( \leq 30\% \) of the control value by CrMP, a metalloporphyrin, which inhibits both HO-1 and HO-2 (29). That CO release from pulmonary intralobar arteries of rats treated with HO-2 AS-ODN, but not with HO-1 AS-ODN, is exceeded by
that from vessels of animals treated with the corresponding scrambled antisense oligodeoxynucleotides implies that HO-2, rather than HO-1, is linked to CO release under the conditions of our study. HO-2-dependent formation of CO was documented previously in rat renal interlobar arteries and gracilis muscle arterioles (10, 37).

The major finding of our studies on isometric tension development, in isolated rat pulmonary intralobar arteries, is that acute hypoxia elicits contraction of vessels pretreated with CrMP to inhibit HO isoforms, and of vessels taken from rats pretreated with HO-2 AS-ODN to decrease HO-2 expression, but not of untreated vessels or of vessels obtained from rats pretreated with HO-2 S-ODN. Hypoxia also was found to elicit slight contraction of vessels taken from rats pretreated with HO-1 AS-ODN but not with HO-1 S-ODN. Previous reports (14) showed that mild precontraction of isolated rat pulmonary arteries with an agonist such as PGF$_{2\alpha}$ enables these vessels to respond to acute hypoxia with a further contraction. In our study, before hypoxia, the basal isometric tension of untreated pulmonary vessels and of vessels obtained from rats treated with CrMP and vessels taken from rats pretreated with HO-2 S-ODN tended to be exceeded (not significantly) by that of vessels pretreated with CrMP or HO-2 AS-ODN on hypoxia-induced contraction of rat pulmonary arteries. However, this may not be the case because we found that pretreatment with CrMP intensifies hypoxia-induced contraction of pulmonary arterial vessels already precontracted with PGF$_{2\alpha}$. Our finding that interventions, which decrease the activity or the expression of HO isoforms, have an enabling or intensifying influence on hypoxia-induced contraction of rat pulmonary arteries that may be taken to indicate that one or more HO products interfere with the mechanism(s) underlying the effect of hypoxia on pulmonary vascular tone. A similar conclusion was inferred from observations that interventions that induce pulmonary HO-1 prevent the increase in pulmonary arterial pressure observed in rats exposed to normobaric hypoxia for 1 wk (4).

The second key finding of our study, in rat pulmonary intralobar arteries treated with CrMP, is that the contractile response to acute hypoxia is attenuated by the inclusion of exogenous CO but not of biliverdin into the bathing buffer. Exogenous CO also was reported to inhibit hypoxic vasoconstriction in an in situ, blood-perfused, lung preparation (26). Hence, CO is the product of pulmonary vascular HO activity most likely to interfere with the mechanism underlying hypoxia-induced contraction of pulmonary arterial vessels. Basal levels of CO production may be sufficient to disrupt such a mechanism because acute hypoxia did not increase CO release from pulmonary arterial vessels.

In agreement with some previous studies (14, 16, 38), we found that the contractile response to acute hypoxia is less intense in pulmonary intralobar arteries denuded of endothelium than in arteries with intact endothelium. Such an observation suggests participation of an endothelium-derived constrictor factor in the implementation of hypoxia-induced pulmonary vasoconstriction. Pulmonary vascular endothelial cells produce ET-1 (31), which elicits pulmonary vasoconstriction acting on ET$_A$ and ET$_B$ receptors (8). Participation of ET-1 in the pulmonary vasoconstrictor response to hypoxia is suggested by reports that pharmacological blockade of ET-1 receptors prevents hypoxia-induced pulmonary hypertension (23). However, reports (9, 16) that ET-1 antagonists inhibit contractile responses to hypoxia in isolated pulmonary arterial vessels conflict with reports that they do not (13). According to our study, in rat pulmonary intralobar arteries treated with CrMP, the ET$_A$ and ET$_B$ receptor blocker L-754142 attenuates the contractile response to hypoxia. Hence, during conditions of HO inhibition, hypoxia-induced contraction of pulmonary arterial vessels relies, at least in part, on a mechanism involving ET-1.

The third key finding of our study is that interventions that inhibit HO activity or HO-2 expression promote elevation of ET-1 levels in pulmonary intralobar arteries. Moreover, we also found that HO inhibition sensitizes the vascular smooth muscle to the contracting action of ET-1. That treatment with HO-2 AS-ODN or with CrMP increases ET-1 levels in pulmonary arterial vessels, particularly during hypoxic conditions, is in line with a report (18) that inhibition of HO augments the production of ET-1 by human umbilical vein endothelial cells cocultured with vascular smooth muscle cells during hypoxic conditions. These observations suggest that a product of HO activity, presumably CO, inhibits endothelial cell synthesis of ET-1, an action that may counteract the stimulatory action of hypoxia on ET-1 production (11). That CrMP sensitizes pulmonary intralobar arteries to ET-1-induced contraction is attributable to reduction of vascular CO because the sensitizing effect could be offset by exogenous CO. Altogether, these observations suggest that CO manufactured by pulmonary arterial vessels is an inhibitory modulator of both the vascular formation and constrictor action of ET-1.

Previous studies (10, 30, 37) documented that endogenous CO reduces the sensitivity of systemic small arterial vessels to constrictor stimuli, an action that was attributed to activation of large conductance K$_{Ca}$ channels in vascular smooth muscle. On the other hand, in the pulmonary circulation of rats, activation of soluble guanylyl cyclase rather than of K$_{Ca}$ channels is
involved in the vasodilatory effect of exogenous CO (21). Whether or not the inhibitory influence of HO-derived CO on the responsiveness of pulmonary intralobular arteries to ET-1 involves activation of K+ channels is unknown. The possibility merits consideration in view of observations linking hypoxia-induced contraction of pulmonary vascular smooth muscle cells to a reduction in K+ currents, which may involve synergistic interactions with ET-1 (16, 25, 34).

The regulatory influence of HO-derived CO on hypoxia-induced pulmonary vasoconstriction is not limited to isolated vascular preparations. In this regard, our study in anesthetized rats documented that the elevation of pulmonary artery pressure caused by breathing a hypoxic gas mixture was intensified by pretreatment with CrMP to inhibit HO. That concurrent treatment with the ET-1 receptor antagonist L-745142 blunts the pulmonary pressor response to acute hypoxia in such animals suggests that endogenous CO minimizes the in vivo activity of an ET-1-dependent pulmonary pressor mechanism induced by acute hypoxic conditions.

In summary, this study demonstrates that pulmonary arterial vessels express HO-1 and HO-2 and manufacture HO. HO-derived CO interferes with the expression of hypoxia-induced pulmonary vasoconstriction both ex vivo and in vivo, in part by reducing vascular ET-1 levels and the sensitivity of the smooth muscle to the contracting action of the peptide. Hence, a heme-HO system intrinsic to the pulmonary vasculature modulates the constrictor response of pulmonary arterial vessels to acute hypoxia.

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