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# Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide

**(endothelium-dependent relaxation/vascular smooth muscle/cyclic GMP)**

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**ABSTRACT** **The objective of this study was to determine whether nitric oxide (NO) is responsible for the vascular smooth muscle relaxation elicited by endothelium-derived relaxing factor (EDRF). EDRF is an unstable humoral substance released from artery and vein that mediates the action of endothelium-dependent vasodilators. NO is an unstable endothelium-independent vasodilator that is released from vasodilator drugs such as nitroprusside and glyceryl trinitrate. We have repeatedly observed that the actions of NO on vascular smooth muscle closely resemble those of EDRF. In the present study the vascular effects of EDRF released from perfused bovine intrapulmonary artery and vein were compared with the effects of NO delivered by superfusion over endothelium-denuded arterial and venous strips arranged in a cascade. EDRF was indistinguishable from NO in that both were labile ($t_{1/2}$ = 3–5 sec), inactivated by pyrogallol or superoxide anion, stabilized by superoxide dismutase, and inhibited by oxyhemoglobin or potassium. Both EDRF and NO produced comparable increases in cyclic GMP accumulation in artery and vein, and this cyclic GMP accumulation was inhibited by pyrogallol, oxyhemoglobin, potassium, and methylene blue. EDRF was identified chemically as NO, or a labile nitroso species, by two procedures. First, like NO, EDRF released from freshly isolated aortic endothelial cells reacted with hemoglobin to yield nitrosylhemoglobin. Second, EDRF and NO each similarly promoted the diazotization of sulfanilic acid and yielded the same reaction product after coupling with *N*-(1-naphthyl)-ethylenediamine. Thus, EDRF released from artery and vein possesses identical biological and chemical properties as NO.**

Both artery and vein are capable of releasing endothelium-derived relaxing factor (EDRF) in response to chemically diverse vasodilators (1–9). Endothelium-dependent relaxation of artery and vein appears to be mediated by increases in tissue cyclic GMP levels (10–13), and such effects are inhibited by methylene blue, hemoglobin, and myoglobin (13–15). Nitroso compounds, organic nitrate and nitrite esters, and inorganic nitrite cause vascular smooth muscle relaxation and cyclic GMP accumulation by endothelium-independent mechanisms, and these actions are attributed to the release of nitric oxide (NO) (16–19). NO itself is a labile substance that causes transient relaxation and cyclic GMP accumulation in both artery and vein (16–20). Moreover, NO directly activates soluble guanylate cyclase from vascular smooth muscle, and the effects of NO are inhibited by methylene blue and hemoproteins (16–20).

Thus, EDRF and NO possess very similar biological and chemical properties. We also reported that arterial and venous EDRF, like NO, directly activates purified soluble guanylate cyclase (7). Similar observations were made by others (21, 22). In studies designed to compare the actions of EDRF and NO in artery and vein, we found that EDRF and NO possessed virtually indistinguishable properties and hypothesized that EDRF is NO§ (23, 24). A similar hypothesis based on experiments of a different experimental design was recently advanced (25). The objective of the present study was to compare more closely the biological and chemical properties of NO to those of EDRF released from perfused artery, vein, and freshly isolated aortic endothelial cells and to ascertain chemically whether EDRF and NO are the same substance. During the preparation of this manuscript a report appeared confirming the biological and chemical identification of EDRF released from cultured endothelial cells as NO (26).

## MATERIALS AND METHODS

**Reagents.** Acetylcholine chloride, phenylephrine hydrochloride, A23187, pyrogallol, hemoglobin (human), and superoxide dismutase (bovine liver) were obtained from Sigma. Glyceryl trinitrate (10% wt/wt triturate in lactose) was a gift from Imperial Chemical Industries (Macclesfield, England), and propylbenzylylcholine mustard was provided by the National Institute for Medical Research (Mill Hill, London). NO (99% pure) was obtained from Matheson. A saturated solution of NO (1–2 mM) in oxygen-free water (prepared by vacuum evacuation and nitrogen flushing) was prepared by injecting about 50 ml of NO gas into 2 ml of water contained in a small tube fitted with an air-tight serum cap through which syringe needles can be inserted for delivery and escape of gases. Appropriate dilutions were similarly made in oxygen-free water with the aid of Hamilton gas-tight syringes. NO concentrations are approximate and are based on a starting stock NO concentration of 1–2 mM. Solutions of NO prepared in this manner were stable for several hours. Deoxyhemoglobin was prepared from hemoglobin by reduction with dithionite in deoxygenated Krebs-bicarbonate solution (27).

**Bioassay Cascade Superfusion Technique.** The details of this procedure have been described previously (9) and represent a modification of the original procedure described by Vane (28). Briefly, segments of main bovine intrapulmonary artery and vein were perfused (3.5 ml/min) with oxygenated Krebs-bicarbonate solution at 37°C. The perfusate was allowed to superfuse three isolated, helically cut, precontracted strips of endothelium-denuded artery (4 g of tension) or vein (2 g of tension) arranged in a cascade (mounted independently one on top of another) at 37°C. In addition, vascular strips were superfused (3.5 ml/min) at 37°C from a separate line. Vas-

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Abbreviation: EDRF, endothelium-derived relaxing factor.

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cular strips were precontracted by 10 μM phenylephrine (artery) or 0.01 μM U46619 (vein) delivered by superfusion. Changes in tension were monitored and recorded as described (9). Perfusion and superfusion media contained 10 μM indomethacin to prevent prostaglandin formation, and in experiments employing acetylcholine, arterial strips were pretreated with 0.01 μM propylbenzylylcholine mustard to prevent the direct contractile effects of acetylcholine (8, 9). Glyceryl trinitrate, a stable endothelium-independent vasodilator, was superfused over the strips in order to standardize the preparations (9, 29). In some experiments only one (Fig. 3) or two (Fig. 2), rather than three, vascular strips were superfused.

**Isolation of Aortic Endothelial Cells.** Bovine aortic endothelial cells were isolated by a collagenase digestion procedure as described previously (30, 31). Fresh segments of bovine thoracic aorta were rinsed and treated with 0.2% (wt/vol) collagenase (protease-free) in phosphate-buffered saline (pH 7.6) at 37°C as described (30, 31). Isolated cells were suspended in Krebs-bicarbonate solution (degassed under vacuum) to yield approximately $2 \times 10^6$ endothelial cells per ml and were used within 30 min.

**Reactions Between Hemoglobin and EDRF or NO.** Solutions of 5 μM deoxyhemoglobin in Krebs-bicarbonate medium buffered to pH 7.4 with 25 mM Tris·HCl were prepared (4 ml) and scanned at 25°C (LKB Ultrospec II) under atmospheric conditions before and after addition of NO. NO was delivered into cuvette solutions by slowly injecting 10 ml of NO gas directly into the solutions. NO caused a characteristic shift in the Soret absorbance peak of hemoglobin from 433 nm ($\varepsilon$ = 133 $mM^{-1} \cdot cm^{-1}$) to 406 nm ($\varepsilon$ = 120 $mM^{-1} \cdot cm^{-1}$). Aortic endothelial cells (1 ml containing $10^6$ cells) were added to 3 ml of 6.25 μM deoxyhemoglobin solution in the absence or presence of 1 μM A23187. After the suspension was mixed gently for 90 sec at 25°C, the cell-free liquid component was aspirated into a Pasteur pipet fitted at the tip with a small piece of Nytex nylon cloth to exclude the cells, transferred to a cuvette, and scanned. Atmospheric oxygen did not interfere appreciably with the stabilities of either deoxyhemoglobin or nitrosylhemoglobin, in that no significant amounts of oxyhemoglobin (Soret peak: 416 nm) were detected under the experimental conditions employed.

**Determination of Cyclic GMP Levels.** Cyclic GMP determinations were made in arterial and venous strips that had been equilibrated under tension and precontracted. Tone was monitored until the time of quick freezing. A modification (9) of the procedure described previously (18) was employed.

**Chemical Determination of NO.** The concentration of NO in the superfusion media collected during superfusion of vascular strips with either NO or perfusion media from intact artery or vein was determined by a modification of the procedure described by Bell *et al.* (32). The assay is based on the diazotization of sulfanilic acid by NO at acidic pH and subsequent coupling with *N*-(1-naphthyl)-ethylenediamine to yield an intensely colored product that is measured spectrophotometrically. Although the diazotization reaction is specific for NO, nitrogen oxides that generate NO under acidic conditions will be detected. These include $NO_2^-$, which forms HONO and NO, and any labile nitroso compound (R-NO) that can spontaneously release NO. The chemical assay is 100-fold more sensitive for NO and labile nitroso compounds than for $NO_2^-$. The concentrations of NO in the superfusion media were calculated after comparison of absorbance values with those of a standard curve constructed with various concentrations of authentic NO. Briefly, 25 ml of superfusion medium was collected into a vessel containing 2.5 ml of 4 M HCl. An aliquot of 7 ml was added to 1 ml of 2 M HCl, followed by 1 ml of sulfanilic acid (2 mg/ml). After 10 min, 1 ml of *N*-(1-naphthyl)-ethylenediamine (1 mg/ml) was added. After 30 min, 4 ml was transferred to a cuvette, and the absorbance at 548 nm was measured.

## RESULTS

**Similarity of EDRF and NO in Bioassay Cascade Superfusion.** Perfusion of an endothelium-intact segment of artery or vein caused small relaxant responses in the first two of three vascular strips arranged in cascade (Fig. 1). Arterial or venous perfusion with acetylcholine or A23187, respectively, caused further relaxation of the three strips. The decrement in magnitudes of relaxation down the cascade of strips is attributed to the very short half-life of EDRF released from artery and vein. The approximate half-life of arterial and venous EDRF was calculated from the tracings (Fig. 1) and found to be 3–5 sec. This was identical to that observed with NO, which was superfused directly over the vascular strips (Fig. 1). Direct superfusion of acetylcholine or A23187 over the strips produced no responses. Pyrogallol, which generates superoxide anion from oxygen-containing solutions (33), inhibited the relaxant actions of EDRF and NO, whereas superoxide dismutase enhanced and prolonged such relaxant responses (Fig. 1). The chemical stability of both EDRF and

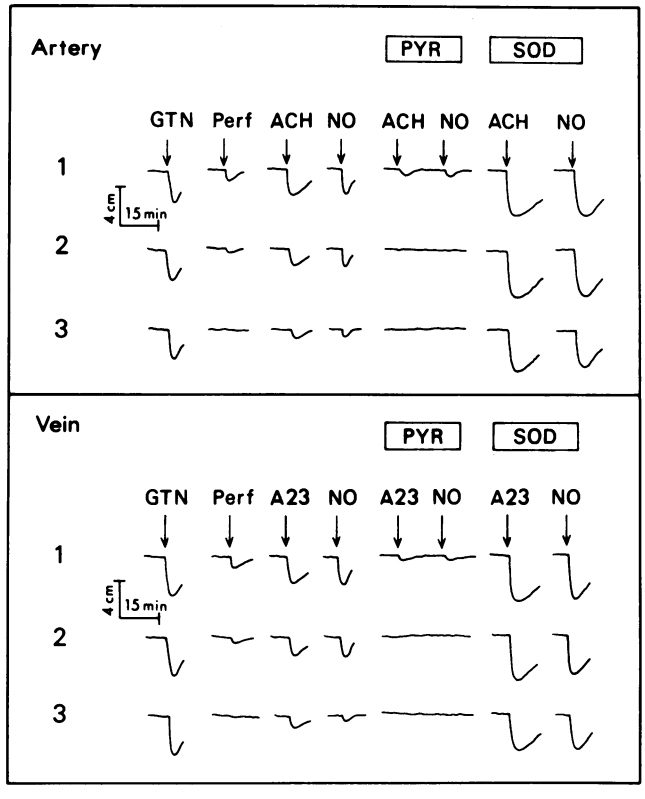


FIG. 1. Similarity between EDRF released from perfused artery and vein and superfused NO on vascular smooth muscle relaxation. Endothelium-denuded strips of pulmonary artery or vein, respectively, were precontracted with 10 μM phenylephrine or 0.01 μM U46619 delivered by superfusion. The numbers 1, 2, and 3 signify the order of the strips in the cascade. Time delay of superfusion between strips was adjusted to 2 sec. Glyceryl trinitrate (0.1 μM; GTN) was superfused over the strips for 1 min. Perf signifies perfusate from the intact segment of artery or vein. Breaks in the tracings represent periods of tissue equilibration. Acetylcholine (1 μM; ACH) and A23187 (0.1 μM; A23) were perfused through the vessel segments for 3 min as indicated. NO (0.1 μM) was superfused over the strips for 3 min. Pyrogallol (20 μM; PYR) was superfused over the strips, and superoxide dismutase (100 units/ml; SOD) was perfused through the vessel segments for the time durations indicated by box length. Two experiments are illustrated, each representing a total of four separate experiments.

NO was increased by superoxide dismutase, as reflected by their longer half-lives (about 30–40 sec). Superfusion of glyceryl trinitrate caused relaxation responses of comparable magnitudes in all three strips, thus reflecting the chemically stable nature of the drug, and responses were not altered by pyrogallol or superoxide dismutase.

**Cyclic GMP Accumulation in Target Tissues.** The superfusion of endothelium-denuded strips of artery or vein with perfusion media from intact artery or vein caused small but significant relaxant responses and increases in cyclic GMP levels in the superfused strips (Fig. 2). Perfusion of intact artery or vein with A23187 caused further relaxation and cyclic GMP accumulation. Similarly, superfusion of vascular strips with NO caused relaxant responses and cyclic GMP accumulation that were comparable in magnitudes to the responses elicited by perfused A23187 (Fig. 2). The relaxant and cyclic GMP-accumulating actions of arterial and venous EDRF and superfused NO were markedly inhibited by 1 $\mu$M methylene blue, 1 $\mu$M oxyhemoglobin, 3 $\mu$M pyrogallol, and 20 mM KCl (data not shown), each delivered by superfusion.

**Chemical Identification of EDRF as NO.** In this series of experiments only one instead of three vascular strips were mounted for superfusion to delay the apparent tissue inactivation or removal of EDRF and NO. Fig. 3 illustrates the relaxant responses and amounts of NO recovered when arterial and venous strips were superfused with different concentrations of NO. Perfusion of intact artery or vein with A23187 caused relaxant responses, and the EDRF released from both artery and vein was detected chemically as NO (Fig. 3). At concentrations of A23187 and NO that elicited relaxant responses of comparable magnitudes, the quantities

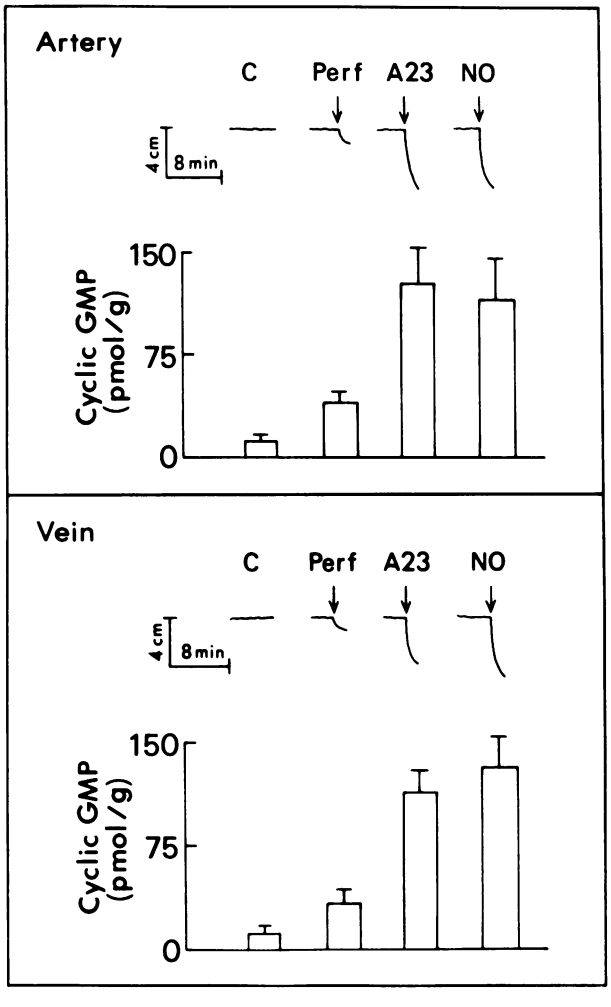


FIG. 2. Similarity between EDRF released from perfused artery and vein and superfused NO on cyclic GMP accumulation in vascular smooth muscle. Endothelium-denuded strips of pulmonary artery or vein, respectively, were precontracted with 10 $\mu$M phenylephrine or 0.01 $\mu$M U46619 delivered by superfusion. Two vascular strips were arranged in a cascade, and the time delay of superfusion between the strips was adjusted to 0.3–0.5 sec. The tracings recorded only from the first of two strips are illustrated. Control (C) strips signify absence of arterial or venous perfusion or superfusion with NO, and strips were quick-frozen at the time of peak contractile response. Perf signifies perfusate from the intact segment of artery or vein, and strips were quick-frozen 2 min after vessel perfusates were allowed to superfuse the strips. A23187 (1 $\mu$M; A23) was perfused through the vessel segments, whereas NO (1 $\mu$M) was superfused over the strips, and strips were quick-frozen 2 min after the onset of relaxation. Representative tracings from two experiments are illustrated, whereas the cyclic GMP data represent the mean ± SE of eight strips from four separate experiments.

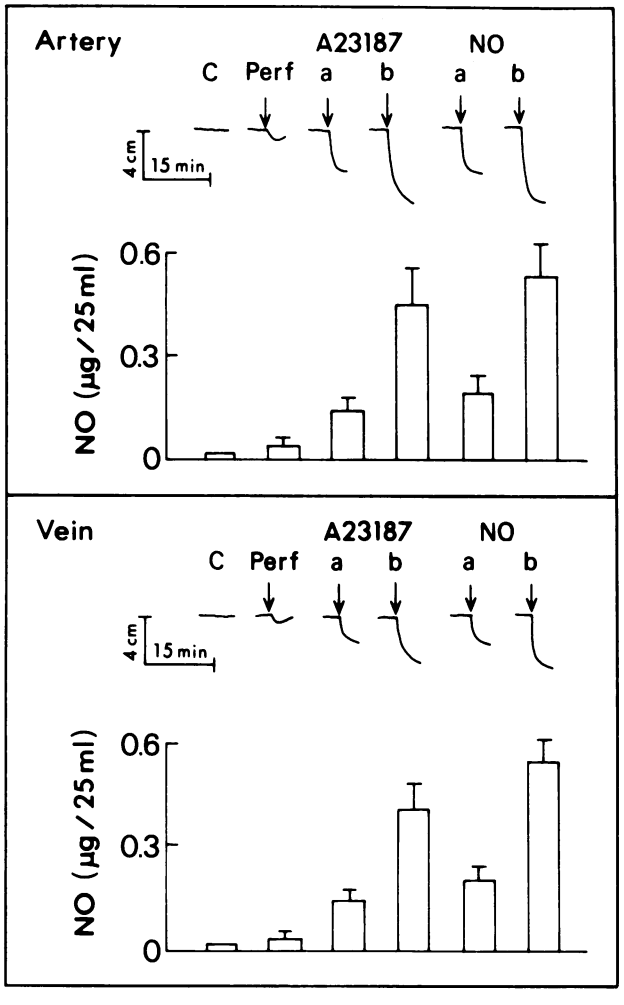


FIG. 3. Release of NO from perfused artery and vein stimulated by A23187. Endothelium-denuded strips of pulmonary artery or vein, respectively, were precontracted with 10 $\mu$M phenylephrine or 0.01 $\mu$M U46619 delivered by superfusion. One vascular strip was arranged for superfusion, and the tracings recorded from the strip are illustrated. Control (C) strips signify absence of arterial or venous perfusion or superfusion with NO. Perf signifies perfusate from the intact segment of artery or vein. A23187 was perfused at concentrations of 0.3 $\mu$M (*a*) or 1 $\mu$M (*b*) through the vessel segments, and NO was superfused at the same concentrations over the strips. The superfusion medium from each strip was collected (25 ml), starting at the onset of relaxation, into a flask containing 2.5 ml of 4 M HCl. Superfusion medium from the control strip was collected in a similar manner. The time delay between the perfused vessel and the collecting flask was ≈2 sec. Representative tracings from two experiments are illustrated, whereas the NO assay data represent the mean ± SE from four separate experiments.

of NO released from artery and vein by A23187 were closely similar to the quantities of NO recovered from superfusion of the strips with NO. Perfusion of artery and vein with only Krebs-bicarbonate solution caused the release of small but significant amounts of NO (Fig. 3). Control strips without endothelium failed to release appreciable quantities of NO. These observations indicate that the EDRF released from both artery and vein is NO or some closely related unstable nitroso compound.

Another chemical identification of EDRF as NO or an unstable nitroso compound was made by ascertaining that EDRF can react with hemoglobin to yield nitrosyl- (or NO-) hemoglobin. NO reacts with hemoglobin to cause a rapid shift in the Soret region absorbance maximum from 433 nm to 406 nm (27, 34, 35). This characteristic reaction is illustrated in Fig. 4*A*. The affinity of hemoglobin for NO greatly exceeds that for oxygen, and the formation of oxyhemoglobin was undetectable under atmospheric conditions. Freshly isolated bovine aortic endothelial cells reacted with A23187 to release a substance that caused an identical spectral shift as that caused by authentic NO (Fig. 4*B*). In the absence of added A23187, the endothelial cells alone failed to produce an observable change in the absorbance characteristics of hemoglobin. Once again, these observations indicate that the EDRF released from arterial endothelial cells closely resembles NO.

## DISCUSSION

The observations in this report provide pharmacological and chemical evidence that EDRF released from both artery and vein is NO or a very labile nitroso compound that readily releases NO. Moreover, the present data also indicate clearly that not only artery but also vein can release EDRF. During preparation of this manuscript, a report appeared that demonstrated that the EDRF released from cultured aortic endothelial cells is NO, or a closely related labile substance (26). In the present study, the bioassay cascade/superfusion technique revealed the virtually identical pharmacological properties of arterial and venous EDRF and NO. Both EDRF and NO are biologically very labile substances ($t_{1/2}$ = 3–5 sec), and their inactivation is accelerated by superoxide anion and slowed by superoxide dismutase. Both EDRF and NO stimulate cyclic GMP accumulation in artery and vein in a manner that correlates well with their concomitant relaxant responses. Furthermore, both vascular relaxation and cyclic GMP accumulation elicited by EDRF and NO are markedly inhibited by methylene blue, oxyhemoglobin, pyrogallol, and potassium. The chemical evidence that EDRF and NO are closely similar derives from the finding that EDRF, like NO, causes the diazotization of sulfanilic acid and reacts with hemoglobin to generate its nitrosylheme adduct.

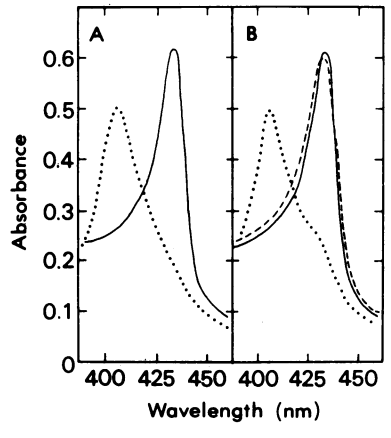


FIG. 4. Spectrophotometric analysis of the reaction between reduced hemoglobin and NO or EDRF released from aortic endothelial cells. (*A*) ——, 5 μM deoxyhemoglobin; ·····, 5 μM deoxyhemoglobin gassed with 10 ml of NO. (*B*) ——, 5 μM deoxyhemoglobin; – – –, 5 μM deoxyhemoglobin after reaction with $10^6$ endothelial cells; ·····, 5 μM deoxyhemoglobin after reaction with $10^6$ endothelial cells plus 1 μM A23187. Data illustrated are from one of three separate experiments.

EDRF released from artery and vein was recently shown to activate purified soluble guanylate cyclase in a manner that was similar to the enzyme activation caused by NO (7). Other reports also showed that arterial EDRF activates soluble guanylate cyclase (21, 22). Methylene blue, an inhibitor of soluble guanylate cyclase from vascular tissue (16–18, 36), inhibited enzyme activation elicited by EDRF (7) and NO (16) and inhibited the relaxant and cyclic GMP-accumulating responses to EDRF and NO in the present study. Hemoglobin, previously shown to inhibit vascular relaxation elicited by NO (16–18) and by endothelium-dependent vasodilators (14, 15), also inhibited relaxation and cyclic GMP accumulation produced by EDRF and NO in the bioassay cascade/superfusion procedure. Pyrogallol, which generates superoxide anion from oxygenated solutions (33), was recently reported to inactivate EDRF released from cultured aortic endothelial cells (29), and in the present study pyrogallol similarly inactivated arterial and venous EDRF and NO. In contrast, superoxide dismutase markedly protected both EDRF and NO against rapid inactivation. When 20 mM KCl was used instead of phenylephrine to precontract the vascular strips, the relaxant and cyclic GMP-accumulating responses to both EDRF and NO were similarly attenuated. These observations are consistent with earlier reports that endothelium-dependent vascular smooth muscle relaxation is difficult to observe in the presence of high potassium concentrations (1, 3, 5). The above observations indicate the closely similar pharmacological properties of arterial and venous EDRF and NO.

The observations that both superoxide anion and hemoglobin antagonize the biologic actions of EDRF could have important physiological implications with respect to the mechanisms by which the actions of EDRF are terminated. Hyperoxia as well as ischemia followed by reperfusion, and hypoxia, respectively, could be associated with increased and decreased amounts of superoxide locally (37), and such conditions could influence the local actions of EDRF. Elevated concentrations of superoxide will inactivate NO by catalyzing its oxidation to $NO_3^-$, which is inactive as a relaxant (16). The hemoglobin present within the erythrocytes could act as a trap for locally released EDRF, thereby preventing any downstream vasodilatory effect. Smooth muscle myoglobin could similarly localize the action of EDRF.

The amounts of NO released from perfused arteries and veins appeared to account for the relaxation of vascular strips elicited by EDRF. That is, the same amounts of authentic NO superfused over the strips and quantified chemically produced the same magnitudes of relaxation as did EDRF. The reaction between arterial EDRF and hemoglobin was nearly identical to the reaction between NO and hemoglobin to generate nitrosylhemoglobin. Thus, substantial pharmacological and chemical evidence have been obtained to support our original hypothesis that EDRF is NO, or some labile nitroso compound (23, 24). *S*-Nitrosothiols are unstable in aqueous solution and rapidly release NO, and several *S*-nitrosothiols have been shown to be potent vasodilators (19). The chemical procedures described here and in a recent report (26) for detecting NO are not entirely specific for NO *per se* and are incapable of distinguishing NO from a labile nitroso compound. The possibility exists, therefore, that EDRF is a labile nitroso substance that spontaneously releases NO. EDRF cannot be $NO_2^-$ because $NO_2^-$ is a

relatively stable species that causes vascular smooth muscle relaxation only at very high concentrations.

In much the same manner that other pharmacologically active substances have been subsequently discovered to occur endogenously, NO, which was first described as one of the most potent vascular smooth muscle relaxants in 1979 (16), now appears to exist naturally in mammalian cells as EDRF. The endogenous NO receptor, unlike most other receptors, is located intracellularly and is the heme group bound to soluble guanylate cyclase. NO is highly lipophilic and readily permeates vascular smooth muscle cells to activate soluble guanylate cyclase, elevate tissue cyclic GMP levels, and relax the muscle (16–19). Moreover, the requirement of guanylate cyclase-bound heme for enzyme activation by NO is well documented (27, 36, 38–40). The present observations suggesting that arterial and venous EDRF are NO are consistent with the classical criteria proposed by Dale (41) for arguing that a chemical agent is a biological mediator. For example, the active substance (EDRF) has been recovered from perfusates during application of the stimulus and has been identified pharmacologically and chemically as NO. Authentic NO produced identical actions to those of EDRF, and the actions of both were antagonized by common inhibitors. Finally, both EDRF and NO were inactivated by superoxide anion and stabilized by superoxide dismutase.

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