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BIOSYNTHESIS AND METABOLISM OF ENDOTHELIUM-DERIVED NITRIC OXIDE

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INTRODUCTION

Studies on the biological actions of nitric oxide (NO) essentially began with the observations that NO gas, generated from an acidified nitrite solution, activated crude soluble preparations of guanylate cyclase (1, 2). In a series of pioneering experiments, Murad et al observed that NO could account for the ability of numerous chemically diverse, nitrogen-containing compounds to activate cytosolic guanylate cyclase and elevate tissue levels of cyclic GMP (3). The mechanism of heme-dependent activation of guanylate cyclase by NO and labile nitroso compounds that spontaneously liberate NO was discovered by Craven & DeRubertis (4). The first observation that NO is a potent inhibitor of platelet aggregation came from our laboratory (5). In addition, we extended the initial hypothesis on the requirement of tissue thiols for the vasodilator action of nitroglycerin, forwarded by Needleman et al (6, 7), with the findings that nitroglycerin reacts with cysteine to yield *S*-nitrosocysteine, which is a labile but potent vascular smooth-muscle relaxant that works through the action of liberated NO (8). *S*-Nitrosothiols were found to be labile precursors of NO that activate cytoplasmic or cytosolic guanylate cyclase, elevate vascular and platelet levels of cyclic GMP, and cause vascular smooth-muscle relaxation, inhibition of platelet aggregation, and profound hypotension in anesthetized animals (8–12).

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Less than 1 year after the demonstration that authentic NO is a vascular smooth-muscle relaxant (13), Furchgott & Zawadzki (14) reported that the endothelium was obligatory for acetylcholine-elicited relaxation of isolated rabbit aortic preparations. Acetylcholine was postulated to interact with muscarinic receptors on the surface of endothelial cells to stimulate the release of an endothelium-derived relaxing factor (EDRF) that diffused into and interacted with the underlying vascular smooth muscle (14, 15). After several years of studying endothelium-independent NO-elicited and endothelium-dependent relaxation of bovine pulmonary artery and vein, we recognized the remarkable similarities between EDRF-elicited and NO-elicited relaxation of artery and vein and forwarded the hypothesis, at an international conference in 1986, that EDRF is either the same as NO or a labile nitroso precursor that spontaneously decomposes with the liberation of NO (16). The proceedings of that conference were not published until 1988 (17). A similar hypothesis was forwarded independently by Fuchgott (18). The chemical and pharmacological identification of EDRF from cultured aortic endothelial cells as NO was provided in 1987 by Palmer et al (19), and we independently provided both chemical and pharmacological evidence that EDRF released from intact artery and vein is NO (20, 21). As we originally cautioned, however, neither the chemical nor the pharmacological assays used can discriminate between NO and labile nitroso compounds that rapidly liberate NO. Thus, it is not yet clear whether EDRF is NO, a labile nitroso precursor, or a mixture of both.

The present focus of attention has been on the biosynthesis and metabolism of endothelium-derived nitric oxide (EDNO). NO is chemically unstable, with a half-life of 3–5 s in aqueous solution under physiological conditions of concentration, temperature, pH, and oxygen tension. The lability of NO, however, may be much greater in the actual tissues in vivo. In aqueous solution NO spontaneously oxidizes primarily to NO_2^-, which is 5 or 6 orders of magnitude less potent than NO as a vasodilator (11, 13). Good direct and indirect evidence exists that EDNO is synthesized from endogenous L-arginine by an enzymatic process that can be inhibited by close structural analogs of arginine (22–27). One of these arginine analogs, N^G-monomethyl-L-arginine, elicits a sustained increase in systemic blood pressure after intravenous bolus injection into anesthetized rabbits and guinea pigs, and subsequent administration of L-arginine causes a rapid reversal of the hypertension (28, 29). The objective of this review is to highlight the important chemistry and pharmacology of EDNO, authentic NO, and *S*-nitrosothiols and to update the information and views on the biosynthesis and metabolism of NO derived not only from vascular endothelial cells but also from other cell types.

PHARMACOLOGY AND CHEMISTRY OF NITRIC OXIDE AND *S*-NITROSOTHIOLS

Chemistry of Nitric Oxide

NO can undergo numerous reactions, as it can act as both a Lewis acid and a Lewis base. Since NO possesses an intermediate oxidation state, it can act both as an oxidizing and a reducing agent. NO is a colorless gas containing an odd number of electrons (paramagnetic; free radical), and its physical properties differ strikingly from those of other molecules having an odd number of electrons. The more important of these differences are its lack of color in the gaseous state, its lack of dimerization in the gaseous state or in solution, and its relatively low chemical reactivity. The electronic structure of NO has been represented as both of the following:

I II

The resonating structure II is more favorable than structure I because structure I favors dimerization of NO, which is not observed (30). The unpaired electron is in a molecular orbital involving both the nitrogen and oxygen atoms and is delocalized over these atoms. The internuclear distance is 1.151 Å (0.115 nm), intermediate between the double- and triple-bond distances. NO gas is only slightly soluble in water at standard temperature and pressure, yielding a saturated solution of 1–3 mM.

NO can be formed nonenzymatically in numerous laboratory reactions that would be most unlikely to occur in biological tissues. The two most common reactions are

$$Fe^{2+} + NO_2^- + 2\,H^+ \rightarrow Fe^{3+} + NO + H_2O$$

and

$$2\,NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O.$$

In each of the above reactions, large amounts of NO can be generated that exceed its solubility limit in aqueous medium, and therefore the NO evolves in the gaseous state. Enzymatic chemical reactions by which NO is generated are the likely source of NO in biological tissues. Enzymatic reactions known to generate NO involve azide anion (N_3^-), hydroxylamine (NH_2OH), hydrazine (NH_2NH_2), and L-arginine. N_3^- and NH_2OH undergo nitrogen oxidation to NO in a complex enzymatic reaction catalyzed by catalase (31, 32). A

similar reaction with hydrazine requires addition of a peroxide. In a newly described enzymatic reaction to be discussed in detail below, L-arginine can be converted to NO plus citrulline in a reaction catalyzed by an NADPH-dependent monooxygenase (33).

NO undergoes a number of chemical reactions, many of which occur under biological conditions. Some of the more important chemical reactions involving NO are discussed below:

1. Reactivity with oxygen to yield NO_2 gas or NO_2^- in solution:

$$2NO + O_2 \rightarrow 2NO_2^-.$$

2. Reactivity with superoxide anion (O_2^-) to yield the unstable intermediate peroxonitrite anion (^-OONO), which rearranges to form NO_3^- (34):

$$NO + O_2^- \rightarrow {}^-OONO \rightarrow NO_3^-.$$

This reaction represents one of the few examples of a radical-radical coupling of O_2^- with another odd-electron species to generate a diamagnetic product.

3. Reactivity with ozone (O_3) to yield an activated or high-energy state NO_2. The activated NO_2 can be readily detected by chemiluminescence (35), and this principle has been used to assay NO (19).

4. Reactivity with oxyhemoglobin to yield methemoglobin and inorganic nitrate according to the overall reaction

$$HbO_2 + NO \rightarrow metHb + NO_3^-.$$

Looking at this reaction in more detail, the component reactions are

$$Hb^{3+}O_2^- + NO \rightarrow metHb + {}^-OONO$$

and

$$^-OONO \rightarrow NO_3^-.$$

Molecular oxygen binds as superoxide anion to the heme iron atom of hemoglobin (36), and this species reacts rapidly with NO to yield the peroxonitrite anion, which rapidly isomerizes to NO_3^- (34). This principle of reacting NO with HbO_2 to yield metHb has been used as a spectrophotometric assay of NO (37).

5. Reactivity with thiols (R-SH) to yield *S*-nitrosothiols (R-SNO). One example is the reaction between cysteine and NO to yield *S*-nitrosocysteine.

These reactions occur readily either when NO is provided directly (8) or when it is generated from NO_2^- in acidic aqueous solution (38):

$$R\text{-}SH + NO \rightarrow R\text{-}SNO + H^+$$

and

$$R\text{-}SH + NO_2^- + H^+ \rightarrow R\text{-}SNO + H_2O.$$

The reaction between R-SH and NO can occur under biological conditions of pH and temperature, whereas the second reaction requires a low pH to generate detectable quantities of product. NO can react with -SH groups on amino acids, amines, organic acids, sugars, peptides, and proteins to yield the corresponding *S*-nitrosothiol.

6. Reactivity with heme iron to yield nitrosyl-heme adducts (39–41). Reduced iron (Fe^{2+}), complexed with protoporphyrin IX to form heme, has a high binding affinity for and reactivity with NO. This is especially true for proteins containing the heme moiety (hemoproteins). Indeed, the binding affinity of hemoglobin for NO exceeds its binding affinity for carbon monoxide by several orders of magnitude. Thus, hemoproteins such as hemoglobin, myoglobin, cytochrome *c*, and soluble guanylate cyclase react readily with NO to yield the corresponding nitrosyl-heme (NO-heme) adduct. The NO reacts with the Fe^{2+} ion of heme to yield the paramagnetic NO-heme species. Solutions of reduced and oxidized hemoproteins are dark red and brown, respectively, whereas NO-hemoproteins are bright pink-red. This chemistry was used in the development of a spectrophotometric assay of NO (21). Interactions between hemoproteins and NO occurring in the atmosphere or in ingested chemicals result in the in vivo formation of relatively large amounts of NO-hemoproteins (42–44). This reaction is used in the meat industry, in which $NaNO_2$ and ascorbic acid are added to freshly cut meat to preserve its pink-red color. In the presence of ascorbic acid the NO_2^- generates HONO and NO, which react with the myoglobin to yield nitrosyl-myoglobin. NO, like carbon monoxide, can form complexes with transition metals unassociated with heme, such as in ferricyanide, other transition metal complexes, and non-heme-iron-containing proteins such as lipoxygenase (45).

7. Other nitrosation reactions involving NO. Numerous N-nitrosation reactions involving NO are likely to occur in mammalian cells (46). The best examples are the synthesis of *N*-nitrosamines by cytotoxic activated macrophages. In one fairly well studied reaction, murine macrophages activated by lipopolysaccharide and interferon gamma are capable of synthesizing *N*-nitrosomorpholine from morpholine. Other products of the reaction are

NO, NO_2^-, and NO_3^-. Finally, in a diazotization reaction, NO combines with sulfanilic acid at low pH, and the diazo product can be coupled with *N*-(1-naphthyl)ethylenediamine to yield an intense chromophore. This method has been used to assay NO (20).

Chemistry of S-*Nitrosothiols*

S-Nitrosothiols, also termed thionitrites, are extremely unstable in the solid form or in aqueous solution and spontaneously decompose with the liberation of NO gas and formation of the corresponding disulfide:

$$2R\text{-}SNO \rightarrow R\text{-}SS\text{-}R + 2NO.$$

The only known exception to this lability is S-nitroso-*N*-acetylpenicillamine (SNAP), which is stable indefinitely as dry crystals maintained at 0–5°C. The half-life of aqueous solutions of SNAP ranges from 500 h under nitrogen to 4–5 h in air at 37°C (8). *S*-Nitrosocysteine undergoes rapid spontaneous decomposition in the solid form and therefore cannot be maintained in the crystalline state. Concentrated solutions in acidified methanol (1% [vol/vol] 1 N HCl in absolute methanol) at −20°C are stable indefinitely. Concentrated aqueous solutions have a half-life of 1.1. h in air at 37°C and 15 min in oxygen at 37°C. Dilute oxygenated aqueous solutions of *S*-nitrosocysteine (less than 1 μM) at 37°C have a half-life of less than 30 s. The *S*-nitroso derivatives of 2-mercaptoethylamine, 3-mercaptopropionic acid, penicillamine, dithiothreitol, glutathione, thioglucose, and *N*-acetylcysteine have chemical stability characteristics in between those of SNAP and *S*-nitrosocysteine (8–11). The reason for the relative chemical stability of SNAP is the presence of the *N*-acetyl group, which structurally hinders the spontaneous formation of the corresponding disulfide. The same is true for *S*-nitroso-*N*-acetylcysteine, but this *S*-nitrosothiol is still unstable in the crystalline state.

S-Nitrosothiols can be readily formed by the reaction of thiols not only with NO or acidic NO_2^- (HONO) but also with *N*-nitroso derivatives such as *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (9, 47), nitroprusside (8), and organic nitrate and nitrite esters (8). The reactions involving organic nitrate esters such as glyceryl trinitrate or nitroglycerin, isosorbide dinitrate, and pentaerythritol tetranitrate have an absolute requirement for cysteine (8). The chemical reactions described above occur at sufficient rates under near-physiological conditions of temperature and pH to generate biologically active quantities of *S*-nitrosothiols

Pharmacology of Nitric Oxide and S-*Nitrosothiols*

The earliest evidence that NO elicits important actions was the finding that NO and nitroso compounds could activate cytoplasmic or cytosolic guanylate

cyclase and stimulate cyclic GMP formation in mammalian tissues (1–4). Various nitrogen-containing substances were found to produce the same effects, and NO was suggested to be the common factor responsible (3). In experiments designed to ascertain whether cyclic GMP could mediate the vascular smooth-muscle relaxant effect of nitroglycerin, nitroprusside, and related vasodilators, NO itself was tested for its capacity to cause relaxation (13). NO caused a marked and potent but transient relaxation of isolated strips of bovine coronary artery, and this was associated with activation of guanylate cyclase. The actions of NO were inhibited or abolished by added hemoproteins or methylene blue. Earlier studies had revealed that guanylate cyclase activation by NO and related agents was inhibited by reduced hemoproteins and methylene blue (3). The novel observation that NO gas caused vascular smooth-muscle relaxation was extended to include nitroso compounds and other compounds related to NO (8, 13, 48–51). Moreover, NO stimulated cyclic GMP formation and relaxed not only artery but also vein (52). In that study, bovine intrapulmonary vein was more sensitive than the corresponding underlying artery to the effects of NO, nitroso compounds, and nitroglycerin. A series of labile *S*-nitrosothiols produced potent hypotensive responses in anesthetized cats (8). The onset (immediate) and duration (1–3 min) of hypotension were identical to those caused by nitroglycerin; little or no change in cardiac output accompanied the decrease in systemic arterial pressure; and the vasodilator responses were not influenced by propranolol, indomethacin, or antihistamines.

In experiments designed to elucidate the action of cyclic GMP on human platelet function, NO elicited potent and marked inhibitory effects on platelet aggregation (5). Platelet responses were associated with marked but transient cyclic GMP accumulation and were antagonized by hemoproteins and methylene blue. This novel action of NO was mimicked by nitroprusside, labile *S*-nitrosoguanidines, and cigarette smoke (which contains 600–800 ppm of NO in the filtered gaseous phase). The concomitant observation that 8-bromo-cyclic GMP inhibited platelet aggregation prompted us to advance the hypothesis that cyclic GMP mediates the inhibition of platelet aggregation elicited by NO (5). More recent studies revealed that NO not only inhibits platelet aggregation but also inhibits platelet adhesion to vascular endothelial surfaces (53).

Studies on the pharmacology of *S*-nitrosothiols began after the observations that the enhancement of guanylate cyclase activation by certain nitroso compounds, nitrite, nitrite esters, and nitrate esters is attributed to the generation of labile *S*-nitrosothiols (8). The pharmacology of *S*-nitrosothiols is essentially that of NO and other labile nitroso compounds. Thus, *S*-nitrosothiols activate cytosolic guanylate cyclase (9–12) by heme-dependent mechanisms (12, 54), elevate vascular (8, 52) and platelet (12) cyclic GMP levels, relax

artery and vein (8, 52), inhibit platelet aggregation (12), and elicit potent vasodilator responses in vivo (8).

NO, *S*-nitrosothiols, and related chemical agents that generate NO activate cytosolic guanylate cyclase by heme-dependent mechanisms. The heme-dependent enzyme activation by NO, nitrosoguanidines, and other nitrogen-containing substances was first described by Craven et al (4, 55). These initial observations with crude enzyme fractions were confirmed by using purified soluble enzyme from lung (54, 56, 57), liver (58), and platelets (12) and were extended to include *S*-nitrosothiols as well (12, 54). The requirement of heme is attributed to the formation of the paramagnetic species, NO-heme, which is responsible for the enzyme activation (4, 55). Heme-deficient purified guanylate cyclase is not activated by NO alone but is activated by either the addition of both NO and heme or the addition of preformed NO-heme complex (56, 57). The molecular mechanism by which NO-heme activates guanylate cyclase is unknown but is indistinguishable from that of protoporphyrin IX (59, 60). The binding of heme to guanylate cyclase involves the complexing of iron to enzyme protein, and this binding prevents enzyme activation. When NO reacts with heme iron to form NO-heme, the bond between heme iron and enzyme protein is broken but the porphyrin ring remains tightly bound to the enzyme. Thus, a protoporphyrin IX–like binding interaction occurs, and this is responsible for enzyme activation (57, 60).

Additional pharmacological actions of NO have been observed or suspected in other cell types. Cytotoxic activated murine macrophages synthesize NO, which is rapidly oxidized to both nitrite and nitrate, and these biochemical transformations are associated with killing of phagocytized microorganisms or adjacent target cells (33, 61, 62). Authentic NO appears to mimic the cytotoxic actions of activated macrophages (33, 62). Following the demonstration that rat peritoneal exudate neutrophils release a relaxing factor with pharmacological properties similar to those of NO (63), the formation of NO by human neutrophils was demonstrated (64). In addition to the above cell types, evidence exists that NO is generated by EMT-6 adenocarcinoma cells (65) and hepatic Kupffer cells (66). The function of NO in these cells is unknown but is probably related to a cytotoxic action on invading cells and microorganisms. NO is a potent nitrosating agent, and this reaction may occur intracellularly to alter the structure of peptides, proteins, and other macromolecules.

In addition to the cytostatic or cytotoxic role of NO generated by macrophages, neutrophils, Kupffer cells, and other scavenger cells, the NO released from these cells may elicit local effects on the microcirculation and platelet function. Locally released NO would cause vasodilation of arterioles and venules and would inhibit platelet adhesion to vascular endothelial surfaces as well as inhibiting platelet aggregation. Such effects would lead to

improved local blood flow to the sites of NO release, and this may be a part of the overall inflammatory response. Thus, NO released from the vascular endothelium and associated cell types could act in a complementary manner to facilitate local blood flow during wound healing.

DISCOVERY OF NITRIC OXIDE AS EDRF

As studies on EDRF progressed after its discovery, numerous properties were observed that were very similar to those of authentic NO that we had observed earlier [see Ignarro (67) for a recent review]. Endothelium-dependent relaxation of artery and vein was associated with cyclic GMP accumulation, and both vascular responses were antagonized by methylene blue (68), an inhibitor of soluble or cytosolic guanylate cyclase. The subsequent finding that hemoglobin and myoglobin antagonize endothelium-dependent relaxation (69) was especially intriguing, as these hemoproteins had earlier been shown to abolish NO-elicited vascular smooth-muscle relaxation (13) and cyclic GMP accumulation (49). This observation prompted us to investigate whether EDRF could activate guanylate cyclase, because we knew of only one chemical species whose biological action could be inhibited by both methylene blue and hemoproteins, and this was NO. After finding that arterial and venous EDRF activated purified guanylate cyclase (70, 71), we discovered that this enzyme activation is heme dependent (21). The latter observation, actually made in 1986, together with the earlier findings of Griffith et al (72, 73) that the biological half-life of EDRF is only several seconds, prompted us to propose that EDRF released from arterial and venous endothelium was NO or a labile nitroso compound (16). Soon afterward, we recognized a report that EDRF could inhibit platelet aggregation (74), an action of NO that we had demonstrated in 1981 (5).

Several laboratories set out to ascertain both pharmacologically and chemically whether EDRF is actually NO. In 1987 Palmer et al (19) first showed that NO, measured by chemiluminescence, could account for the biological action of EDRF released from cultured porcine aortic endothelial cells in response to bradykinin. We reported, independently, that NO or a labile nitroso compound, measured by spectrophotometric monitoring of the formation of NO-hemoglobin from deoxyhemoglobin (21) or the diazotization of sulfanilic acid (20), could account for the biological action of EDRF released from perfused artery, vein, and freshly harvested bovine aortic endothelial cells. The latter observations were subsequently confirmed by Schmidt et al (24). Many pharmacological properties of EDRF and authentic NO were similar, if not identical (53, 75–78). Subsequently, another procedure was used to reveal the apparent identity of EDRF and NO. On the basis of the reactions described above, in which NO reacts with oxyhemoglobin to form

Table 1 Identical pharmacological and chemical properties of EDRF, NO, and S-nitrosocysteine

1.	Chemically unstable with half-lives of 3–5 s under assay conditions
2.	Spontaneous inactivation in the presence of oxygen or superoxide anion
3.	Chemical stabilization by the addition of superoxide dismutase or low pH
4.	Identical reaction with sulfanilic acid and ozone (reactivity of *S*-nitrosocysteine is due to liberated NO)
5.	Lipophilic and readily able to permeate biological membranes (NO is more lipophilic than *S*-nitrosocysteine)
6.	High binding affinity for and reactivity with heme iron in hemoglobin, myoglobin, and soluble guanylate cyclase to form the corresponding nitrosyl-heme adduct
7.	Rapid termination of biological actions by hemoglobin and myoglobin
8.	Heme-dependent activation of cytosolic guanylate cyclase; inhibition by methylene blue
9.	Stimulation of cyclic GMP formation in vascular tissue and platelets
10.	Relaxation of arterial and venous smooth muscle
11.	Inhibition of platelet aggregation and adhesion to endothelial surfaces

methemoglobin, EDRF released from cultured arterial endothelial cells was identified as NO (37).

Table 1 lists the common properties of EDRF and NO. It is noteworthy that a labile nitroso compound such as *S*-nitrosocysteine possesses similar properties to those of NO.

METABOLISM OF EDNO

The biological half-life of EDNO under the conditions of bioassay is approximately 3–5s, which is identical to that of authentic NO superfused over the vascular strips at concentrations (10–100 nM) that elicit equivalent relaxant responses to those elicited by EDNO released from intact artery or vein and cultured aortic endothelial cells (19, 20). The biological inactivation of authentic NO in oxygenated physiological salt solution can be accounted for by spontaneous oxidation of NO to NO_2^-. At least 90% of the NO is converted to NO_2^-, with little or no formation of NO_3^- (37, 79; L. J. Ignarro, unpublished observations). The same can be observed of EDNO released from endothelial cells or intact blood vessels. Extracellular NO_2^- is incapable of causing vascular smooth-muscle relaxation at concentrations below 0.1 mM at pH 7.4.

The biological half-lives of EDRF and authentic NO vary inversely as a function of oxygen tension and superoxide anion concentration (80–84). Moreover, the biological half-life of authentic NO varies directly with its concentration in aqueous solution (L. J. Ignarro, G. M. Buga, unpublished observations). NO concentrations of 10–50 nM have half-lives of 3–5 s,

whereas concentrations in excess of 300 nM have biological half-lives of longer than 30 s. We have noted repeatedly that under conditions when large amounts of ENDO are released from cultured endothelial cells, bovine pulmonary artery, or human umbilical vein, the half-life of EDNO is in excess of 30 s, which is similar for a pharmacologically equivalent concentration of authentic NO. It is also possible that the endothelium generates and releases both NO and a nitroso compound that is somewhat more chemically stable than NO itself.

There is no question that the presence of superoxide anion results in the rapid and nearly complete inactivation of EDRF or EDNO (77, 83, 85, 86). Superoxide dismutase prevents this inactivation, which is triggered by superoxide anion generated in oxygenated aqueous media (77, 85, 86). The occurrence of superoxide anion–mediated inactivation of EDNO in biological tissues is unknown. It appears to me to be unlikely that tissue-generated superoxide anion contributes significantly to the biological inactivation of EDNO. First, the ubiquitous distribution of high activities of superoxide dismutase would make it unlikely that enough superoxide anion could accumulate to inactivate NO. Second, because superoxide anion catalyzes the oxidation of NO to NO_3^- without intermediate formation of NO_2^- (34) and because authentic NO and EDNO are converted almost exclusively to NO_2^-, it appears that superoxide anion does not play a physiological role in the inactivation of EDNO. Pathophysiological conditions, however, could conceivably set the stage for a role of superoxide anion.

From the above observations, it appears that the major mode of EDNO inactivation occurs via oxygen-catalyzed spontaneous oxidation of NO to NO_2^-. What actually occurs in the passage of EDNO or authentic NO through viable tissues is as yet unknown, and it is entirely possible that the half-life of NO within tissues is much shorter than 3–5 s, perhaps as short as a fraction of a second. Oxidizing equivalents generated by cellular oxidative reactions could facilitate the inactivation of EDNO. Alternatively, cellular hemoproteins could act as a sink for NO and rapidly bind even traces of NO, thereby shortening the half-life of elimination of NO. Experiments designed to compare EDNO with authentic NO in perfused highly vascularized organs (heart, lungs, and kidneys) should answer this question.

BIOSYNTHESIS OF EDNO

The discovery that at least one form of EDRF is NO set the stage for the studies that followed on the biosynthesis of EDNO. This required some novel thought, as NO had not been previously known to be present in or released from mammalian cells. Even prior to the finding that vascular endothelial cells release NO, Stuehr & Marletta had demonstrated that murine mac-

rophages synthesize NO_2^- and NO_3^- in response to *Escherichia coli* lipopolysaccharide (87). These observations were extended to include macrophages activated by *Mycobacterium bovis* BCG infection, lymphokines, or gamma interferon (88). In these initial studies, however, no mention was made even of the possibility that NO was a labile intermediate precursor of NO_2^- and NO_3^-. An unrecognized clue to this possibility was provided from a report, by the same group (89), that activated murine macrophages catalyze the nitrosation of amines. Although NO_2^- can serve as a nitrosating agent under fairly strongly acidic conditions, NO or HONO is much more active and potent a nitrosating agent than NO_2^- at pH 7.0–7.4. The authors did speculate, however, that a reactive nitrosating species may have been formed prior to formation of NO_2^- and NO_3^-.

Macrophage cell culture experiments revealed that an L-arginine–dependent biochemical pathway as involved in the biosynthesis of NO_2^- and L-citrulline and that this pathway was inhibited by N^G-monomethyl-L-arginine, a close structural analog of L-arginine (90). Related analogs of L-arginine also inhibited the formation of NO_2^- and citrulline, whereas addition of excess L-arginine was able to override the inhibition in a competitive manner (91). In an elegant series of experiments Iyengar et al (92) showed that L-arginine was obligatory for the formation of NO_2^-, NO_3^-, and *N*-nitrosylated amines by cytotoxic activated murine macrophages. Gas chromatography–mass spectrometry experiments with L-[*guanido*-^{15}N]arginine established that the NO_2^-, NO_3^-, and the nitroso moiety of *N*-nitrosylated amines were derived exclusively from one or both of the terminal guanidino nitrogens of L-arginine. In addition, L-citrulline was confirmed as representing the other reaction product, and L-canavanine, a structural analog of L-arginine, inhibited this biochemical reaction.

In discussions of the precise mechanism by which L-arginine is converted to NO_2^- plus citrulline, one group postulated that an arginine deiminase catalyzed the formation of L-citrulline plus ammonia, followed by oxidation of the ammonia to NO_2^- (90). Another group argued, however, that arginine deiminase does not occur in mammalian cells and that ammonia is not a precursor to NO_2^- and NO_3^- formation, suggesting that an alternative pathway is responsible for arginine oxidation (92).

The above classic experiments demonstrating the synthesis of NO_2^-, NO_3^-, and citrulline from L-arginine by cytotoxic activated macrophages led to the discovery that vascular endothelial cells also utilize L-arginine in the biosynthesis of NO (22). The principal objective of this study, however, was to ascertain whether NO is the immediate product derived from L-arginine. The formation of ^{15}NO from either [U-^{15}N]arginine or [G-^{15}N]arginine was detected by mass spectrometry, and NO formation was confirmed by chemiluminescence. This arginine-dependent formation of EDNO was specific for

L-arginine and was inhibited competitively by N^G-monomethyl-L-arginine (26). The clear demonstration of NO formation from L-arginine by vascular endothelial cells, together with the knowledge that NO undergoes rapid spontaneous oxidation to NO_2^-, prompted Hibbs et al to reexamine the macrophage system, and they reported that NO was, after all, the immediate product of L-arginine and that NO_2^- was, in turn, derived from NO (33). Moreover, NO was shown to be the actual cytotoxic activated macrophage effector molecule.

Additional studies from other laboratories confirmed and extended the above observations that structural analogs of L-arginine cause a competitive antagonism of endothelium-dependent relaxation and that such antagonism is reversed by the addition of excess L-arginine (23–25, 27). L-Argininosuccinic acid, a naturally occurring structural analog of L-arginine, was found to inhibit endothelium-dependent relaxation, but the mechanism of inhibition could not be reversed by addition of excess L-arginine (27). The striking commonality of observations that close structural analogs of L-arginine antagonize endothelium-dependent relaxation, that addition of L-arginine overrides such antagonism, and that arginine analogs cause endothelium-dependent vascular smooth-muscle contraction, together with the findings from experiments with macrophages and endothelial cells that NO is derived from the basic amino-nitrogen atom of the guanidino function of L-arginine in a manner that is inhibited by L-arginine analogs, suggests that a specific enzyme is involved in the catalytic conversion of L-arginine to NO.

In addition to using structural analogs of L-arginine to inhibit NO formation from L-arginine, we embarked on still another approach to test the strict requirement of endogenous L-arginine for EDNO formation and endothelium-dependent relaxation. We wanted to find the effect on endothelium-dependent relaxation if the endothelium was depleted of endogenous L-arginine. Initial experiments indicated that when isolated bovine aortic endothelial cells are grown in the absence of L-arginine for the final 24 h of cell culture, they fail to generate or release EDNO in response to bradykinin or A23187 (a calcium ionophore), as assessed by the bioassay cascade technique. We also noted that repeated additions of A23187 to isolated arterial or venous ring preparations mounted under tension in bath chambers results in the development of refractoriness or tolerance not only to A23187 but also to all other endothelium-dependent relaxants (27). Cross-tolerance does not develop, however, to endothelium-independent relaxants such as NO or nitroglycerin. Very interestingly, although the addition of L-arginine does not usually cause relaxation of healthy, endothelium-intact vascular rings, it does cause complete endothelium-dependent relaxation of rings that had been rendered tolerant to endothelium-dependent relaxation by pretreatment with A23187. In more recent experiments we found that incubation of healthy vascular ring prepara-

tions for 18–24 h in oxygenated Krebs bicarbonate solution at 37°C results in the development of complete or nearly complete refractoriness or tolerance to endothelium-dependent but not -independent relaxation (93). L-Arginine failed to relax refractory rings but relaxed tolerant rings and also restored the capacity of acetylcholine, bradykinin, and A23187 to relax tolerant rings. Preliminary experiments with intact arterial rings indicate that arginine levels in tissue are threefold lower in tolerant than control arterial rings (control rings, 300–350 nmol g^{-1} or 0.30–0.35 mM; tolerant rings; 90–110 nmol g^{-1} or 0.09–0.11 mM). A greater difference in arginine levels probably exists in the endothelial cells than in whole vascular ring preparations. We have found that the concentration of endogenous L-arginine in cultured bovine aortic endothelial cells grown in normal medium is about 9 nmol 10^7 $cells^{-1}$ or 0.8 mM; however, when the endothelial cells are grown in arginine-free medium during the final 24 h, the cellular concentration of L-arginine is less than 1 nmol 10^7 $cells^{-1}$.

Studies have now begun to focus on the identification and characterization of the enzyme system responsible for the conversion of endogenous L-arginine to NO in mammalian cells. Some limited progress has been made with the vascular endothelial cell system up to submission of this review, but more advances have been made with the macrophage system. A novel citrulline-forming enzyme activity present in the cytosolic fraction of vascular endothelial cells was found, and the authors implicated its involvement in the formation of NO from L-arginine (94). The soluble fraction catalyzed the conversion of L-arginine to citrulline in an NADPH-dependent manner, and conversion was inhibited by N^G-monomethyl-L-arginine. Thus, this activity closely resembles the activity that is believed to convert L-arginine to NO. This similarity may be superficial, however, as Palmer & Moncada (94) were unable to demonstrate the concomitant formation of NO in the same reaction mixtures that formed citrulline from L-arginine. In cytotoxic activated macrophages the soluble fraction possessed enzymatic activity that catalyzed the conversion of L-arginine to NO plus citrulline (61). This activity required NADPH and L-arginine; it was enhanced by Mg^{2+} and inhibited by N^G-monomethyl-L-arginine. Similar observations with activated macrophages were reported by Hibbs et al (33). The macrophage enzyme is believed to be an NADPH-dependent monooxygenase that first converts L-arginine into a labile N^G-hydroxy intermediate, which then undergoes oxidation to the -N=O derivative followed by fragmentation to NO. The remaining amino acid moiety engages in nucleophilic attack by H_2O to form citrulline.

The only enzyme study on vascular endothelial cells reported to date (94) provides information that is somewhat inconsistent with that provided by the studies on activated macrophages, in that the concomitant formation of equal amounts of NO and citrulline from L-arginine in reactions involving the

soluble fraction from aortic endothelial cells could not be demonstrated. Thus, it is possible that the alleged NADPH-dependent monooxygenase in vascular endothelial cells is not present in the cytosolic or soluble fraction. It is possible that other citrulline-forming enzymes that do not generate NO as a second reaction product are present in the soluble fraction, whereas the monooxygenase that generates both NO and citrulline is present in the plasma membrane fraction. For example, a novel enzymatic activity present in a rat kidney 10,000 × *g* supernatant fraction catalyzes the direct conversion of N^G,N^G-dimethyl-L-arginine or N^G-monomethyl-L-arginine to L-citrulline plus dimethylamine or methylamine without concomitant formation of NO (95).

Studies on vascular endothelial cells should focus on a careful analysis of the subcellular distribution of enzymatic activities capable of concomitant generation of equal amounts of NO and citrulline from L-arginine. The distribution of this enzymatic activity may be different in vascular endothelium than in the macrophage. Indeed, it is conceivable to me that the NO-synthetase or NADPH-dependent monooxygenase resides in the plasma membrane fraction rather than in the cytosolic compartment of the endothelial cell. It is the interaction between the extracellular endothelial cell surface and external factors such as endothelium-dependent vasodilators and shear stress forces that increases the formation and/or release of EDNO. A plasma membrane–bound, NADPH-dependent monooxygenase system would be much more amenable to regulation by external factors and forces. Moreover, the NO generated at or near the membrane surface would have more immediate access to the extracellular environment. The demonstration of NADPH-dependent conversion of L-arginine to NO plus citrulline in washed membrane fractions, confirmed by appropriate enzyme marker analyses, may require the use of an NADPH-regenerating system and reduced incubation temperatures to maintain the NADPH concentration at levels compatible with the K_m of the enzyme for NADPH. Subcellular membrane-enriched fractions are very active in utilizing NADPH for numerous redox reactions. The K_m of the enzyme for L-arginine should be similar to the endogenous intracellular endothelial concentration of L-arginine (0.5–1.0 mM; M. E. Gold, L. J. Ignarro, unpublished observations), and the K_m should be the same whether the reaction product being measured is NO or citrulline. The high arginine concentration in endothelial cells is consistent with the findings that fresh vascular rings do not relax in response to L-arginine, whereas 24-h-incubated tolerant rings relax up to 100% (93). As unpurified membrane fractions may be contaminated with entrapped or bound arginine, it may be wise to treat the fractions with arginase (bovine liver; 5–10 U ml^{-1}) before conducting reactions in which L-arginine is added back to reaction mixtures. Reactions should be conducted under air, oxygen, or nitrogen to ascertain the requirement of oxygen. Too high a pO_2, however, may require the addition of superoxide

dismutase to prevent superoxide anion–catalyzed oxidation of NO to NO_3^-. The dependence upon calcium for endothelium-dependent relaxation probably lies at the level of coupling of endothelial plasma membrane perturbation to monooxygenase activity rather than of catalysis itself. Nevertheless, comparisons should be made of reaction mixtures containing various concentrations of Ca^{2+} and/or Mg^{2+}. Finally, enzyme activity should be inhibited (competitively with respect to L-arginine substrate) by N^G-monomethyl-L-arginine. L-Argininosuccinic acid should cause irreversible inhibition, and SKF 525-A should also inhibit enzyme activity if the enzyme is truly an NADPH-dependent monooxygenase.

IS EDRF A MIXTURE OF NO PLUS AN *S*-NITROSOTHIOL?

Although NO has clearly been identified as an endothelium-derived relaxing factor, the question has been asked whether NO does really account completely for the relaxant effects of EDRF. The studies of Palmer et al, in which the chemiluminescence technique is used to assay NO, suggest that NO alone can account for the vascular smooth-muscle relaxant effects of EDRF released from cultured porcine aortic endothelial cells (19). This technique, however, cannot distinguish between authentic NO and a labile nitroso compound that spontaneously liberates NO in aqueous solution. The bioassay cascade, however, can distinguish between the compounds, and when the two techniques are used in conjunction, they show that NO may account for the actions of EDRF. The bioassay cascade, however, can distinguish between NO and a labile nitroso compound only if their biological half-lives are appreciably different. NO and *S*-nitrosocysteine cannot be distinguished by these procedures because the biological half-lives of 0.01–0.1 μM concentrations of these agents in oxygenated Krebs solution are very similar ($t_{1/2}$ = 3–5 s).

Myers et al have used the chemiluminescence procedure together with a bioassay to characterize the properties of EDRF released from bovine aortic endothelial cells (96). In contrast to the conclusions drawn by Palmer et al (19), these investigators argued that NO alone cannot account for the complete relaxant effects of EDRF. Although basally released NO was detected at a time when the endothelial cell perfusion medium caused relaxation of a bioassay detector arterial segment, the further relaxant effect of EDRF released in response to bradykinin or A23187 was not accompanied by sufficient additional release of NO to account completely for the relaxation elicited. The authors concluded that a labile nitroso compound may be released in addition to NO or that a labile nitroso compound that is more potent than NO itself could account for the EDRF activity. Another possibil-

ity, of course, is that technical problems with the chemiluminescence procedure and/or preparation and delivery of authentic NO standards could account for the divergent results obtained by these two groups. Although a detailed discussion of these procedures is beyond the scope of this review, some comments for the purpose of clarification are in order. A presumed authentic NO standard that is unknowingly contaminated with NO_2^- would yield erroneously high EC_{50} values in the bioassay but would be detected as 100% NO by chemiluminescence if the usual reflux reduction system is used, which quantitatively converts NO_2^- to NO prior to detection. Since NO, but not NO_2^-, is detected by chemiluminescence, the absence of a reduction system would allow NO_2^- to go undetected. Experience with this procedure indicates that it is wise to assay samples in both the absence and the presence of the reduction system and to compare the calculated amounts of NO with the results obtained in a bioassay to estimate more accurately the concentration of NO.

Additional reports have surfaced that provide indirect evidence for the possibility that EDRF is not entirely NO. Berkowitz and coworkers argue that the pharmacological properties of EDRF released from cultured arterial endothelial cells are not identical to those of authentic NO tested in the same bioassay (97–99). However, we could not confirm the principal difference, that NO but not EDRF relaxes nonvascular smooth muscle (100). Instead, we found that EDRF released from bovine pulmonary artery and human umbilical vein mimicked the relaxant action of authentic NO on strips of bovine tracheal smooth muscle, rat stomach fundus, rabbit taenia coli, and guinea pig ileum. Nevertheless, other differences were found that have led some to question the identity of EDRF as NO. In another study, in which electron paramagnetic resonance spectroscopy was used, EDRF released from cultured arterial endothelial cells failed to generate a spectrum that was charateristic of that for authentic NO radical (101). It may be of interest that, unlike its spontaneously generated product NO, *S*-nitrosocysteine itself is not paramagnetic (K. S. Wood, L. J. Ignarro, unpublished observations).

As discussed above, NO, *S*-nitrosocysteine, and EDRF are virtually indistinguishable in terms of pharmacology. High-pressure liquid chromatography techniques are available that can distinguish among various *S*-nitrosothiols and their corresponding free thiols, but the techniques are not yet sensitive enough to measure *S*-nitrosothiols at nanomolar concentrations. Other experiments conducted in this laboratory suggest indirectly that EDRF generated and released from bovine pulmonary artery and vein is a mixture of NO plus a labile *S*-nitrosothiol. Treatment of vascular rings with 0.1 mM nitroglycerin for 30–60 min results in the development of marked tolerance (1000–fold increase in EC_{50}) to the relaxant effect of nitroglycerin, with little or no cross-tolerance to the relaxant effects of NO or *S*-nitrosothiols. Partial

cross-tolerance (10- to 50-fold increase in EC_{50}), however, develops to the relaxant effects of endothelium-dependent vasodilators (acetylcholine, bradykinin, and A23187). On the basis of the view that nitroglycerin causes depletion of intracellular thiols (6, 7) and that cysteine is obligatory for the relaxant action of nitroglycerin (8, 11), these observations are consistent with the hypothesis that endothelium-dependent relaxants stimulate the formation and release of an *S*-nitrosothiol, perhaps *S*-nitrosocysteine. Studies from several laboratories wih N^G-monomethyl-L-arginine as a test agent indicate clearly that this arginine analog inhibits endothelium-dependent relaxation only partially. If EDRF could be accounted for entirely by NO, one might expect that high enough concentrations of N^G-monomethyl-L-arginine (1–3 mM) would abolish endothelium-dependent relaxation, but such is not the case. This observation is consistent with the veiw that NO is only one component of EDRF and that another component may be a preformed nitroso precursor to NO. We have found that the addition of N^G-monomethyl-L-arginine to arterial rings rendered tolerant to nitroglycerin nearly abolishes endothelium-dependent relaxation. These observations suggest that EDRF is a mixture of NO plus *S*-nitrosothiol in which the S-nitrosothiol serves as a preformed, temporarily stored nitroso precursor of NO. Perhaps the *S*-nitrosothiol is stored in acidic lysosomelike granules, where *S*-nitrosothiols are very stable, and is discharged from endothelial cells by a secretory process. Immediate contact with the extracellular environment, in which the pH is above 7, would result in the rapid, spontaneous liberation of NO. Indirect evidence for a secretory process was provided by the finding that agents that interfere with exocytosis or secretion cause partial inhibition of endothelium-dependent relaxation (102).

CONCLUSIONS AND FUTURE DIRECTION

Experimental evidence from several laboratories indicates clearly that the EDRF described originally by Furchgott & Zawadzki (14) is NO and/or a labile nitroso precursor of NO. It is highly likely that NO is the final active species responsible for the cyclic GMP-mediated vasodilator and antiplatelet actions of EDRF. EDNO appears to be synthesized from L-arginine via an enzymatic reaction catalyzed by an NADPH-dependent monooxygenase. Very little is known about this enzyme system, including its subcellular distribution and mechanism of activation. Acquisition of such knowledge will undoubtedly lead to a better understanding of the regulatory mechanisms involved in the formation of EDNO. Many questions must be answered, such as the precise roles of calcium and oxygen in endothelium-dependent relaxation, the mechanism of continuous or basal formation of EDNO from L-arginine, the coupling of endothelium-dependent vasodilator receptors on the

endothelial cell membrane to the enzyme system responsible for converting L-arginine to NO, the source of endogenous endothelial L-arginine, and the fate of NO_2^- that accumulates as a result of NO oxidation.

Numerous mechanisms can be proposed for the enzymatic conversion of L-arginine to NO in vascular endothelial cells. The current view by some is that a cytosolic, NADPH-dependent monooxygenase is responsible for the conversion of L-arginine to NO. Alternative hypotheses, however, are possible and, perhaps, probable. A hypothetical depiction of one such plausible mechanism is given in Figure 1. NO-synthetase (an NADPH-dependent monooxygenase) is conceived to be membrane-bound and associated with the endothelial plasma membrane. The enzyme possesses a low level of basal catalytic activity (basal formation of EDNO) and is not dependent on calcium. Endothelium-dependent vasodilators interact with selective endothelial cell surface receptors to mobilize calcium (extracellular and intracellular), which

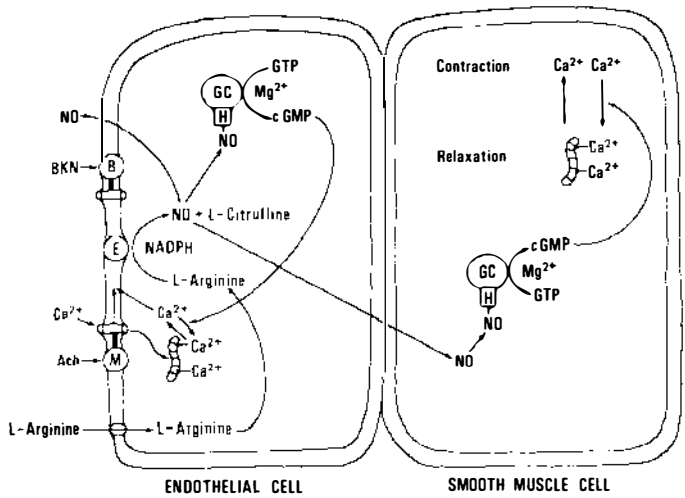

Figure 1 Schematic illustration of possible regulation of NO biosynthesis and action in endothelial cells. L-Arginine is converted to NO plus L-citrulline by a plasma membrane–bound, NADPH-dependent monooxygenase (E) that possesses basal catalytic activity in the absence of calcium. L-Arginine is derived from other tissues and enters endothelial cells via amino acid carriers or transporters. Endothelium-dependent vasodilators such as acetylcholine (Ach) or bradykinin (BKN) interact with selective endothelial cell surface receptors (M, muscarinic; B, bradykinin) to trigger calcium influx and mobilization of intracellular calcium. Increased levels of free calcium lead to the activation of the monooxygenase and thereby enhance NO formation. NO diffuses out of the cell in all directions. In the adjacent smooth-muscle cells, NO binds to the heme moiety (H) of cytosolic guanylate cyclase (GC), causing enzyme activation, and the elevated cyclic GMP (cGMP) levels lead to smooth-muscle relaxation by stimulation of intracellular binding of free calcium. In endothelial cells, NO activates guanylate cyclase by similar mechanisms and the cyclic GMP stimulates intracellular binding of free calcium, thereby preventing stimulation of NO synthesis but still allowing basal NO formation.

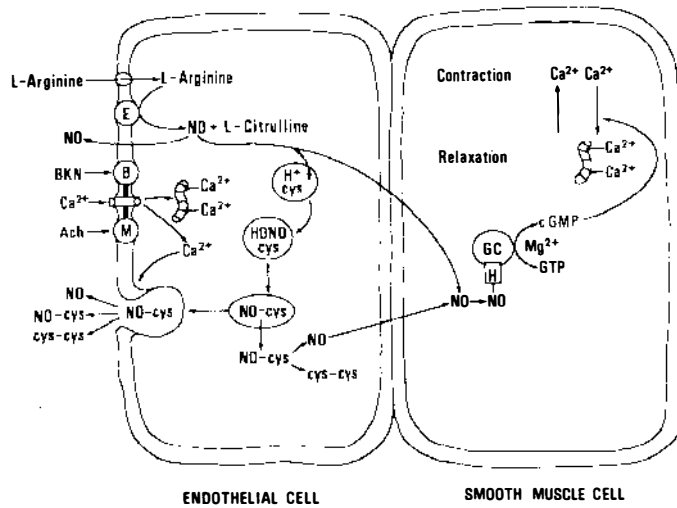

Figure 2 Schematic illustration of the biosynthesis of NO and *S*-nitrosocysteine in endothelial cells. L-Arginine is converted to NO plus L-citrulline by a plasma membrane–bound NADPH-dependent monooxygenase (E) that possesses basal catalytic activity in the absence of calcium. L-Arginine is derived from other tissues and enters endothelial cells via amino acid carriers or transporters. As NO is formed, it diffuses in all directions, and within the endothelial cell, it diffuses into lysosomes or other acidic granules containing cysteine (cys), where it is converted to nitrous acid (HONO) and readily reacts with cysteine to form *S*-nitrosocysteine (NO-cys). The *S*-nitrosocysteine is less lipophilic than NO and is stored temporarily within lysosomes, with only slow outward diffusion into the cytosol. Endothelium-dependent vasodilators such as acetylcholine (Ach) or bradykinin (BKN) interact with selective endothelial cell surface receptors (M, muscarinic; B, bradykinin) to trigger calcium influx and mobilization of intracellular calcium. Increased levels of free calcium facilitate the fusion of lysosomes with the plasma membrane, thereby promoting the exocytosis or secretion of granule contents (*S*-nitrosocysteine) into the extracellular environment. *S*-Nitrosocysteine decomposes spontaneously into NO and cystine (cys-cys). Secretion of *S*-nitrosocysteine can occur at either the luminal or abluminal surface of the endothelial cell (only the luminal surface is illustrated). In the adjacent smooth-muscle cells, NO derived either directly from L-arginine or from *S*-nitrosocysteine binds to the heme moiety (H) of cytosolic guanylate cyclase (GC), causing enzyme activation, and the elevated cyclic GMP (cGMP) levels to smooth-muscle relaxation by stimulation of intracellular binding of free calcium.

in turn leads to activation of NO-synthetase and accelerated conversion of L-arginine to NO plus citrulline. The source of endothelial cell L-arginine is likely to be extracellular, as endothelial L-arginine can be easily depleted. EDNO diffuses into adjacent endothelial cells, smooth muscle cells, and the lumen. EDNO causes heme-dependent activation of cytosolic guanylate cyclase and cyclic GMP accumulation in both endothelial and vascular smooth muscle cells. In vascular smooth muscle cyclic GMP causes relaxation, presumably by accelerating the intracellular binding of free calcium, thereby lowering the free calcium concentration. In endothelial cells cyclic GMP may

work by a similar mechanism in decreasing intracellular free calcium levels and thereby inhibiting the stimulation of NO formation from L-arginine. Such a negative feedback mechanism could control basal EDNO formation.

An alternative hypothesis (Figure 2) involves the notion that EDRF is a mixture of NO and an *S*-nitrosothiol. Basal formation of NO from L-arginine may proceed as described above, whereas the enhanced formation of EDRF elicited by endothelium-dependent relaxants may be attributed to a calcium-dependent exocytosis of recently preformed *S*-nitrosothiol. The *S*-nitrosothiol could be synthesized within acidic granules as a result of a simple nitrosation reaction between NO (HONO at low pH) and a thiol (perhaps cysteine) and stored temporarily in a relatively stable form in acidic granules. Although *S*-nitrosocysteine is less lipophilic than NO, some *S*-nitrosocysteine would continually leak out of the granules and undergo decomposition to NO and cystine (disulfide of cysteine). Exocytotic discharge of *S*-nitrosocysteine would result in the liberation of NO from the *S*-nitrosothiol, but a chemical analysis of endothelial cell perfusates within 5–10 s of stimulated release of EDRF should reveal the presence of both NO and *S*-nitrosothiol. Future experiments should be directed toward the elucidation of the precise chemical composition of EDRF, as well as the biosynthesis, storage, and release of its components.

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