Role of endothelial carbon monoxide in attenuated vasoreactivity following chronic hypoxia

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Caudill, Timothy K., Thomas C. Resta, Nancy L. Kanagy, and Benjimen R. Walker. Role of endothelial carbon monoxide in attenuated vasoreactivity following chronic hypoxia. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1025–R1030, 1998.—Chronic hypoxic exposure has been previously demonstrated to attenuate systemic vasoconstrictor activity to a variety of agents. This attenuated responsiveness is observed not only in conscious animals but in isolated vascular preparations as well. Because hypoxia has been documented to increase heme oxygenase (HO) levels and the subsequent production of the vasodilator CO in vitro, we hypothesized that the blunted reactivity observed with chronic hypoxia (CH) may be in part due to increased HO activity. In thoracic aortic rings from CH rats, cumulative dose-response curves to phenylephrine (PE) in the presence of the nitric oxide (NO) synthase inhibitor N\(^{-}\)-nitro-L-arginine (L-NNA) and the HO inhibitor zinc protoporphyrin 9 (ZnPPIX) elicited increased contractility compared with CH rings treated with only L-NNA. Similar results were observed in rings incubated overnight with the HO-inducing agent sodium m-arsenite. In contrast, contractile responses in rings from control rats were unaffected by the HO inhibitor. Furthermore, endothelium-denuded rings from either control or CH rats did not exhibit an increase in reactivity to PE following ZnPPIX incubation. ZnPPIX had no effect on relaxant responses to the NO donor S-nitroso-N-penicillamine, suggesting that its actions were specific to HO inhibition. Finally, aortic rings exhibited dose-dependent relaxant responses to exogenous CO that were endothelium independent and blocked by an inhibitor of soluble guanylyl cyclase. The other products of HO enzyme activity, iron and biliverdin, were without effect on vasoreactivity. Thus we conclude that the attenuated vasoreactivity to PE following CH is likely to involve the induction of endothelial HO and the subsequent enhanced production of CO.

rat; thoracic aorta; heme oxygenase; nitric oxide synthase; endothelium

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contributes to CH-mediated attenuation of vasoactivity by adrenergic stimulation in this preparation.

METHODS

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine.

Experimental Groups

Male Sprague-Dawley rats (250–300 g; Harlan) were divided into CH and control groups. Rats in the CH group were housed in a hypobaric chamber maintained at 380 mmHg, whereas rats in the control group were housed at ambient barometric pressure (~630 mmHg). CH rats were acclimated to hypobaria for 4 wk before experimentation. The chamber was briefly opened three times per week to provide animals with clean bedding and fresh food and water.

Aortic Ring Preparation

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the descending thoracic aorta was isolated and placed in 5°C physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 0.4 NaH2PO4, 19 NaHCO3, 1.8 CaCl, and 5.5 glucose (all from Sigma) (pH 7.4). Once cleaned of adventitia, the aorta was cut into six to seven 3-mm segments. Endothelium was removed in some rings by gently rubbing the lumen with a clean forceps. Aortic rings were threaded between two 600-µm-diameter hooks and suspended in tissue baths (37°C) filled with 20 ml PSS. The top hook was connected to a Grass FT03 force-displacement transducer, and the bottom hook was anchored to an immovable support. Tissue baths were continuously bubbled and maintained transducer, and the bottom hook was anchored to an immovable support. Tissue baths were continuously bubbled with a 21%O2:5.5%CO2:balance N2 gas mixture. Rings were stretched to resting tension of 1.5 g and allowed to equilibrate for 60 min before experimentation.

Demonstration of Attenuated Vasoreactivity to Phenylephrine in Aortic Rings from CH Rats

After equilibration, endothelium-intact rings from control and CH rats were challenged with the α1-adrenoceptor agonist phenylephrine (PE; 1 µM). At the peak of contraction, endothelial integrity was verified by noting the relaxant response to ACh (10 µM). In endothelium-intact rings, at least 50% relaxation was necessary for inclusion in experiments. Relaxation in response to ACh averaged 97.06 ± 7.66% (control) and 91.04 ± 6.34% (CH). Rings were rinsed with PSS and returned to baseline tension, followed by generation of a concentration-response curve to PE (10⁻¹⁰ to 10⁻⁶ M).

Effect of HO Blockade on PE Responsiveness in Aortic Rings From Control and CH Rats

To determine whether endogenously produced CO contributes to reduced contractile responsiveness following CH, PE concentration-response curves were generated in aortic rings from each group of animals after treatment with the HO inhibitor ZnPPIX. All experiments were performed in the presence of the NOS inhibitor N-nitro-L-arginine (L-NNA; 100 µM) (7) to eliminate any confounding effects of NO. After initial contraction and endothelial test, a concentration-response curve to PE was generated as a further test of ring viability. Rings were next rinsed, followed by a 1-h incubation with either ZnPPIX (10 µM) + L-NNA (100 µM) or vehicle + L-NNA (100 µM). Due to the photosensitivity of protoporphyrin compounds, all rings in this and subsequent experiments were incubated in the dark, inside foil-wrapped baths. After the incubation period, a second concentration-response curve was generated.

Role of Endothelium in Generating CO in Aortic Rings from Control and CH Rats

Experiments were performed using endothelium-denuded vessels to determine the potential contribution of endothelium-derived CO in attenuated vasoactivity following CH. The experimental design was identical to the preceding protocol except that ZnPPIX and its vehicle were administered in the absence of L-NNA. In endothelium-denuded rings, relaxation in response to ACh averaged 2.04 ± 3.18% (control) and 1.87 ± 3.35% (CH).

Efficacy and Specificity of ZnPPIX Inhibition of HO Activity

Additional experiments were conducted to test the effectiveness and selectivity of ZnPPIX in this preparation.

Effectiveness of ZnPPIX in sodium m-arsenite-treated aortic rings. To establish the efficacy of ZnPPIX in blocking HO activity, endothelium-intact control aortic rings were incubated for 18 h in minimum essential medium (Sigma) containing either 12.5 µM sodium m-arsenite, an agent shown to enhance HO-1 gene expression (2), or its vehicle. After incubation, rings were treated for 1 h with either ZnPPIX (10 µM) + L-NNA (100 µM) or vehicle + L-NNA (100 µM) as above, and a PE concentration-response curve was generated.

Specificity of ZnPPIX. Whereas ZnPPIX is a potent inhibitor of HO activity, at higher concentrations it may also inhibit NOS and sGC activities (25). Because our experiments eliminate the contribution of NO by administration of an NOS inhibitor or by physical endothelial removal, any nonspecific actions of ZnPPIX on NOS activity are not of concern. However, to confirm that the actions of ZnPPIX were not due to inhibition of sGC, we performed additional experiments to evaluate the effects of the inhibitor on relaxation due to stimulation of sGC by the NO donor S-nitroso-N-acetylpenicillamine (SNAP). After incubation for 1 h with either ZnPPIX (10 µM; n = 5) or vehicle (n = 6), endothelium-intact aortic rings from control rats were contracted with 10⁻⁵ M PE and cumulative vasorelaxant responses to 10⁻⁶ to 10⁻⁴ M SNAP were determined.

Vasorelaxant Responses to HO Products

Experiments were performed to evaluate the ability of CO to elicit relaxation in PE-contracted aortic rings from control rats. Both endothelium-intact (n = 5) and -denuded (n = 4) aortic rings were contracted with 1 µM PE and then administered increasing volumes of PSS equilibrated with CO gas. The CO solution was prepared by bubbling CO gas into sealed, air-tight glass test tubes containing PSS for 3 min on ice. The saturated solution was drawn into Hamilton gas-tight glass syringes, and increasing volumes were added quickly to the PSS in the tissue baths containing the contracted arteries. CO relaxations reached a plateau in 2 min and then the next volume of saturated solution was added (10–2,500 µl to a 25-ml bath volume). Additional experiments examined responses of intact (n = 5) and denuded (n = 4) rings to CO in the presence of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µM). In addition to CO, the metabolism of heme by HO also produces biliverdin and iron. Thus experiments were performed to evaluate the ability of these products to reverse PE contraction in endothelium-intact aortic segments. After a stable contraction was established, increasing concentrations of
FeCl₂ (n = 3), FeCl₃ (n = 3), and biliverdin (n = 3) were added and the change in tension was recorded.

Solutions

All experimental drugs were prepared on the day of experimentation. PE and acetylcholine (Sigma) were dissolved in normal saline. L-NNA, sodium m-arsenate, FeCl₂, FeCl₃, and biliverdin (all from Sigma) were dissolved in water, whereas ZnPPIX (Porphyrin Products) was dissolved in normal saline containing 5 mM Na₂CO₃. SNAP (Cayman) was first dissolved in ethanol and diluted with water. ODQ (Calbiochem) was dissolved in DMSO and diluted with PSS. Final minimum essential medium contains 10.2 g α-MEM (Sigma), 0.02 M HEPES buffer (Sigma), 0.05 M NaHCO₃ (Sigma), and 2.4% penicillin-streptomycin (Life Technologies).

Calculations and Statistics

For contractile experiments, maximum tensions for PE were compared by either Student's t-test or a two-way ANOVA. If differences were detected by ANOVA, individual groups were compared using the Student-Newman-Keuls test. SigmaPlot (Jandel Scientific) was used to perform a nonlinear regression analysis on each individual ring experiment to calculate EC₅₀ values. Vasorelaxant responses for SNAP and CO were calculated as percent reversal of maximum PE-induced contraction. Percent data underwent arcsin transformation before statistical analysis. Results were compared by Student's t-test. Data are presented as means ± SE. Differences were considered significant if P < 0.05.

RESULTS

Demonstration of Attenuated Vasoreactivity in Aortic Rings From CH Rats

Figure 1 shows concentration-response curves to PE in endothelium-intact aortic rings from control (n = 7) and CH (n = 6) rats. As we have previously shown (6), aortic rings from CH rats exhibited attenuated contractile responses to pressor agents compared with control rings. However, EC₅₀ values did not differ between control (1.87 ± 0.53 x 10⁻⁷ M) and CH (2.37 ± 0.28 x 10⁻⁷ M) groups.

Effect of HO Blockade

Figure 2 shows PE concentration-response curves for aortic rings pretreated with either L-NNA and ZnPPIX or L-NNA and vehicle from both control (n = 7) and CH (n = 6) rats. Rings from both control and CH rats treated with L-NNA alone appeared to exhibit greater contractility than untreated rings in the previous protocol (Fig. 1), although these groups were not statistically compared because of differences in the protocol design. Interestingly, however, L-NNA-treated rings from CH rats still demonstrated reduced responsiveness to PE compared with similarly treated control rings, suggesting involvement of an NO-independent mechanism in the sustained alteration in reactivity following hypoxia. HO inhibition with ZnPPIX had no effect on contractile responses to PE in rings from control rats, and EC₅₀ values of concentration-response curves did not differ between L-NNA + ZnPPIX (5.39 ± 0.85 x 10⁻⁸ M) and L-NNA + vehicle (5.11 ± 0.76 x 10⁻⁸ M) groups. However, aortic rings from CH rats incubated with L-NNA and ZnPPIX exhibited increased contractility compared with those incubated with L-NNA and vehicle, suggesting that HO-derived CO plays a role in diminished vasoreactivity in rings from CH rats. EC₅₀ values of concentration-response curves did not differ between L-NNA + ZnPPIX (4.53 ± 0.45 x 10⁻⁸ M) and L-NNA + vehicle (5.63 ± 0.62 x 10⁻⁸ M) groups of aortic rings from CH rats.

Role of Endothelium in Generating CO

After incubation with either ZnPPIX or vehicle, endothelium-denuded aortic rings from control (n = 7) and CH (n = 6) rats showed no difference in vascular responsiveness to PE (Fig. 3). This finding suggests that HO-generated CO, which appears to play a role in diminished vascular responsiveness following CH, is produced by the endothelium. Neither the EC₅₀ values
of the concentration-response curves from control [2.92 ± 0.51 × 10⁻⁸ M (ZnPPIX) vs. 4.37 ± 0.77 × 10⁻⁸ M (vehicle)] nor CH [3.06 ± 0.59 × 10⁻⁸ M (ZnPPIX) vs. 3.49 ± 1.06 × 10⁻⁸ M (vehicle)] aortas were different.

Efficacy and Specificity of ZnPPIX Inhibition of HO Activity

Effectiveness of ZnPPIX in sodium m-arsenite-treated aortic rings. After incubation with 12.5 µM sodium m-arsenite, aortic rings treated with ZnPPIX and L-NNA (n = 6) exhibited significantly greater contractility to PE than rings treated with vehicle and L-NNA (n = 6) (Fig. 4A). EC₅₀ values did not differ between L-NNA + ZnPPIX (5.69 ± 0.94 × 10⁻⁸ M) and L-NNA + vehicle (5.72 ± 0.62 × 10⁻⁸ M) groups. In contrast, no differences in contractility were observed between groups treated with the vehicle for sodium m-arsenite (n = 6) (Fig. 4B). EC₅₀ values of concentration-response curves also did not differ in these control rings between L-NNA + ZnPPIX (6.18 ± 1.04 × 10⁻⁸ M) and L-NNA + vehicle (5.10 ± 0.89 × 10⁻⁸ M) groups. These data demonstrate the efficacy of ZnPPIX as an HO inhibitor in this preparation.

Specificity of ZnPPIX. Vasorelaxant responses to SNAP in control rings pretreated with either ZnPPIX (n = 5) or its vehicle (n = 6) are shown in Fig. 5. There were no differences between groups in vasorelaxant responses or in the amount of tension initially generated by PE (0.54 ± 0.12 vs. 0.53 ± 0.04 g/mg tissue, respectively). The data strongly suggest that 10 µM ZnPPIX does not affect sGC activity in this preparation.

Vasorelaxant Responses to HO Products

Administration of CO-equilibrated PSS to PE-contracted rings elicited similar dose-dependent relaxant responses in both endothelium-intact and -denuded aortic rings (Fig. 6). Both responses were completely eliminated in the presence of 10 µM of the sGC inhibitor ODQ (Fig. 6). In contrast, FeCl₂, FeCl₃, and biliverdin were without effect on PE contraction in endothelium-intact rings.

Fig. 3. Concentration-response curves to PE in endothelium-denuded aortic rings from control rats (n = 7) incubated with either L-NNA and ZnPPIX (●) or L-NNA and vehicle (○) and from CH rats (n = 6) incubated with either L-NNA and ZnPPIX (○) or L-NNA and vehicle (□). Data are means ± SE. There were no significant differences.

Fig. 4. A: concentration-response curves to PE in endothelium-intact aortic rings treated with 12.5 µM sodium m-arsenite (n = 6 rats). Curves following 1-h incubation with either L-NNA and ZnPPIX (●) or L-NNA and vehicle (○). Data are means ± SE. *P < 0.05 vs. L-NNA + vehicle. B: concentration-response curves to PE in endothelium-intact aortic rings treated with vehicle for arsenite (n = 6 rats). Curves following 1-h incubation with either L-NNA and ZnPPIX (●) or L-NNA and vehicle (○). Data are means ± SE. There are no significant differences.

Fig. 5. Vasorelaxant responses to S-nitroso-N-penicillamine (SNAP) in ZnPPIX (●) (n = 5)- and vehicle (○) (n = 6)-treated rings precontracted with 100 µM PE. Data are means ± SE. There were no differences between groups.
The major findings of the present study are 
1) the HO inhibitor ZnPPIX augments contractile responses to PE in L-NNA-treated aortic rings from CH rats but not controls, 
2) administration of ZnPPIX causes no change in contractility in endothelium-denuded aortic rings from either control or CH rats, 
3) 10 µM ZnPPIX does not affect NO-dependent vasorelaxation, and 
4) exogenous CO elicits vasorelaxation in control aortic rings that is blocked by an inhibitor of sGC. These data suggest that increased aortic endothelial HO activity contributes to the decreased vasoreactivity to PE following exposure to CH.

Recently, a hypoxia-responsive element has been identified in the mouse HO-1 gene (12), and the expression of hypoxia-inducible factor 1 has been implicated in mediating hypoxia-induced HO-1 expression. After a CH stimulus, Northern blotting has shown a fivefold increase in HO-1 mRNA levels in rat aorta (12) and a sevenfold increase in HO-1 mRNA levels in cultured rat aortic smooth muscle cells (16). These findings, coupled with data implicating a physiological role for CO in vascular control (11, 24), suggest that a portion of the altered vasoreactivity following CH may be linked to increased production of CO. Compared with NOS blockade alone, the increase in contractility to PE following combined NOS and HO blockade in endothelium-intact aortas from CH rats suggests that levels of HO enzyme and/or activity are increased in these vessels. However, inhibition of both NOS and HO returned contractile responses to levels still slightly below their corresponding controls, which may be interpreted as evidence for involvement of other factors such as alterations in smooth muscle sensitivity to vasoconstrictors or an increase in release of other vasodilatory compounds in the attenuation of reactivity following CH.

From our initial observations, it was unclear whether the apparent increase in HO activity following CH was localized to the endothelial or vascular smooth muscle layer. Previous studies have shown that inhibition of HO activity by another HO inhibitor, tin protoporphyrin 9, reverses the component of endothelium-dependent relaxation of porcine distal pulmonary arteries not reversed by an inhibitor of NOS (25). Thus CO, like NO, may contribute to endothelium-dependent vasorelaxation in the pulmonary circulation. Immunocytochemical localization of HO-1 expression in rat aorta after lipopolysaccharide (LPS) administration revealed increased staining in both the endothelial and vascular smooth muscle layers (24), and ZnPPIX caused a reduction of LPS-induced hypotension. In addition, HO-1 has been localized by immunological staining to the endothelial layer of the sheep ductus arteriosus (3). Our results suggest that the endothelium, but not vascular smooth muscle, produces physiologically relevant NO and CO in the rat aorta following CH. The observation that exogenous CO elicits endothelium-independent vasorelaxation in aortic rings suggests that the response to CO indeed resides within the smooth muscle and is not due to the secondary release of a separate endothelium-derived dilator. Furthermore, our findings confirm a primary role of activation of sGC in the vascular response to CO. We speculate that CO, like NO, acts in a paracrine manner to affect the underlying vascular smooth muscle.

Because ZnPPIX is not specific for either the HO-1 or HO-2 isozyme of HO, it is uncertain which isoform is being affected by the inhibitor in our study. HO-2 is abundant in the brain, testes, and endothelial lining of the aorta (25). This isoform is generally considered to be constitutive (25), although its expression can be induced by glucocorticoids (13). However, because hypoxia upregulates HO-1 (12), elevated expression of this form of the enzyme is more likely to be responsible for the effect on contractility observed in our experiments. Furthermore, HO-1 expression is also enhanced by mechanical forces such as increased cyclic strain or shear stress in cultured vascular smooth muscle cells (22), although it is unclear whether a similar response is observed in intact endothelium. Although cardiac output does not appear to change following CH (1, 23), polycythemia associated with this stimulus could cause
elevated shear stress on endothelial cells in the systemic vasculature, thereby potentially contributing to an increase in HO-1 expression. Nevertheless, the specific isoform responsible for the physiological actions documented in our study could not be determined because of the ineffectiveness of available antibodies to HO-1 and HO-2 in Western blot analysis in aortic tissue (3). In the absence of definitive data demonstrating upregulation of HO enzyme in the vasculature following CH, an alternative possibility is that the level of vascular heme substrate is elevated under these conditions, thereby affecting enzyme activity. However, the extensive data illustrating enhanced expression of HO-1 under conditions associated with CH suggest that enhanced gene expression is more likely responsible for the current findings.

In summary, the current study provides evidence that the previously observed attenuation of vasoconstrictor reactivity following prolonged hypoxic exposure is partially due to the likely enhanced release of CO from the vascular endothelium.

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