Endothelium-Derived Relaxing Factor From Pulmonary Artery and Vein Possesses Pharmacologic and Chemical Properties Identical to Those of Nitric Oxide Radical

Louis J. Ignarro, Russell E. Byrns, Georgette M. Buga, and Keith S. Wood

The objective of this study was to elucidate the close similarity in properties between endothelium-derived relaxing factor (EDRF) and nitric oxide radical (NO). Whenever possible, a comparison was also made between arterial and venous EDRF. In vascular relaxation experiments, acetylcholine and bradykinin were used as endothelium-dependent relaxants of isolated rings of bovine intrapulmonary artery and vein, respectively, and NO was used to relax endothelium-denuded rings. Oxyhemoglobin produced virtually identical concentration-dependent inhibitory effects on both endothelium-dependent and NO-elicited relaxation. Oxyhemoglobin and oxymyoglobin lowered cyclic guanosine monophosphate (cGMP) levels, increased tone in unrubbed artery and vein, and abolished the marked accumulation of vascular cGMP caused both by endothelium-dependent relaxants and by NO. The marked inhibitory effects of oxyhemoglobin on arterial and venous relaxant responses and cGMP accumulation as well as its contractile effects were abolished or reversed by carbon monoxide. These observations indicate that EDRF and NO possess identical properties in their interactions with oxyhemoproteins. Both EDRF from artery and vein and NO activated purified soluble guanylate cyclase by heme-dependent mechanisms, thereby revealing an additional similarity in heme interactions. Spectrophotometric analysis disclosed that the characteristic shift in the Soret peak for hemoglobin produced by NO was also produced by an endothelium-derived factor released from washed aortic endothelial cells by acetylcholine or A23187. Pyrogallol, via the action of superoxide anion, markedly inhibited the spectral shifts, relaxant effects, and cGMP accumulating actions produced by both EDRF and NO. Superoxide dismutase enhanced the relaxant and cGMP accumulating effects of both EDRF and NO. Thus, EDRF and NO are inactivated by superoxide in a closely similar manner. We conclude, therefore, that EDRF from artery and vein is either NO or a chemically related radical species. (Circulation Research 1987;61:866-879)
perimental, and chemical properties of EDRF and NO. The aims of this study were severalfold. A detailed comparison of the pattern of inhibitory effects of increasing concentrations of oxyhemoglobin on arterial and venous relaxation produced by EDRF and NO was made. Carbon monoxide (CO), NO, and oxygen all bind to a common heme site on hemoglobin and, therefore, have the potential of competition for a common binding site. Carboxyhemoglobin was recently reported to be slightly less active than hemoglobin in inhibiting EDRF-mediated arterial relaxation, but the instability of carboxyhemoglobin in oxygenated medium precluded meaningful interpretation of the data. In the present study, the effects of continuous delivery of CO into bathing media on relaxation and cGMP accumulation in response to arterial and venous EDRF and to NO were compared. As guanylate cyclase–bound heme is required for enzymatic activation by NO, it was of importance to determine whether a similar requirement was true for the direct activation of guanylate cyclase by EDRF. In view of the knowledge that NO reacts with the heme of hemoglobin to form the corresponding NO-heme adduct, a spectral analysis was made to ascertain whether EDRF released from washed aortic endothelial cells could react similarly with hemoglobin. Finally, the unstable nature of EDRF and NO were compared, and the effects of superoxide anion on relaxation, cGMP accumulation, and hemoglobin spectral shifts produced by both EDRF and NO were evaluated.

Materials and Methods
Preparation of Rings of Bovine Intrapulmonary Artery and Vein
Bovine lungs were obtained from cows 5 years of age or older and transported to the laboratory as described previously. Intrapulmonary arterial and venous branches were rapidly isolated; gently cleaned of parenchyma, fat, and adhering connective tissue; and placed in cold preoxygenated Krebs-bicarbonate solution. Segments of the 2nd arterial branch and underlying 2nd venous branch extending into the larger lobe were isolated. Outside diameters were 4–6 mm (artery) and 6–8 mm (vein). Vessel segments were sliced into rings (4 mm wide) using a specially designed microtome. Rings prepared in this manner possessed an intact or functional endothelium as assessed by 80–100% relaxation responses to 10–7–10–4 M acetylcholine (arteries) or 10–4 M bradykinin (veins). These vascular rings are referred to in the text as unrubbed. Endothelial cells were largely removed from vessel segments prior to slicing by gently passing a moistened stick from a cotton swab into the lumen and rubbing against the intima for 30–40 seconds without stretching the vessel walls. These endothelium-denuded rings sharply contracted in response to 10–4 M acetylcholine (arteries) or 10–8 M bradykinin (veins).

Mounting Rings and Recording of Muscle Tension
Arterial and venous rings were mounted by means of fine nichrome wires in jacketed, 25-ml-capacity, drop-away chambers containing Krebs-bicarbonate solution (37°C) gassed with 95% O2-5% CO2. The upper nichrome wire of each ring was attached to a force-displacement transducer (model FT03C, Grass Instrument Co., Quincy, Mass.), and changes in isometric force were recorded on a Grass polygraph (model 79D). Length-tension relations were determined initially for unrubbed and endothelium-denuded rings of artery and vein. Tension was adjusted to the optimal length for maximal isometric contractions to potassium by progressively stretching the rings and repeatedly obtaining contractile responses to 80 mM KC1, with washing and 15 minutes of equilibration between each contractile response. Arterial rings generally required a greater optimal tension than venous rings. Optimal tensions did not vary significantly as a function of intimal rubbing. The optimal tensions determined in these initial experiments were employed in all subsequent experiments. Optimal tensions and maximal contractile tensions developed in response to potassium chloride at optimal lengths, respectively, were 6 g and 20–24 g for arterial rings and 4 g and 18–22 g for venous rings.

Arterial and venous rings were routinely depolarized by addition of 120 mM KC1 following 2 hours of equilibration at optimal tension and were subsequently washed and allowed to equilibrate for 45 minutes prior to initiating any given protocol. This procedure increases and stabilizes any subsequent submaximal precontractile responses to phenylephrine and related contractile agents, presumably by loading the smooth muscle cells with calcium. This procedure has been employed routinely for bovine pulmonary vessels in this laboratory.

Phenylephrine, a selective α1-adrenergic receptor agonist, was employed to elicit precontractile responses in arterial rings whereas U46619, a thromboxane A2-mimetic eicosanoid, was used to precontract venous rings. The reason for using U46619 to contract veins is that contractile responses were highly reproducible and well maintained. In contrast, contractile responses of venous rings to phenylephrine varied in magnitude and were inconsistent. This was particularly evident with unrubbed venous rings and may be attributed to the presence of a very active EDRF that tends to override the contractile effects of phenylephrine but not potassium. In this regard, U46619 behaved in part like potassium. However, U46619 differed from potassium in that endothelium-dependent relaxant responses to bradykinin were fully evident in veins that were precontracted with U46619 but were markedly attenuated in veins that were precontracted with potassium. The mechanism by which U46619 mobilizes calcium and causes vascular smooth muscle contraction is unknown.

Determination of Cyclic Nucleotide Levels
cGMP and cyclic adenosine monophosphate (cAMP) determinations were made in arterial and venous rings that had been equilibrated under tension and depolarized with potassium chloride. Tone was
monitored until the time of freeze-clamping. The use of drop-away bath chambers, freeze-clamping of rings, preparation and extraction of tissues for cyclic nucleotide determinations, and radioimmunoassay procedures were described previously. Cyclic GMP and cAMP levels were determined in aliquots from the same ring extract. None of the test agents added to bath chambers interfered directly with antigen-antibody binding in the radioimmunoassay procedures. Recoveries of standard amounts of added cyclic nucleotides were determined periodically, and the values ranged from 92 to 104%. Therefore, no corrections for sample recoveries were made.

**Chemicals, Solutions, and Delivery of NO and CO**

Acetylcholine chloride, bradykinin triacetate, A23187, phenylephrine hydrochloride, potassium cyanide, methemoglobin, myoglobin, catalase, hemin, protoporphyrin IX, sodium dithionite, dithiothreitol, pyrogallol, superoxide dismutase (bovine liver), and Sephadex G-25 were obtained from Sigma Chemical Co., St. Louis, Mo. The crystalline, purified metalloporphyrins tin-, cobalt-, and nickel-protoporphyrin IX were obtained from Porphyrin Products, Logan, Utah. U46619 ([15S]-hydroxy-11α,9α-[epoxymetha-

Carbon monoxide (CO; 99%) was obtained from Matheson Gas and was delivered as the gas into bath chambers as follows. The CO gasline was connected to the main O₂-CO₂ gasline that supplies the tissue bath chambers through fritted glass disks located at the bottom of the chambers. Approximately equal volumes of CO and the O₂-CO₂ mixture were delivered. Delivery of CO into oxygenated Krebs-bicarbonate solution by this procedure did not alter the pH of the medium (pH 7.4). Failure to maintain adequate delivery of the O₂-CO₂ mixture, however, caused the pH of the medium to decline rapidly and the medium to turn cloudy.

Crystalline methemoglobin was used as supplied. Deoxyhemoglobin was prepared from methemoglobin by reduction with dithionite in deoxygenated Krebs-bicarbonate solution. Deoxymyoglobin was prepared from the commercially available myoglobin, which is approximately 75% metmyoglobin, by the same procedure. Oxyhemoglobin and oxymyoglobin solutions were prepared by slowly bubbling oxygen into the deoxyhemoprotein solutions for 20 minutes at 4°C. Cyanohemoglobin was prepared by dissolving methemoglobin in 10 mM triethanolamine-HCl, pH 7.4, containing a fiftyfold molar excess of KCN at 25°C. After 30 minutes, the excess KCN was removed by gel filtration chromatography using a 0.7 X 5 cm column of Sephadex G-25 that had been preequilibrated with 10 mM triethanolamine-HCl, pH 7.4, containing 0.1 M NaCl. The void volume was determined by precalibrating the column using blue dextran. The identification and concentration of cyanohemoglobin, as well as the verification of oxyhemoglobin and oxymygoblin, were determined by visible absorption spectroscopy.

Ferro-protoporphyrin IX (heme) was prepared from crystalline hemin by dissolving the hemin in 0.1 N NaOH containing 50 mM dithionite. Dilutions were made in 10 mM triethanolamine-HCl, pH 7.4, just before use. Tin-, cobalt-, and nickel-protoporphyrin IX were dissolved in 0.1 N NaOH, and dilutions were made in 50 mM triethanolamine-HCl, pH 7.6. All concentrations indicated in the text are expressed as final bath concentrations.

Krebs-bicarbonate solution consisted of (in mM): NaCl 118, KCl 4.7, CaCl₂ 1.5, NaHCO₃ 25, MgSO₄ 1.1, KH₂PO₄ 1.2, and glucose 5.6. Depolarizing potassium chloride solution had a composition similar to Krebs-bicarbonate solution except the sodium chloride was replaced with an equimolar concentration of potassium chloride. The salt solution in which the bovine lungs were transported from the slaughterhouse to the laboratory (30 minutes) had the following composition (in mM): Tris-HCl (pH 7.4) 23.8, NaCl 125, KCl 2.7, CaCl₂ 1.8, and glucose 11. Lungs were excised from animals and immediately submerged in ice-cold salt solution contained in a plastic bag. The bag was sealed, placed in a cooler packed with ice, and brought to the laboratory.

**Spectrophotometric Analyses**

Fresh bovine aortic endothelial cells were employed as the source of EDRF and were isolated by a
modification of the collagenase digestion procedure reported previously.24,25 Fresh segments of bovine thoracic aorta (30–40 cm in length) were rinsed with phosphate-buffered saline (pH 7.4) at 25°C and small arterial branches were ligated to reduce leakage. A solution of collagenase (protease free; 0.2% wt/vol in buffered saline) was added into the lumen of each aorta (sealed at one end) and maintained for 15–20 minutes with periodic agitation. The media were collected and the above procedure was repeated four times. Pooled media were centrifuged, and cell sediments were resuspended and washed twice in buffered saline and resuspended in un oxygenated Krebs-bicarbonate solution to yield a cell concentration of approximately 10^6 endothelial cells/ml. Cell suspensions were kept at 37°C for 1–3 hours before use.

A solution of 5 μM (0.325 mg/ml) deoxyhemoglobin in pH 7.4 buffered (25 mM Tris-HCl) Krebsbicarbonate solution was prepared and scanned at 25°C using an LKB Ultrospec II Model 4050 recording spectrophotometer. A sharp absorbance peak in the Soret region at 433 nm (OD = 0.6; E = 132/mM/cm) was consistently observed. NO was delivered by injection of 1 ml or 10 ml of purified NO gas into a quartz cuvette containing 4 ml of deoxyhemoglobin, and the solution was immediately scanned. A sharp absorbance peak at 406 nm (OD = 0.5; E = 120/mM/cm) was observed when excess NO (10 ml) was reacted with the deoxyhemoglobin. An aliquot of aortic endothelial cells (1 ml containing either 2 × 10^6 cells or 10^6 cells) was added to 3 ml of 6.25 mM deoxyhemoglobin in the absence or presence of added test agents, and the suspension was mixed gently at 25°C for 90 seconds. The cell-free liquid component was aspirated into a Pasteur pipette fitted at the tip with a small piece of Nytex nylon cloth, which served to exclude the cells. The cell-free solution was transferred to a cuvette and immediately scanned.

**Purification and Assay of Bovine Lung Soluble Guanylate Cyclase**

Soluble guanylate cyclase from bovine lung was purified to apparent homogeneity in either the hemeccontaining or heme-deficient form exactly as described previously.25 Before use of enzyme in each experiment, enzyme fractions were subjected to gel filtration chromatography to remove dithiothreitol and glycerol, both of which inactivate EDRF.3 Guanylate cyclase activity was determined by measuring the formation of [3H]cGMP from α-[3H]GTP as described.4 The effects of EDRF released from isolated rings of bovine intrapulmonary artery or vein into reaction mixtures containing guanylate cyclase were assessed as recently described.5

**Calculations and Statistical Analysis**

Relaxation was measured as the decrease in tension below the elevated tension elicited by precontracting arterial or venous smooth muscle with phenylephrine or U46619, respectively, as indicated. All values in the text are expressed as the mean ± SEM and represent unpaired data. Comparisons were made using either the Duncan's multiple range test where comparisons with a common control were made (Figure 5) or the Student's t-test for unpaired values for all other comparisons. The level of statistically significant difference was p<0.05.

**Results**

**Characteristics of Endothelium-Dependent and NO-Elicited Relaxation and cGMP Accumulation in Artery and Vein**

Acetylcholine and bradykinin were employed as endothelium-dependent relaxants of isolated rings of bovine intrapulmonary artery and vein, respectively. Acetylcholine relaxes phenylephrine-precontracted arterial rings and causes a concomitant increase in tissue levels of cGMP but not cAMP, whereas venous rings are further contracted by acetylcholine.10 Therefore, endothelium-dependent relaxation of veins was assessed by addition of bradykinin, which relaxes precontracted venous rings and causes tissue accumulation of cGMP but not cAMP.2 Bradykinin was not used to relax arteries because bradykinin stimulates both cGMP and cAMP formation in bovine pulmonary artery, and EDRF appears to relax vascular smooth muscle through an action involving only cGMP.3 NO was used to elicit endothelium-independent relaxation of both artery and vein. Endothelium-denuded rings were used to eliminate any possible influence of endothelium on intrinsic tone, cGMP levels, and relaxant responses to NO. Acetylcholine and bradykinin elicited maximal relaxant responses at 10^-8 M and 10^-7 M, respectively. Higher concentrations often caused contractile responses because of a direct action on the underlying vascular smooth muscle, and were therefore avoided. The limit of solubility of NO in water as well as the procedure used to deliver NO into bath chambers precluded the testing of bath concentrations in excess of 10^-6 M.

**Effects of Hemoproteins and Metalloporphyrins on Endothelium-Dependent and NO-Elicited Relaxation of Artery and Vein**

Oxyhemoglobin produced a concentration-dependent, rightward shift of the concentration-relaxation curves to acetylcholine, bradykinin, and NO (Figures 1 and 2). Lower concentrations of oxyhemoglobin (below 3 × 10^-8 M) produced a parallel shift of the concentration-relaxation curves without depressing maximal responses, while higher concentrations markedly depressed maximal responses. Oxyhemoglobin produced inhibitory effects that were very similar to those of oxyhemoglobin (not shown). The deoxy-reduced forms of hemoglobin and myoglobin, which presumably become oxygenated in the tissue bathing medium, also produced inhibitory effects, but preliminary experiments showed that such hemoproteins were about tenfold less potent than their preoxygenated reduced forms. Therefore, the deoxy-reduced hemoproteins were converted to the oxy forms just prior to use. Ferro-protoporphyrin IX (heme) partially antag-
Influence of increasing concentrations of oxyhemoglobin on endothelium-dependent and NO-elicited relaxation of artery. Unrubbed (+ endothelium) and endothelium-denuded (-endothelium) arterial rings were mounted under 6 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, oxyhemoglobin was added at a final concentration of $3 \times 10^{-9}$ M (a), $10^{-8}$ M (b), $3 \times 10^{-8}$ M (c), $10^{-7}$ M (d), or $10^{-6}$ M (e) for 10 minutes. Control rings (•) received no oxyhemoglobin. Phenylephrine was then added to rings at concentrations that elicited contractile responses equivalent to 65-75% of maximal. At peak contractions, cumulative additions of acetylcholine or NO were made as indicated. Values represent the mean ± SEM using 15 rings from 5 animals (3 rings per animal).

Reversal by CO of Inhibitory Effects of Oxyhemoglobin on Endothelium-Dependent and NO-Elicited Relaxation of Artery and Vein

The inhibitory effect of hemoproteins on NO-elicited vascular smooth muscle relaxation is attributed to the direct binding of NO to the hemoprotein in the bathing medium because the affinity of hemoglobin and myoglobin for NO is very high. The close similarity between the inhibitory effect of oxyhemoproteins on relaxant responses to NO and to acetylcholine or bradykinin suggests that EDRF and NO possess similar properties with respect to binding to hemoproteins.

CO, which displays a very high binding affinity for hemoglobin or oxyhemoglobin, was tested to determine whether the inhibitory action of hemoproteins on relaxant responses to NO, acetylcholine, and brady-
kinin could be similarly reversed or blocked. Oxyhemoproteins were added to bathing media, and CO was immediately delivered for the duration of a given experimental protocol. Figures 3 and 4 illustrate that continuous CO delivery markedly attenuated the inhibitory effects of oxyhemoglobin on both endothelium-dependent relaxant responses of artery and vein to acetylcholine and bradykinin, respectively, and endothelium-independent relaxant responses to NO. CO also abolished the capacity of oxyhemoglobin to enhance contractile responses in unrubbed rings of artery and vein. In the absence of added hemoprotein, CO failed to alter smooth muscle tone, contractile responses, or relaxant responses. Cyanohemoglobin was synthesized and tested because this form of hemoglobin, with its fifth coordinate ligand occupied, cannot bind to O₂, NO, or CO. Cyanohemoglobin (10⁻⁸ M–10⁻⁶ M) failed to alter endothelium-dependent or endothelium-independent relaxation of artery and vein (not shown). These observations suggest that EDRF binds to and is sequestered by hemoglobin in a manner that is similar to that for NO.

Relation Between cGMP Accumulation and Relaxation in Absence and Presence of Hemoproteins

Tables 1 and 2 illustrate the influence of oxyhemoproteins, cyanohemoglobin, and CO on arterial and venous levels of cGMP in control rings and after addition of acetylcholine, bradykinin, or NO. cGMP levels were consistently about threefold higher in

![Figure 3](image_url)  
**Figure 3.** Reversal by CO of oxyhemoglobin blockade of endothelium-dependent and NO-elicited relaxation of artery. Unrubbed (+ endothelium) and endothelium-denuded (-endothelium) arterial rings were mounted under 6 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, 10⁻⁷ M (▲, △) or 10⁻⁶ M (●, ○) oxyhemoglobin was added for 10 minutes. CO was delivered (▲, ●) as a fine stream of bubbles and was started at the time of addition of oxyhemoglobin. Control rings (●) received neither oxyhemoglobin nor CO. Phenylephrine was then added to rings at concentrations that elicited contractile responses equivalent to 65–75% of maximal. At peak contractions, cumulative additions of acetylcholine or NO were made as indicated. Values represent the mean ± SEM using 24 rings from 4 animals (6 rings per animal).

![Figure 4](image_url)  
**Figure 4.** Reversal by CO of oxyhemoglobin blockade of endothelium-dependent and NO-elicited relaxation of vein. Unrubbed (+ endothelium) and endothelium-denuded (-endothelium) venous rings were mounted under 4 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, 10⁻⁷ M (▲, △) or 10⁻⁶ M (●, ○) oxyhemoglobin was added for 10 minutes. CO was delivered (▲, ●) as a fine stream of bubbles and was started at the time of addition of oxyhemoglobin. Control rings (●) received neither oxyhemoglobin nor CO. U46619 was then added to rings at concentrations that elicited contractile responses equivalent to 65–75% of maximal. At peak contractions, cumulative additions of bradykinin or NO were made as indicated. Values represent the mean ± SEM using 24 rings from 4 animals (6 rings per animal).
unrubbed than in endothelium-denuded arterial and venous rings. Acetylcholine and NO, each at $10^{-6}$ M, caused a tenfold and twentyfold increase in cGMP levels in unrubbed and endothelium-denuded artery, respectively, after 60 seconds (Table 1). Bradykinin (10$^{-8}$ M) and NO (10$^{-6}$ M) caused a sevenfold and tenfold increase in cGMP levels in unrubbed and endothelium-denuded vein, respectively, after 60 seconds (Table 2). Oxyhemoglobin and oxymyoglobin, but not cyanohemoglobin, lowered the intrinsic cGMP levels in unrubbed and endothelium-denuded vein, respectively, after 60 seconds (Table 2). Bradykinin, NO, and oxyhemoglobin, but not cyanohemoglobin, lowered the intrinsic cGMP levels in unrubbed artery and vein to values that approximated those of endothelium-denuded rings. CO markedly reversed the inhibitory effect of oxyhemoglobin and restored cGMP accumulation back toward the normal range. None of the hemoproteins tested significantly altered vascular cAMP levels (not shown).

### Table 1. Influence of Hemoproteins and CO on Arterial Levels of cGMP in the Absence and Presence of Acetylcholine or NO

<table>
<thead>
<tr>
<th>Additions</th>
<th>Controls +E</th>
<th>−E</th>
<th>ACh (10$^{-6}$ M) +E</th>
<th>−E</th>
<th>NO (10$^{-6}$ M) +E</th>
<th>−E</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>37 ± 4.6</td>
<td>11 ± 2.3</td>
<td>362 ± 45</td>
<td>230 ± 37</td>
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<td></td>
</tr>
<tr>
<td>Oxyhemoglobin, 10$^{-6}$ M</td>
<td>9.2 ± 2.1*</td>
<td>10 ± 1.8</td>
<td>21 ± 1.7*</td>
<td>12 ± 1.8*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxymyoglobin, 4 × 10$^{-6}$ M</td>
<td>13 ± 2.7*</td>
<td>10 ± 2.6</td>
<td>26 ± 3.3*</td>
<td>15 ± 2.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanohemoglobin, 10$^{-6}$ M</td>
<td>40 ± 3.9</td>
<td>13 ± 2.5</td>
<td>376 ± 48</td>
<td>271 ± 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyhemoglobin, 10$^{-6}$ M plus CO</td>
<td>31 ± 4.1</td>
<td>14 ± 1.8</td>
<td>296 ± 38</td>
<td>217 ± 25</td>
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</tr>
</tbody>
</table>

Unrubbed (+E) and endothelium-denuded (−E) arterial rings were mounted under 6 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, hemoproteins were added for 10 minutes as indicated. Phenylephrine was then added to all rings at concentrations that elicited contractile responses equivalent to 65–75% of maximal. Carbon monoxide (CO) was delivered as a fine stream of bubbles and was started at the time of addition of oxyhemoglobin. Control rings were quick-frozen 60 seconds later. Values represent the mean ± SEM using 9 to 12 rings isolated from 3 or 4 animals (3 rings per animal). cGMP, cyclic guanosine monophosphate.

*Significantly different (p<0.01) from corresponding values obtained in the absence of added hemoprotein (None).

### Table 2. Influence of Hemoproteins and CO on Venous Levels of cGMP in the Absence and Presence of Bradykinin or NO

<table>
<thead>
<tr>
<th>Additions</th>
<th>Controls +E</th>
<th>−E</th>
<th>BKN (10$^{-6}$ M) +E</th>
<th>−E</th>
<th>NO (10$^{-6}$ M) +E</th>
<th>−E</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>40 ± 5.3</td>
<td>12 ± 1.6</td>
<td>276 ± 38</td>
<td>262 ± 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyhemoglobin, 10$^{-6}$ M</td>
<td>12 ± 1.1*</td>
<td>10 ± 1.2</td>
<td>26 ± 2.1*</td>
<td>19 ± 1.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxymyoglobin, 4 × 10$^{-6}$ M</td>
<td>18 ± 2.5*</td>
<td>12 ± 1.2</td>
<td>24 ± 1.8*</td>
<td>22 ± 1.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanohemoglobin, 10$^{-6}$ M</td>
<td>40 ± 3.7</td>
<td>14 ± 1.4</td>
<td>241 ± 22</td>
<td>251 ± 36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyhemoglobin, 10$^{-6}$ M plus CO</td>
<td>33 ± 4.1</td>
<td>14 ± 1.9</td>
<td>301 ± 26</td>
<td>232 ± 34</td>
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</tbody>
</table>

Unrubbed (+E) and endothelium-denuded (−E) venous rings were mounted under 4 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, hemoproteins were added for 10 minutes as indicated. U46619 was then added to all rings at concentrations that elicited contractile responses equivalent to 65–75% of maximal. CO was delivered as a fine stream of bubbles and was started at the time of addition of oxyhemoglobin. Control rings were quick-frozen 60 seconds later. Values represent the mean ± SEM using 9 to 12 rings isolated from 3 or 4 animals (3 rings per animal). cGMP, cyclic guanosine monophosphate.

*Significantly different (p<0.01) from corresponding values obtained in the absence of added hemoprotein (None).
Arterial Relaxation and cGMP Accumulation in Ignarro et al. Vascular EDRF and Nitric Oxide Radical

Responses to acetylcholine or bradykinin. After rinsing and equilibration, rings were added to guanylate cyclase reaction mixtures in the presence or absence of added acetylcholine, bradykinin, NO, and methylene blue. Endothelium-intact, but not denuded, rings caused a twofold to threefold activation of hemecontaining guanylate cyclase that was markedly enhanced by acetylcholine or bradykinin and abolished by methylene blue (Table 3). Heme-deficient enzyme was not activated significantly by rings in the absence or presence of acetylcholine or bradykinin. NO caused a direct heme-dependent activation of guanylate cyclase that was independent of the presence of vascular rings and was inhibited by methylene blue. Oxyhemoglobin (1 μM) was also tested only in the presence of arterial rings and was found to produce effects on acetylcholine- and NO-elicited enzyme activation that were very similar to the effects of methylene blue.

Effects of Pyrogallol and Superoxide Dismutase on Arterial Relaxation and cGMP Accumulation in Response to Acetylcholine and NO

Pyrogallol, which generates superoxide anion in oxygenated medium,38 and superoxide dismutase were recently reported to inactivate and stabilize, respectively, EDRF released from cultured aortic endothelial cells.39 In the present study using isolated arterial rings, pyrogallol was found to inhibit markedly the relaxant and cGMP accumulating effects of acetylcholine in unrubbed arterial rings and of NO in endothelium-denuded arterial rings (Figure 5). In contrast, superoxide dismutase enhanced these vascular responses and also caused a small degree of endothelium-dependent relaxation and cGMP accumulation when tested alone (Figure 5). In these experiments, concentrations of acetylcholine and NO were selected that produced submaximal vascular responses. The principal action of superoxide dismutase is to inactivate or scavenge superoxide radical. High concentrations of hydroxyl radical scavengers and spin trap reagents, including mannitol, tyrosine, tryptophan, dimethyl pyrroline N-oxide, and several substituted phenyl- and butyl-nitrones, were tested and found to produce no appreciable effects on the vascular responses to acetylcholine, bradykinin, or NO (not shown).

Comparison of Reactivity of Hemoglobin with NO and EDRF

Hemoglobin reacts characteristically with NO to form nitrosyl- or NO-hemoglobin, which can be monitored spectrophotometrically. The addition of excess NO (10 ml) to a dilute solution of deoxyhemoglobin in pH 7.4 buffered Krebs-bicarbonate caused a complete shift in the Soret absorption peak (Figure 6) from 433 nm (deoxyhemoglobin) to 406 nm (NO-hemoglobin). Addition of a less than excess amount of

Table 3. Heme-Dependent Activation of Purified Soluble Guanylate Cyclase by EDRF and NO

<table>
<thead>
<tr>
<th>Additions</th>
<th>Artery</th>
<th>Vein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-E</td>
<td>+E</td>
</tr>
<tr>
<td></td>
<td>Heme-containing guanylate cyclase</td>
<td>Heme-deficient guanylate cyclase</td>
</tr>
<tr>
<td>None</td>
<td>32 ± 2.1</td>
<td>84 ± 6.4*</td>
</tr>
<tr>
<td>Acetylcholine, 10^-6 M</td>
<td>30 ± 1.7</td>
<td>442 ± 28*†</td>
</tr>
<tr>
<td>+ Meth. blue, 10^-4 M</td>
<td>26 ± 2.5</td>
<td>34 ± 3.3†</td>
</tr>
<tr>
<td>Bradykinin, 10^-7 M</td>
<td>41 ± 5.2</td>
<td>43 ± 3.2†</td>
</tr>
<tr>
<td>+ Meth. blue, 10^-4 M</td>
<td>633 ± 45†</td>
<td>690 ± 54†</td>
</tr>
<tr>
<td>NO, 10^-7 M</td>
<td>94 ± 9.7</td>
<td>87 ± 8.8</td>
</tr>
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Unrubbed (+ E) and endothelium-denuded (−E) arterial and venous rings were mounted in bath chambers, equilibrated under tension, and tested for endothelial integrity by determining relaxant responses to acetylcholine (artery) or bradykinin (vein). After rinsing and equilibration, rings were added to enzyme reaction mixtures (0.4 ml) containing 40 mM triethanolamine HCl, pH 7.4, 0.1 mM GTP, 2 mM MgCl₂, 0.3 mM CaCl₂, 0.1 mM EDTA, 4 μg bovine serum albumin, 10 mg glucose, 40 mM NaCl, 0.1 mM Na₂NO₃ purified heme-containing or heme-deficient guanylate cyclase, and the agents indicated under Additions. Reaction mixtures excluding enzyme were preincubulated at 4°C with 95% O₂-5% CO₂ for 10 minutes, warmed to 37°C, and enzyme was added just prior to insertion of rings. Reaction mixtures were incubated at 37°C for 10 minutes following addition of rings. Basal or unstimulated enzyme activities were 30 ± 2.3 and 62 ± 6.7 nmol cGMP/min/mg for heme-containing and heme-deficient guanylate cyclase, respectively. Values represent the mean ± SEM using 6 rings isolated from 3 animals (2 rings per animal). Meth. blue signifies methylene blue; EDRF, endothelium-derived relaxing factor.

*Significantly different (p<0.01) from corresponding values obtained for -E.
†Significantly different (p<0.01) from corresponding values obtained for None.
FIGURE 5. Influence of pyrogallol and superoxide dismutase on endothelium-dependent and NO-elicited arterial relaxation and cGMP accumulation. Unrubbed (+ endothelium) and endothelium-denuded (− endothelium) arterial rings were mounted under 6 g of tension, allowed to equilibrate for 2 hours, and depolarized with potassium chloride. After washing and 45 minutes of equilibration, arterial rings were precontracted by phenylephrine to 65–75% of maximal, and 10−4 M pyrogallol (Pyr) or 100 units/ml of superoxide dismutase (SOD) was added for 60 seconds where indicated. Acetylcholine (10−7 M) or NO (10−7 M) was then added for 60 seconds where indicated. Rings were quick-frozen at 60 seconds after the additions indicated above. Control rings (C) were quick-frozen at the time of peak contraction to phenylephrine or 60 seconds after addition of acetylcholine or NO as indicated. Values represent the mean ± SEM using 12 rings isolated from 3 animals (4 rings per animal). * Significantly different (p<0.01) from corresponding control values.

Discussion

The marked inhibitory effect of various hemoproteins on coronary arterial relaxation elicited by NO was first reported in 1979. Hemoproteins antagonized the effects of NO by binding to and sequestering the NO in the extracellular compartment and thereby preventing entry of NO into the smooth muscle cells. In that study, hemoproteins showed little or no antagonism of the relaxant effects of nitroso compounds or nitroglycerin because the latter vasodilators first penetrate smooth muscle cells and subsequently release NO, which produces cGMP accumulation and relaxation. Hemoproteins are unable to penetrate cells and, therefore, bind NO only in the extracellular compartment. More recently, Martin and coworkers demonstrated that reduced hemoproteins antagonized the relaxant and cGMP accumulating effects of endothelium-derived relaxing factor (EDRF).

NO (1 ml) resulted in only a partial spectral shift to 406 nm, with a distinct absorption peak remaining at 433 nm. Because the observations made in this study suggest that the properties of EDRF closely resemble those of NO, an attempt was made to conduct an analogous reaction between EDRF and hemoglobin. Freshly washed, bovine aortic endothelial cells were employed to generate sufficient amounts of EDRF for subsequent reaction with hemoglobin. In the presence of acetylcholine, the addition of endothelial cells to deoxyhemoglobin solutions caused a partial shift in the Soret peak from 433 to 406 nm (Figure 6). The magnitude of the spectral shift was dependent on the number of cells and the presence of acetylcholine and probably, therefore, the amount of EDRF released for reaction with hemoglobin. The calcium ionophore A23187, a potent endothelium-dependent relaxant, also produced a concentration-dependent shift in the Soret absorption peak of hemoglobin in the presence of endothelial cells (Figure 7). Unlike 10 μM acetylcholine, 1 μM A23187 produced a complete shift of the Soret peak. Neither cells alone nor acetylcholine or A23187 alone altered the spectral properties of hemoglobin (Figures 6 and 7). The knowledge that pyrogallol inactivates EDRF prompted an analysis of the influence of pyrogallol on the hemoglobin spectral shifts produced by A23187 and NO. Pyrogallol nearly abolished the characteristic spectral shift produced by both A23187 and NO (Figure 8). Neither superoxide dismutase nor the oxygen radical scavengers and spin trap reagents discussed above could be tested in the spectrophotometric assays because of their marked interference with absorbance at the wavelengths used.
additional arterial relaxants. Oxidized hemoproteins were partially inhibitory only at higher concentrations. These investigators postulated that hemoproteins inhibited endothelium-dependent relaxation by binding to, and thereby interfering with, the relaxant action of arterial EDRF. Very few studies have been published that address endothelium-dependent venous smooth muscle relaxation, and the influence of hemoproteins on venous tone has not been described. Moreover, a direct comparison between EDRF and NO in the same vessel with respect to the effects of hemoproteins has not been previously made.

The results of the present study illustrate the close similarity between endothelium-dependent arterial and venous smooth muscle relaxants and NO with respect to their actions on isolated rings of bovine intrapulmonary artery and vein. Oxyhemoglobin and oxymyoglobin antagonized the smooth muscle relaxant and cGMP accumulating effects of acetylcholine, bradykinin, and NO in a nearly identical manner, as indicated by the remarkably similar rightward shifts of concentration-relaxation curves in the presence of increasing concentrations of oxyhemoproteins. The inhibitory actions of oxyhemoproteins on both smooth muscle relaxation and cGMP accumulation were similarly and markedly inhibited or reversed by excess carbon monoxide, presumably because of the continuous formation of carboxyhemoproteins, which have a lower affinity than oxyhemoproteins for NO and perhaps EDRF. Although a previous study indicated that preformed carboxyhemoglobin was slightly less effective than hemoglobin as an inhibitor of endothelium-dependent arterial relaxation, the transient nature of this effect, due to the instability of carboxyhemoglobin in oxygenated medium, precluded a more in-depth study.19 In the present study, the continuous delivery of carbon monoxide along with oxygen and carbon dioxide into the bathing media produced consistent reversal of the inhibitory effects of oxyhemoproteins. These observations suggest that EDRF and NO bind to a common site on hemoglobin and myoglobin. This view is consistent with the findings that not only NO but also EDRF from both artery and vein required binding to the heme of purified soluble guanylate cyclase to cause enzyme activation. More direct evidence for an interaction between EDRF and heme derived from spectral studies, which revealed that EDRF reacted with hemoglobin to generate a hemoprotein complex that was indistinguishable from the complex formed in the reaction of hemoglobin with NO under identical conditions. Other similarities between EDRF and NO were their chemical instability in oxygenated media, further inactivation by superoxide anion, and stabilization by superoxide dismutase. Collectively, these observations suggest that EDRF is chemically either NO or some closely related radical species.

The very high affinity of the reduced forms of hemoglobin, myoglobin, and iron-protoporphyrin IX for NO is well known.41 These substances react readily with NO to form the corresponding NO- or nitrosyl-heme complexes. The oxidized forms of these substances also bind NO but higher concentrations of NO are required to first generate the reduced heme iron...
species. Oxyhemoglobin and oxymyoglobin bind NO just as avidly as their deoxy-reduced forms because the affinity of these hemoproteins for NO exceeds by several orders of magnitude their affinity for oxygen. Protoporphyrin IX, which lacks iron, cyanohemoglobin, and certain noniron metalloporphyrins such as tin-, cobalt-, or nickel-protoporphyrin IX are unable to bind appreciable amounts of either NO or oxygen. The present observations on the effects of hemoproteins, metalloporphyrins, and protoporphyrin IX on the responses of arterial and venous ring preparations to NO are highly consistent with the known properties of these substances. In addition, hemoproteins are known to inhibit the activating effect of NO on soluble guanylate cyclase. The effects of the various forms of hemoproteins, metalloporphyrins, and protoporphyrin IX on vascular responses to endothelium-dependent vasodilators were virtually identical to their effects on responses to NO, thus indicating the close similarity between EDRF and NO. The present observations represent a confirmation and extension of those of Martin et al., who reported that reduced hemoproteins inhibited endothelium-dependent arterial relaxation. The latter investigators, however, did not study venous relaxant responses, the relaxant effects of NO, or the effects of noniron metalloporphyrins. The present findings also support those of Griffith et al., who conducted perfusion-superfusion experiments and suggested that hemoglobin inhibits acetylcholine-elicited relaxation by combining with and inactivating EDRF. Recent studies from this laboratory showed that an endothelium-derived substance from artery and vein activated heme-containing guanylate cyclase and that enzyme activation was abolished by the guanylate cyclase inhibitor methylene blue. The present study reveals that such enzyme activation was homedependent. Activation of soluble guanylate cyclase by NO is now well recognized to be heme-dependent. The reason for this is that the activating species is NO-heme, a paramagnetic complex that alters the molecular configuration of guanylate cyclase and thereby facilitates the catalytic conversion of GTP to cGMP. The present observation that guanylate cyclase-bound heme was required for enzyme activation by EDRF provides strong evidence for the closely similar properties of EDRF and NO. Moreover, excess heme in the form of hemoglobin abolished the actions of both EDRF and NO.

Recent studies have suggested that EDRF can be inactivated by oxygen radicals such as superoxide anion. Pyrogallol, which generates superoxide in oxygenated medium, was shown to inactivate EDRF, whereas superoxide dismutase protected EDRF against inactivation. In the present study, pyrogallol inhibited both endothelium-dependent arterial relax
ation and cGMP accumulation in response to acetylcholine. In addition, pyrogallol similarly inhibited endothelium-independent arterial responses to NO. Moreover, superoxide dismutase enhanced both arterial responses to acetylcholine and NO in a similar manner. These observations indicate that EDRF and NO possess virtually identical properties of chemical and pharmacologic responses to acetylcholine and NO in a similar manner. Indeed, the present data suggest that EDRF is a radical species chemically related to NO radical.

In conclusion, the present study provides indirect pharmacologic and direct chemical evidence that EDRF from both intrapulmonary artery and vein very closely resembles NO radical. The evidence is that both EDRF and NO 1) require guanylate cyclase-bound heme for enzyme activation, 2) bind to a common site on hemoglobin and myoglobin, 3) are inactivated by superoxide anion and protected by superoxide dismutase, and 4) react with hemoglobin to form the same heme adduct, which appears to be nitrosyl-hemoglobin. Procedures for the isolation and additional chemical identification of NO, which is a highly unstable substance, could be difficult to perform by conventional techniques. In addition, the question of the formation or source of such a species in vascular tissue must be addressed. A recent report indicates that certain oxides of nitrogen such as nitrate are synthesized independently of microflora in the germfree rat. Although nitrate synthesis appears, therefore, to be a mammalian process, it is unknown whether nitrite formation can likewise occur. Assuming that nitrite formation by mammalian cells can occur, the conversion of nitrite to NO should proceed quite readily in muscle cells because of the presence of myoglobin. Nitrite reacts with myoglobin or oxymyoglobin to generate NO-myoglobin by either nonenzymatic or enzymatic mechanisms. Reducing agents such as ascorbate, monothiols such as cysteine, and reduced iron all accelerate or enhance the formation of NO-myoglobin. Thiols can accelerate the denitration of organic nitrate esters such as nitroglycerin to yield nitrite, and the nitrite would be available for reaction with myoglobin. NO-myoglobin complexes readily dissociate with the release of NO. Considering our present state of knowledge on EDRF, the above metabolic pathway would suppose that vascular endothelial cells generate an unstable, lipophilic organic nitrite or nitrate precursor that readily traverses membrane barriers and reacts with myoglobin to generate EDRF or NO. The presently available information on EDRF does not rule out the possibility of its formation within smooth muscle cells after the release of an endogenous nitrite or nitrate precursor from endothelial cells. Alternatively, the NO may be generated within the endothelial cells by presently unknown mechanisms. Regardless of its site of formation, if EDRF is indeed NO, the metabolic pathways for its formation in vascular tissue will have to be elucidated.

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**KEY WORDS** • endothelium • relaxation • cyclic GMP • nitrite • vasodilation • EDRF • nitric oxide
Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical.

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