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Review

Endothelium-Derived Nitric Oxide: Pharmacology and Relationship to the Actions of Organic Nitrate Esters

Louis J. Ignarro[1]

Vascular smooth muscle relaxation elicited by various endogenous substances results from their interaction with vascular endothelial cells to trigger the formation of endothelium-derived relaxing factor (EDRF). EDRF from pulmonary and peripheral arteries and veins and from cultured and freshly harvested aortic endothelial cells has been identified pharmacologically and chemically as nitric oxide (NO) or a labile nitroso compound. Endothelium-derived NO (EDNO) and authentic NO activate the cytoplasmic form of guanylate cyclase by heme-dependent mechanisms and thereby stimulate intracellular cyclic GMP accumulation in cells including vascular smooth muscle and platelets. Cyclic GMP functions as a second messenger to cause vascular smooth muscle relaxation and inhibition of platelet aggregation and adhesion to vascular endothelial surfaces. EDNO is synthesized from L-arginine and perhaps arginine-containing peptides by an unidentified calcium-requiring process coupled to the occupation of extracellular endothelial receptors. The biological actions of EDNO are terminated by spontaneous oxidation to NO_2^- and NO_3^-. The biological half-life of the very lipophilic EDNO is only 3–5 sec and this allows EDNO to function locally as an autacoid. Nitroglycerin and other organic nitrate esters elicit endothelium-independent relaxation after entering vascular smooth muscle cells and undergoing denitration and formation of NO. The pharmacological actions of nitroglycerin are therefore essentially the same as those of EDNO, and the endogenous NO receptor is the heme group bound to soluble guanylate cyclase. EDNO may serve a biological role to modulate local blood flow and platelet function.

KEY WORDS: endothelium-derived relaxing factor; nitric oxide; cyclic GMP; vasodilation; blood flow; platelet aggregation; thrombosis; artery; vein.

INTRODUCTION

Vasodilation or vascular smooth muscle relaxation *in vitro* in response to acetylcholine and related agents has been appreciated since the turn of the century. Nevertheless, many pharmacologists and physiologists had recognized and come to accept the perplexing observation that acetylcholine elicits contractile responses *in vitro* on isolated preparations of artery or vein. The reason for this difference in behavior remained an enigma until 1980 when careful experimentation revealed that acetylcholine could relax arterial preparations that had been prepared without damaging the endothelial cell layer that lines the intimal surface of all blood vessels (1). Further studies revealed that acetylcholine, bradykinin, other endogenous vasodilators, and the calcium ionophore A23187 contracted endothelium-denuded but relaxed endothelium-intact arterial preparations (see Ref. 2 for a review). The hypothesis was forwarded that endothelium-dependent relaxants interact with vascular endothelial cells to trigger the generation or release of a relaxing factor (1), later termed endothelium-derived relaxing factor or EDRF (3). Several years later EDRF was reported to inhibit the aggregation of platelets (4). Thus, it became clear that EDRF possessed at least two distinct and important biological actions.

Prior to the discovery of EDRF, nitric oxide (NO) was reported to elicit a marked but transient relaxation of coronary arterial smooth muscle (5), and shortly thereafter NO was shown to inhibit platelet aggregation and to cause disaggregation of platelets (6). Those studies with NO gas were initiated as a result of earlier experiments revealing that NO as well as nitrogen oxide-containing vasodilators (nitrovasodilators) activated cytoplasmic guanylate cyclase and stimulated tissue cyclic GMP accumulation (7–11). Thus, it was clear by 1981 that NO produced at least two distinct pharmacological actions.

The early to mid 1980s witnessed studies that revealed a close similarity in the pharmacological actions of EDRF, authentic NO, and certain nitrovasodilators (see Ref. 12 for a review). These observations led to the proposal in 1986 that EDRF may actually be NO (13,14). This proposal was soon followed by the acquisition of pharmacological and chemical evidence that EDRF is NO or a labile nitroso compound that spontaneously generates NO (15–17). Evidence now exists for the endogenous formation of NO not only by mammalian vascular endothelial cells (18) but also by cytotoxic activated murine macrophages (19) and possibly acti-

[1] Department of Pharmacology, University of California, Los Angeles, California 90024.

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vated neutrophils (20). The discovery that EDRF is NO or a closely related molecule provides a better understanding and appreciation of the long-standing pharmacological and clinical actions of nitroglycerin, other organic nitrate esters, organic nitrite esters, and nitroprusside. This Review addresses the pharmacological and biochemical properties, and mechanisms of action of EDRF and NO, our current knowledge on the biosynthesis, release, and metabolism of endothelium-derived NO (EDNO), and the physiological significance of EDNO and its relationship to the clinical actions of nitrovasodilators.

PHARMACOLOGICAL PROPERTIES OF EDRF

The existence of EDRF was first revealed in experiments where acetylcholine caused endothelium-dependent relaxation of precontracted strips of rabbit aorta, and this was antagonized by atropine but not by indomethacin or aspirin, and was diminished by potassium, quinacrine, eicosatetraynoic acid, and anoxic conditions (1). On the basis of a tissue sandwich technique, the authors proposed that acetylcholine stimulates the release of a relaxing factor from the vascular endothelium. Chemically diverse endogenous and other substances have been shown to cause endothelium-dependent relaxation by mechanisms involving EDRF (see Refs. 2 and 21 for reviews). The unequivocal release of EDRF from vascular tissue was first demonstrated by bioassay (22,23). It is now appreciated that EDRF can be released from the endothelium of intact artery or vein and from freshly harvested or cultured vascular endothelial cells. Bioassay techniques have been instrumental in evaluating the pharmacological properties of EDRF (see Ref. 12 for a review). One such bioassay technique is schematically illustrated in Fig. 1. A wide variety of naturally occurring substances interacts selectively with receptors on the endothelial cell surface that are coupled to intracellular processes associated with EDRF generation and/or release. Calcium and ATP-derived energy sources are required for EDRF formation, whereas magnesium is a competitive inhibitor of the actions of calcium (24,25).

EDRF activates cytoplasmic guanylate cyclase (26,27) by heme-dependent mechanisms (17), and this accounts for the capacity of EDRF to stimulate cyclic GMP accumulation in artery and vein (see Ref. 28 for a review). The biological actions of EDRF and thus of many endothelium-dependent vasodilators are antagonized by substances that inhibit guanylate cyclase activity (methylene blue; 29), bind or sequester EDRF (hemoglobin; 30), accelerate the inactivation of EDRF (catecholamines, superoxide anion, antioxidants; 22, 31–33), and inhibit the synthesis of EDRF from arginine or arginine-containing peptides (34–36). Endothelium-dependent relaxation can also be at least partially attenuated by substances that interfere with phospholipid or arachidonic acid metabolism and monooxygenase activity, including quinacrine, nordihydroguaiaretic acid, eicosatetraynoic acid, SKF-525A, gossypol, and related agents (see Ref. 37 for a review). The latter observations suggest that phospholipid metabolism and/or monooxygenase enzymes are involved in the biosynthesis of EDNO from arginine or arginine-containing peptides.

The chemical and pharmacological properties of EDRF

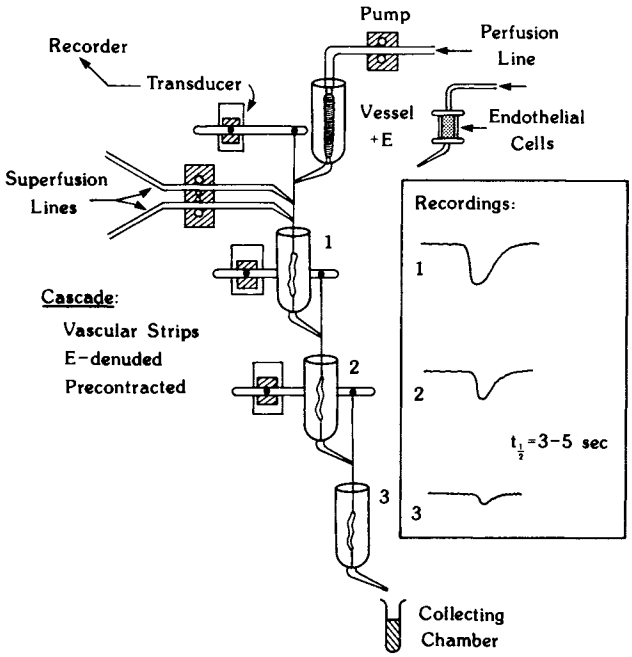

Fig. 1. Schematic of a bioassay cascade apparatus employed to study EDRF and other vasoactive factors. Krebs–bicarbonate buffer is perfused through a segment of artery or vein or a column of microcarrier beads on which endothelial cells have been grown. The perfusate is directed over three helical strips of precontracted endothelium-denuded artery or vein separated by about 3 sec in flow time. Changes in smooth muscle tone are monitored by transducers and recorded. EDRF release is monitored by ensuing decremental relaxant responses down the cascade of strips (see inset), attributed to the short half-life of EDRF. (Reproduced with permission from Ref. 80.)

from perfused artery or vein and from freshly harvested or cultured vascular endothelial cells are indistinguishable as assessed by bioassay (see Ref. 12 for a review). Thus, one common EDRF is likely to be responsible for the vascular smooth muscle relaxation observed. This EDRF is very unstable ($t_{1/2}$ = 3–5 sec), is further inactivated by superoxide anion, is protected from rapid inactivation by SOD or low oxygen tension, activates soluble guanylate cyclase by heme-dependent mechanisms, elevates smooth muscle levels of cyclic GMP, and relaxes both vascular and nonvascular smooth muscle, and its biological actions are antagonized by methylene blue and hemoglobin. Another similarity among EDRFs from several species is the property of inhibition of platelet aggregation (see Ref. 38 for a review).

PHARMACOLOGICAL PROPERTIES OF NO

NO is an unstable gas that is soluble in cold water to the extent of 1–3 m*M*. In the gaseous state and in dilute aqueous solution (less than 1 μ*M*) where oxygen is not excluded, NO is very labile and is spontaneously oxidized to NO_2 (gas) or NO_2^- (solution) and higher oxides of nitrogen. More concentrated solutions of NO are stable for minutes to hours depending on the NO concentration, oxygen tension, and temperature. Early experimentation involved the use of small aliquots of NO gas diluted in oxygen-free nitrogen or argon. More recently, dilute aqueous solutions of NO in ox-

ygen-free medium have been employed. The half-life of biologically active concentrations (10–100 n*M*) of NO in oxygen-containing media at 37°C and pH 7.4 is 3–5 sec (15–17).

Perhaps the earliest evidence that NO could elicit biologically important actions was the finding that NO and nitroso compounds could activate cytoplasmic guanylate cyclase and stimulate cyclic GMP formation in mammalian tissues (39,40). Since certain nitroso compounds had been shown to relax vascular smooth muscle, the hypothesis that cyclic GMP is a mediator of vascular smooth muscle relaxation was tested by ascertaining whether authentic NO can relax vascular smooth muscle (5). Low concentrations of NO were found to elicit marked but transient relaxation of precontracted strips of bovine coronary artery in a manner that was inhibited by methylene blue and hemoproteins such as hemoglobin, myoglobin, and methemoglobin (5). Typical responses to NO are illustrated in Fig. 2. NO also activated soluble guanylate cyclase prepared from bovine coronary artery and elevated vascular cyclic GMP levels, and both responses were inhibited by methylene blue and hemoproteins (5,41). These vascular actions were pharmacologically

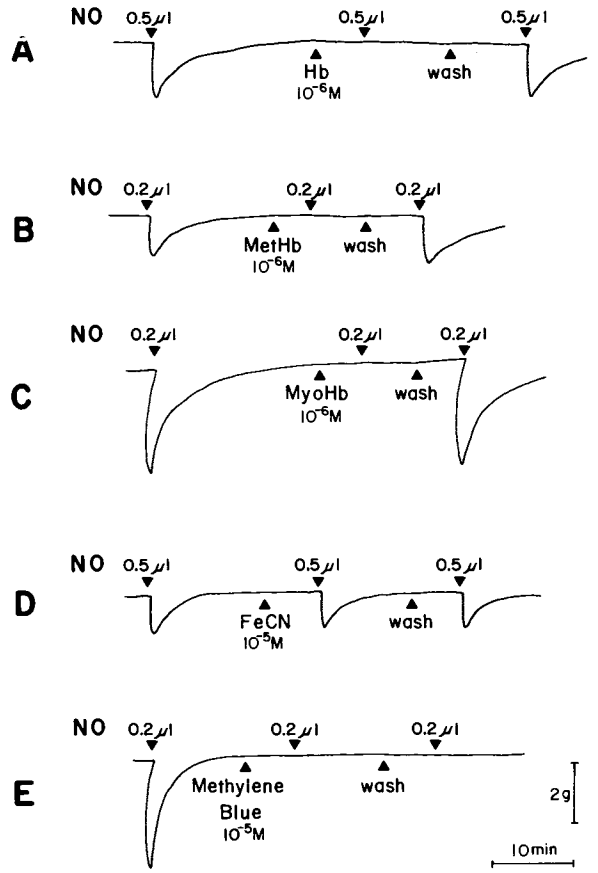

Fig. 2. Relaxation of bovine coronary artery by NO and inhibition by hemoproteins and methylene blue. Isolated strips of bovine coronary artery were mounted under tension and precontracted by addition of phenylephrine or KCl. Dilutions of NO in nitrogen were delivered in the gaseous state by injection into the bath chambers. A volume of 0.5 μl represents an approximate concentration of 0.01 μ*M* NO in the bathing medium. Hb, hemoglobin; MetHb, methemoglobin; myoHb, myoglobin; FeCN, potassium ferricyanide. (Reproduced with permission from Ref. 5.)

similar to those produced by nitrovasodilators, particularly on soluble guanylate cyclase (42). Certain nitrovasodilators activated guanylate cyclase only in the presence of thiols, and organic nitrate esters possessed a selective requirement for cysteine (42). The reaction between thiols and nitrovasodilators resulted in the formation of highly unstable *S*-nitrosothiols that underwent spontaneous decomposition with the generation of NO. The cardiovascular hemodynamic actions of nitroglycerin were indistinguishable from those of several *S*-nitrosothiols after i.v. infusion into anesthetized, closed-chest cats (42). Additional experiments showed that NO, *S*-nitrosothiols, and related nitrovasodilators relaxed both arterial and venous smooth muscle and that veins were more responsive than arteries to such actions (43).

In experiments originally designed to ascertain the biological actions of cyclic GMP on human platelet function, NO was discovered to elicit potent and marked inhibitory effects on platelet aggregation and also to reverse platelet aggregation (6). Platelet responses were associated with rapid and marked but transient cyclic GMP accumulation and were inhibited by methylene blue and hemoproteins. The inhibitory action of NO on platelet function was mimmicked by exogenously added 8-bromo-cyclic GMP. Thus, it was clear that stimulation of platelet cyclic GMP accumulation was associated with and might cause inhibition of platelet aggregation. Additional studies revealed that a series of unstable *S*-nitrosothiols produced effects on platelets that were virtually indistinguishable from those produced by NO (44). Both NO and *S*-nitrosothiols activated purified platelet soluble guanylate cyclase by heme-dependent mechanisms (44). These observations illustrated clearly that not only prostacyclin via the second-messenger actions of cyclic AMP, but also NO via the second-messenger actions of cyclic GMP elicited the same biological action on platelets. Additional studies showed that platelet accumulation of both cyclic GMP and cyclic AMP produced synergistic inhibitory effects on platelet aggregation (45).

Additional pharmacological actions of NO are currently being found or suspected in other cell types. Cytotoxic activated murine macrophages have been shown to synthesize nitrite and nitrate and such biochemical transformations are associated with cytotoxicity or the killing of phagocytized microorganisms (46–51). More recently, experiments have revealed that NO is the likely cytotoxic agent synthesized and that nitrite and nitrate are merely oxidation products of NO (19,52). Evidence for the possible formation and release of NO from activated neutrophils has been provided (20), although the role for such a substance in neutrophil function has not been established. Whether or not the NO generated by activated macrophages and perhaps neutrophils can elicit local effects on vascular smooth muscle and platelets is presently unknown.

CHEMICAL AND PHARMACOLOGICAL IDENTIFICATION OF EDRF AS NO

Earlier studies suggested that EDRF might be either a lipoxygenase or cytochrome P450 product of arachidonic acid metabolism (see Ref. 28 for a review). Another view was that EDRF was an unstable aldehyde, ketone, or lactone

(22), but these observations have not been extended or confirmed. Other studies revealed that the cleavage of phospholipids and release of fatty acids may be involved in or closely associated with the process of EDRF formation (see Ref. 37 for a review), but additional experiments indicated that most of the lipoxygenase and cytochrome P450 inhibitors tested produced highly inconsistent and nonselective effects on relaxant responses to various endothelium-dependent vasodilators and that the interpretation of such data is difficult (53). Nevertheless, this author believes that membrane phospholipid metabolism is linked in some manner to the synthesis and/or release of EDRF, perhaps via the second-messenger actions of intracellular calcium.

The previous sections of this Review highlighted the fact that the pharmacological actions of EDRF and NO are very similar. In 1986 two laboratories proposed independently that EDRF is NO or a labile nitroso compound. The proceedings of the symposium at which the proposals were presented were published almost 2 years later (13,14). These proposals were based on indirect observations on the similar pharmacological properties of EDRF and NO. The most convincing evidence to this author was the observation that like NO, EDRF activated purified soluble guanylate cyclase and that this enzyme activation was heme-dependent and inhibited by methylene blue or hemoglobin. Soluble guanylate cyclase is activated by only few compounds including NO, phenyl radical, protoporphyrin IX, and certain polyunsaturated fatty acids and peroxides (see Ref. 37 for a review). Only NO and phenyl radical activate soluble guanylate cyclase by heme-dependent mechanisms and only the action of NO is inhibited by methylene blue or hemoglobin. Thus, the actions of EDRF and NO are indistinguishable with regard to the mechanism of activation of soluble guanylate cyclase.

The chemical identification of EDRF was provided independently in 1987 by two laboratories. One group showed that cultured aortic endothelial cells could generate NO in response of the cells to bradykinin, and NO was detected and quantified by a chemiluminescence procedure (15). The basis of this procedure is that NO reacts with added ozone to generate an excited state of NO_2 which can be detected by chemiluminescence. Cultured endothelial cells were grown on microcarrier beads and perfused within a small column in a bioassay cascade system where the perfusates were directed over several isolated strips of endothelium-denuded artery. The addition of bradykinin to the medium perfusing the endothelial cells caused the release of EDRF, which was detected by its relaxant action on the superfused arterial strips. The superfusion medium contained amounts of NO that could account for the relaxant action of EDRF. This technique is very sensitive but cannot distinguish between authentic NO and labile nitroso compounds that spontaneously liberate NO.

EDRF released from endothelium-intact pulmonary artery and vein and human umbilical vein was identified as NO by a chemical procedure based on the NO-catalyzed diazotization of sulfanilic acid and subsequent coupling of the diazo product with *N*-naphthylethylenediamine to yield an intensely colored complex that can be monitored spectrophotometrically (16). A bioassay cascade technique similar to that described above was employed here as well in order to characterize the pharmacological profiles of EDRF and authentic NO. This chemical technique is less sensitive than the chemiluminescence method and cannot distinguish between NO and labile nitroso compounds.

Another procedure used to identify EDRF as NO was based on the high reactivity between NO and hemoglobin to yield NO-hemoglobin. Hemoglobin was found to react with EDRF released from freshly harvested aortic endothelial cells in a manner that was identical for the same reaction involving NO (17). Under conditions of low oxygen tension NO reacts rapidly with the heme moiety of hemoglobin to yield the NO- or nitrosyl-adduct of hemoglobin. NO-hemoglobin possesses a distinct absorbance in the Soret region and can be identified and quantified to a limited extent. This technique is not as sensitive as the two described above and cannot distinguish between NO and labile nitroso compounds. Moreover, the presence of dissolved oxygen can sometimes generate oxyhemoglobin that is rapidly oxidized to methemoglobin by any NO present. A significant amount of methemoglobin can obscure any absorbance peaks characteristic of NO-hemoglobin.

It is clear from the above methodology that it has not been possible to conclude unequivocally that EDRF is NO and not a labile nitroso compound. For example, EDRF may be a labile *S*-nitrosothiol that undergoes spontaneous oxidation with the liberation of NO and formation of the corresponding disulfide. It is likely, however, that the important working end of the molecule is NO. Table I lists the pharmacological and chemical similarities between EDRF and NO. Thus, in the same way that other potent pharmacological agents have been subsequently discovered to occur endogenously, NO, which was described as a potent vasodilator and inhibitor of platelet aggregation 10 years ago, now has been identified to occur endogenously as EDRF in mammalian blood vessels. The potent vasodilator nitroglycerin, which has been used clinically for over a century, has an endogenous counterpart in NO since NO is the pharmacologically active species of nitroglycerin.

Table I. Identical Chemical and Pharmacological Properties of EDRF and NO

1. Chemically unstable: $t_{1/2}$ = 3–5 sec under bioassay conditions
2. Accelerated instability by oxygen and superoxide anion
3. Chemical instability attributed to spontaneous oxidation to NO_2^- and NO_3^-
4. Chemical stability and duration of action prolonged by superoxide dismutase and acidic pH
5. Lipophilicity and rapid diffusion through membrane barriers
6. High binding affinity for and reactivity with reduced hemoproteins; formation of nitrosyl hemoproteins
7. Oxidation of oxyhemoglobin to methemoglobin
8. Reaction with ozone, sulfanilic acid, and hemoproteins
9. Heme-dependent activation of cytoplasmic guanylate cyclase; inhibition by methylene blue
10. Stimulation of cyclic GMP accumulation in and relaxation of both vascular and nonvascular smooth muscle
11. Stimulation of cyclic GMP accumulation in platelets and inhibition of platelet aggregation and adhesion
12. Termination of biological actions by oxygen, superoxide anion, hemoglobin, myoglobin, and methylene blue

MECHANISM OF ACTION OF NO

NO elicits its two principal biological actions by activating soluble guanylate cyclase and thereby stimulating cyclic GMP formation in the target tissues. Cyclic GMP acts as a second messenger in expressing the actions of NO. One likely mechanism by which intracellular cyclic GMP causes vascular smooth muscle relaxation and inhibition of platelet function is by promoting a rapid lowering of the concentration of free uncomplexed calcium, perhaps by stimulating calcium binding to intracellular proteins (54,55).

NO activates soluble guanylate cyclase by heme-dependent mechanisms (40,44,56,57). Many hemoproteins like hemoglobin have a high binding affinity for NO and the heme moiety reacts with NO to form a nitrosyl-heme complex of the hemoprotein. Thus, NO reacts with hemoglobin to generate NO-hemoglobin. A similar reaction occurs with guanylate cyclase, which is a hemoprotein containing 1 mol of heme per mol of holoenzyme (58,59). Guanylate cyclase from which heme is detached is not activated by NO, whereas heme-containing or heme-reconstituted enzyme is markedly activated by NO (56,57). Preformed NO-heme activates heme-deficient guanylate cyclase in a manner that is indistinguishable from the activation of heme-containing enzyme by NO. Heme-dependent activation of soluble guanylate cyclase by NO has been demonstrated for enzyme purified from bovine lung (56,57), rat liver (60), and human platelets (44).

The mechanism by which the NO-heme complex activates guanylate cyclase is unknown, but one possible mechanism is that the binding of NO to heme-iron may cause a change in the conformation of the NO-heme complex in such a manner that the bond between the iron and the enzyme protein may be broken (61). Binding of the underlying porphyrin ring to the enzyme may cause activation. This view is supported by the observation that protoporphyrin IX (heme without iron) activates guanylate cyclase by mechanisms that are identical kinetically to the mechanism by which NO-heme causes enzyme activation (62). Thus, NO-heme activates guanylate cyclase by a protoporphyrin IX-like interaction. Structural analogues of protoporphyrin IX also activate guanylate cyclase, whereas metalloporphyrins such as ferroprotoporphyrin IX (heme), zinc protoporphyrin IX, and manganese protoporphyrin IX completely inhibit enzyme activation by either protoporphyrin IX or NO-heme (63). Zinc and manganese protoporphyrin IX also inhibit basal activity or unactivated guanylate cyclase. The K_I values for the three metalloporphyrins are quite different, thus indicating that the metal directly influences the binding of the metalloporphyrin to guanylate cyclase.

MECHANISM OF ACTION OF ORGANIC NITRATE AND NITRITE ESTERS

Many studies published since the mid 1970s suggest clearly that the organic nitrate and nitrite esters cause vascular smooth muscle relaxation through the actions of NO (see Refs. 28, 39, 64, and 65 for reviews). The initial studies were actually conducted not with esters but rather with inorganic substances such as nitrite ion and nitroprusside. Organic *N*-nitroso compounds were also examined. The activation of soluble guanylate cyclase by these substances resembled closely the enzyme activation observed by authentic NO. Like NO, these compounds elevated tissue levels of cyclic GMP and such effects were inhibited by methylene blue.

In initial experiments we had observed that acidic solutions of sodium nitrite and neutral solutions of sodium nitroprusside or *N*-nitrosoguanidines were unstable and decomposed with the liberation of NO into the closed atmosphere above the respective solutions. Aliquots of the solutions as well as gaseous aliquots of the atmosphere both caused activation of guanylate cyclase and vascular smooth muscle relaxation, and both responses were inhibited by methylene blue. We were unable to observe significant enzyme activation by nitroglycerin in the absence of added thiols, whereas high concentrations of inorganic nitrite and isoamyl nitrite, an organic nitrite ester, did cause enzyme activation (66,67). The addition of thiols to enzyme reaction mixtures markedly enhanced enzyme activation by nitrite and isoamyl nitrite. Nitroglycerin and other organic nitrate esters activated guanylate cyclase only in the presence of cysteine (42,66).

The consistent observations that vascular smooth muscle relaxation elicited by NO was antagonized by hemoglobin or methemoglobin and methylene blue, whereas relaxant responses to organic nitrate and nitrite esters were antagonized only by methylene blue suggested that NO permeates smooth muscle cells to produce its biologic effect intracellularly, whereas the other nitrovasodilators permeate smooth muscle cells and undergo biotransformation to NO within the intracellular compartment (5). Added hemoproteins can act only in the extracellular compartment, whereas methylene blue is a vital biological stain and therefore permeates cells. Thus, extracellular hemoproteins can bind NO only in the extracellular compartment and methylene blue blocks the activation of guanylate cyclase by intracellular NO. A subsequent series of experiments led to the conclusion that the nitrovasodilators in general react with thiols to yield unstable intermediate *S*-nitrosothiols, which decompose spontaneously with the liberation of NO (42). With the exception of the organic nitrate esters, the other nitrovasodilators can react with a variety of thiols to yield the corresponding labile *S*-nitrosothiol. The organic nitrate esters, however, have a selective requirement for cysteine and on reaction with cysteine yield *S*-nitrosocysteine. *S*-Nitrosocysteine is so labile that it cannot exist in the solid form. Aqueous or methanolic solutions of *S*-nitrosocysteine are red in color, and aqueous solutions are extremely labile and spontaneously liberate NO gas. The *in vitro* vascular smooth muscle relaxant effects of *S*-nitrosothiols mimic those of NO, and their *in vivo* hemodynamic effects mimic those of nitroglycerin (42). Thus, it is likely that, *in vivo*, nitroglycerin and other organic nitrate esters interact with free thiols such as cysteine in vascular smooth muscle cells to generate labile intermediate *S*-nitrosothiols that degrade spontaneously to liberate NO (Fig. 3). Organic nitrite esters probably elicit their effects via a very similar interaction with intracellular thiols.

Chronic administration of nitroglycerin to humans can lead to the development of tolerance to the vascular smooth muscle relaxant action of nitroglycerin with only little cross-tolerance to nitroprusside. The mechanism of development

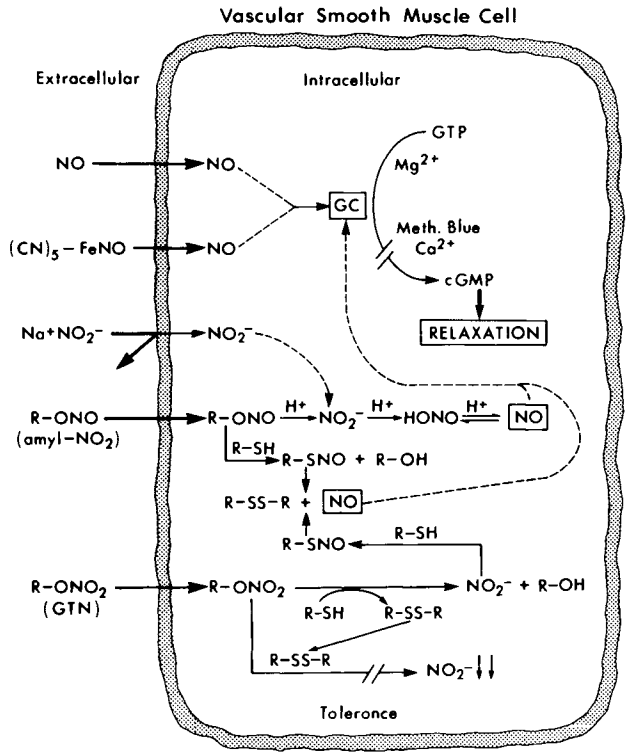

Fig. 3. Schematic of proposed mechanism of vascular smooth muscle relaxation by nitrovasodilators. R-ONO, organic nitrite ester; $R-ONO_2$, organic nitrate ester; GTN, glyceryl trinitrate or nitroglycerin; $(CN)_5$-FeNO, nitroprusside; HONO, nitrous acid; R-SH, thiol; R-SNO, *S*-nitrosothiol; R-OH, denitrated organic nitrite/nitrate ester; R-SS-R, disulfide; GC, cytoplasmic guanylate cyclase; cGMP, cyclic GMP; Meth. Blue, methylene blue. (Reproduced with permission from Ref. 28.)

of tolerance was first attributed to the depletion of smooth muscle sulfhydryl groups or thiols caused by the oxidative action of high concentrations of nitroglycerin (68,69). Tolerance was accompanied by a markedly reduced capacity for generation of nitrite from nitroglycerin in the tissues, but the authors attributed the development of tolerance to a paucity of selective sulfhydryl groups with which nitroglycerin must interact to elicit a biological response. Since nonselective thiol alkylating agents inhibited the actions of both nitroglycerin and nitroprusside, the authors concluded that nitroprusside must interact with a pool of sulfhydryl groups distinct from that which interacts with nitroglycerin. Responsiveness to these vasodilators could be restored by addition of thiol reducing agents.

This hypothesis was confirmed and extended by the observations that nitrovasodilators react with thiols to form labile *S*-nitrosothiols and that cysteine is specifically required for the reaction involving organic nitrate esters (42). Arterial and venous tissues rendered tolerant to nitroglycerin do not show appreciable cross tolerance to added *S*-nitrosothiols or NO itself (64). Recent clinical studies support the above interpretations in that i.v. *N*-acetylcysteine improves the responsiveness of patients tolerant to nitroglycerin for treatment of angina or improvement of coronary sinus blood flow (see Ref. 28 for a review). Additional studies have supported this view in artery and vein.

Biotransformation of nitroglycerin has been shown to occur concurrently with relaxation of rabbit aorta elicited by nitroglycerin (70). One study revealed that NO generation from organic nitrate esters is catalyzed by cysteine and that such a reaction accounts for the capacity of nitrates to activate soluble guanylate cyclase and cause vascular smooth muscle relaxation (71). One of the conclusions reached, however, was the possibility that *S*-nitrosocysteine is not an intermediate and that NO is released directly from the nitrate ester (72). Another study supported the view that organic nitrate esters react with thiols to yield *S*-nitrosothiols (73). This study revealed that *N*-acetylcysteine potentiates the *in vivo* hypotensive action of nitroglycerin, not by any pharmacokinetic mechanism, but rather by reacting with nitroglycerin in the extracellular compartment to generate lipophilic *S*-nitrosothiols. The authors suggested that such a reaction may also account for the capacity of *N*-acetylcysteine to reverse tolerance developed to nitroglycerin.

BIOSYNTHESIS, RELEASE, AND METABOLISM OF EDNO

The chemical properties of NO are compatible with the view that NO functions locally, soon after its formation in vascular endothelial cells. Once synthesized, the NO can diffuse rapidly into the underlying smooth muscle and blood compartment. Because of its unstable nature and lipophilicity, it is reasonable to suppose that NO is not stored in any granules but is available to act immediately after its biosynthesis. Therefore, the requirement of oxygen, extracellular calcium, and ATP-derived energy sources for demonstration of endothelium-dependent relaxation or EDRF release from endothelial cells may be attributed to their roles in promoting the biosynthesis of NO. Multiple steps are likely associated with the overall biosynthesis of NO. One must consider not only the chemical conversion of substrate(s) to NO, but also the biochemical reactions that couple endothelial cell receptor occupancy to stimulation of NO formation.

It is possible, however, that EDNO is derived from a relatively more stable nitroso precursor that may or may not be stored in acidic lysosome-like granules. A small organic *N*-nitroso or *S*-nitroso compound would be more stable than pure NO under such conditions and could slowly decompose spontaneously with the generation of NO. A slow generation of EDNO could account for the basal release of EDRF so often observed by many investigators. More information on the precise biochemical pathway of NO biosynthesis is required before the above concept can be addressed further.

The most likely pathway for the biosynthesis of EDNO is enzymatic oxidation of L-arginine to L-citrulline plus NO (18,74). ^{15}N-Enriched NO was identified as one product of a reaction between cultured aortic endothelial cells and L-arginine enriched with ^{15}N at the basic guanidino nitrogen atom. The enzymatic activity appears to reside primarily in the soluble or cytoplasmic fraction and requires NADPH. In this regard, this conversion of L-arginine to NO resembles that which is believed to occur in cytotoxic activated murine macrophages (19,52) and perhaps other cell lines (75). The enzyme may 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 deriva-

tive and fragmentation to NO. The amino acid moiety which remains undergoes nucleophilic attack by water to yield L-citrulline. Structural analogues of L-arginine (N^G-monomethyl-L-arginine and L-canavanine) that are known to antagonize endothelium-dependent relaxation also inhibit the enzymatic conversion of L-arginine to NO.

Although it appears that L-arginine serves as a precursor to EDNO, the possibility that arginine-containing peptides also serve as precursors has been suggested (76,77). This view was based on an earlier study showing that poly-L-arginine caused endothelium-dependent relaxation via the action of EDRF (78). A study from this laboratory revealed that basic polyamino acids, in general, cause endothelium-dependent relaxation mediated by EDNO (79). Evidence was obtained that these high molecular weight basic peptides stimulate the formation of EDNO from perfused artery and vein. Prolonged contact between peptide and blood vessel caused the development of tolerance or refractoriness or tachyphylaxis to endothelium-dependent relaxation without influencing endothelium-independent, NO-elicited relaxation. Two mechanisms can account for the vascular actions of the basic polyamino acids. One is that they serve as alternate substrates for the enzyme that catalyzes the formation of EDNO and that refractoriness develops as a result of desensitization of the enzyme system. Alternatively, the basic polyamino acids may initially stimulate conversion of endogenous L-arginine to NO and subsequently cause enzyme desensitization. An explanation of the latter possible mechanism is lacking because the interaction between the peptides and the endothelial cells does not appear to be receptor mediated (78). Answers to this question will come from studies designed to elucidate the influence of basic polyamino acids specifically on the enzymatic conversion of L-arginine to NO.

Once the precise biochemical pathway for the biosynthesis of EDNO is elucidated, the next step will be to unravel the mechanism by which endothelial cell receptor occupancy is coupled to EDNO generation. Acetylcholine and bradykinin interact with selective receptors to trigger EDNO release by mechanisms that are dependent on the presence of extracellular calcium, and the calcium ionophore A23187 is a potent endothelium-dependent relaxant that works by provoking EDNO release. Thus, extracellular calcium is involved in turning on the biochemical machinery that is ultimately involved in promoting the formation of EDNO. As discussed above, it is possible that EDNO is formed from a relatively more stable nitroso compound. How this nitroso compound would be formed from L-arginine is another matter. Although unlikely perhaps, NO may first be formed from L-arginine and subsequently trapped by nitrosation of L-cysteine or glutathione, thereby forming an *S*-nitrosothiol. The *S*-nitrosothiol would be stable for several minutes or longer at neutral or slightly acidic pH but would rapidly decompose at pH 7.4 or higher by oxidation to NO and the disulfide cystine.

The mechanism of release of EDNO into the extra-endothelial cell environment is likely one of simple diffusion immediately after biosynthesis. The metabolism or termination of action of EDNO very likely proceeds by spontaneous oxidation to inorganic nitrite and nitrate. The latter anions show little or no capacity to relax vascular smooth muscle at concentrations lower than 1 m*M*. The spontaneous oxidation of NO is accelerated by normal oxygen tension and this accounts for the very short half-life (3–5 sec) of EDNO in biological fluids. This spontaneous inactivation of EDNO is further accelerated by superoxide anion that may be generated in oxygen-containing solutions or tissues (see Ref. 28 for a review). It is possible that the concomitant generation of superoxide anion and NO by vascular endothelial cells could ensure rapid inactivation of NO and thus localization of action of EDNO. It is unnecessary to speculate that enzymatic or tissue reuptake mechanisms are involved in terminating the action of EDNO. These chemical properties endow NO with one of the most important requisites of a local modulator or autacoid, namely, rapid termination of action so that the biological action remain localized. A simplified schematic illustrating the proposed biosynthesis, metabolism, and action of EDNO is presented in Fig. 4.

CONCLUSIONS AND CLINICAL SIGNIFICANCE OF EDNO

EDNO can be added to the growing list of important local mediators of biological action. EDNO causes local vasodilation and inhibition of thrombosis and may play some role in modulating local blood flow. The NO generated within cytotoxic activated murine macrophages, and perhaps activated neutrophils, appears to function as a part of the cytotoxic action of these cells against certain target tissues. It is conceivable that NO generated from such cells could also influence local vascular smooth muscle tone and platelet function and thereby complement the potential pathophysiological function of such cytotoxic cells.

EDNO elicits its biological actions by causing heme-dependent activation of cytoplasmic gyanylate cyclase, stimulation of intracellular cyclic GMP accumulation, and probably a lowering of the intracellular free calcium concentration. The intracellular receptor for EDNO is precisely the same intracellular receptor as that for exogenously administered organic nitrates or nitrites, inorganic or organic nitroso

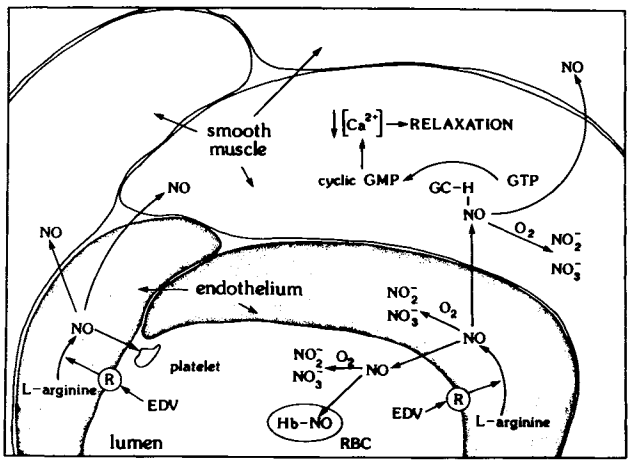

Fig. 4. Schematic representation of the synthesis, metabolism, and action of EDNO. EDV, endothelium-dependent vasodilator; R, selective receptors; H, heme moiety of cytoplasmic guanylate cyclase (GC); Hb, hemoglobin; RBC, red blood cells. (Reproduced with permission from Ref. 80.)

compounds, and NO. This receptor is the heme moiety bound to cytoplasmic guanylate cyclase. Other hemoproteins can compete with guanylate cyclase for NO binding and thereby terminate the action of NO. Since the tissue receptor sites for EDNO and clinically employed nitrovasodilators are indistinguishable, the biological actions of EDNO are identical to those of the nitrovasodilators, the only subtle differences being attributed to pharmacokinetic properties.

What is the biological and clinical significance of the endogenous nitrovasodilator? In view of the novelty of this field of research, one can only offer educated speculation at this time. Some investigators close to this field suppose that EDNO functions as a local tissue hormone or autacoid in modulating or regulating local blood flow in response to chemical agents (bradykinin, histamine, substance P, others) and shear forces developed at the blood vessel wall–blood junction. In the latter instance, a sudden local increase in blood flow would stretch the vessel wall, create shear stress, and trigger EDNO formation, which would act to dilate the vessel and thereby accommodate the sudden local increase in blood volume. Another logical and predictable function of EDNO is to inhibit local platelet adhesion to and aggregation at the vascular endothelial surface. This would especially be true if, as suspected, there is a continual generation and release of EDNO from healthy endothelium.

If the above speculation on the physiological significance of EDNO is true, what happens to local blood flow and platelet function in the event of vascular endothelial damage provoked by pathophysiological or traumatic means? If continual or basal generation of EDNO occurs in order to limit smooth muscle contractile tone and to provide a thrombogenic-free intimal surface, then endothelial damage could provoke local vasoconstriction and thrombosis. Such pathophysiological responses could have deleterious consequences if they should occur in the coronary or cerebral arterial circulation. If studies show this to be the case, then drugs that deliver exogenous NO could be useful as replacement therapy for locally deficient EDNO. Drugs that stimulate EDNO formation would not be useful because damaged endothelium cannot synthesize NO. Perhaps certain presently available drugs such as nitroglycerin, other organic nitrate esters, or nitroprusside would be useful clinically in such situations. An injectable solution of a very labile nitroso compound that rapidly delivers NO itself might be useful in treating certain vascular endothelial disorders. Such a labile nitroso compound could be one of several *S*-nitrosothiols. New concepts pertaining to transdermal delivery of NO could provide novel and effective ways of administering *S*-nitrosothiols or inorganic nitrites by way of patches or pads. For example, a dilute solution of $NaNO_2$ in ascorbic acid or any other weak organic acid slowly generates NO. The NO would dissolve in the aqueous vehicle and rapidly permeate tissues to elicit local biological actions.

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REFERENCES

1. R. F. Furchgott and J. V. Zawadzki. *Nature* (*Lond.*) 288:373–376 (1980).
2. R. F. Furchgott. *Circ. Res.* 53:557–573 (1983).
3. P. D. Cherry, R. F. Furchgott, J. V. Zawadzki, and D. Jothianandan. *Proc. Natl. Acad. Sci. USA* 72:2106–2110 (1982).
4. H. Azuma, M. Ishikawa, and S. Sekizaki. *Br. J. Pharmacol.* 88:411–415 (1986).
5. C. A. Gruetter, B. K. Barry, D. B. McNamara, D. Y. Gruetter, P. J. Kadowitz, and L. J. Ignarro. *J. Cyclic Nucleotide Res.* 5:211–224 (1979).
6. B. T. Mellion, L. J. Ignarro, E. H. Ohlstein, E. G. Pontecorvo, A. L. Hyman, and P. J. Kadowitz. *Blood* 57:946–955 (1981).
7. J. Diamond and K. S. Blisard. *Mol. Pharmacol.* 12:688–692 (1976).
8. S. Katsuki, W. Arnold, C. Mittal, and F. Murad. *J. Cyclic Nucleotide Res.* 3:23–35 (1977).
9. W. P. Arnold, C. K. Mittal, S. Katsuki, and F. Murad. *Proc. Natl. Acad. Sci. USA* 74:3203–3207 (1977).
10. K. D. Schultz, K. Schultz, and G. Schultz. *Nature* (*Lond.*) 265:750–751 (1977).
11. E. Bohme, H. Graf, and G. Schultz. *Adv. Cyclic Nucleotide Res.* 9:131–143 (1978).
12. L. J. Ignarro. *FASEB J.* 3:31–36 (1989).
13. L. J. Ignarro, R. E. Byrns, and K. S. Wood. In P. M. Vanhoutte (ed.), *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium,* Raven Press, New York, 1988, pp. 427–435.
14. R. F. Furchgott. In P. M. Vanhoutte (ed.), *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium,* Reven Press, New York, 1988, pp. 401–414.
15. R. M. J. Palmer, A. G. Ferrige, and S. Moncada. *Nature* (*Lond.*) 327:524–526 (1987).
16. L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns, and C. Chaudhuri. *Proc. Natl. Acad. Sci. USA* 84:9265–9269 (1987).
17. L. J. Ignarro, R. E. Byrns, G. M. Buga, and K. S. Wood. *Circ. Res.* 61:866–879 (1987).
18. R. M. J. Palmer, D. S. Ashton, and S. Moncada. *Nature* (*Lond.*) 333:664–666 (1988).
19. M. A. Marletta, P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok. *Biochemistry* 27:8706–8711 (1988).
20. T. J. Rimele, R. J. Sturm, L. M. Adams, D. E. Henry, R. J. Heaslip, B. M. Weichman, and D. Grimes. *J. Pharmacol. Exp. Ther.* 245:102–111 (1988).
21. R. F. Furchgott. *Annu. Rev. Pharmacol. Toxicol.* 24:175–197 (1984).
22. T. M. Griffith, D. H. Edwards, M. J. Lewis, A. C. Newby, and A. H. Henderson. *Nature* (*Lond.*) 308:645–647 (1984).
23. T. M. Griffith, A. H. Henderson, D. H. Edwards, and M. J. Lewis. *J. Physiol.* 351:13–24 (1984).
24. H. A. Singer and M. J. Peach. *Hypertension* 4:II19–II25 (1982).
25. D. D. Ku and H. S. Ann. *J. Pharmacol. Exp. Ther.* 241:961–966 (1987).
26. L. J. Ignarro, R. G. Harbison, K. S. Wood, and P. J. Kadowitz. *J. Pharmacol. Exp. Ther.* 237:893–900 (1986).
27. U. Forstermann, A. Mulsch, E. Bohme, and R. Busse. *Circ. Res.* 58:531–538 (1986).
28. L. J. Ignarro. *Circ. Res.* (in press).
29. L. J. Ignarro, T. M. Burke, K. S. Wood, M. S. Wolin, and P. J. Kadowitz. *J. Pharmacol. Exp. Ther.* 228:682–690 (1984).
30. W. Martin, G. M. Villani, D. Jothianandan, and R. F. Furchgott. *J. Pharmacol. Exp. Ther.* 232:708–716 (1985).
31. G. M. Rubanyi, R. R. Lorenz, and P. M. Vanhoutte. *Am. J. Physiol.* 249:H95–H101 (1985).
32. G. M. Rubanyi and P. M. Vanhoutte. *Am. J. Physiol.* 250: H822–H827 (1986).

33. S. Moncada, P. M. J. Palmer, and R. J. Gryglewski. *Proc. Natl. Acad. Sci. USA* 83:9164–9168 (1986).
34. R. M. J. Palmer, D. S. Ashton, and S. Moncada. *Nature (Lond.)* 333:664–666 (1988).
35. H. H. H. W. Schmidt, M. M. Klein, F. Niroomand, and E. Bohme. *Eur. J. Pharmacol.* 148:293–295 (1988).
36. I. Sakuma, D. Stuehr, S. S. Gross, C. Nathan, and R. Levi. *Proc. Natl. Acad. Sci. USA* 85:8664–8667 (1988).
37. U. Forstermann. *J. Cardiovasc. Pharmacol.* 8(Suppl. 10):S45–S51 (1986).
38. S. Moncada, M. W. Radomski, and R. M. J. Palmer. *Biochem. Pharmacol.* 37:2495–2501 (1988).
39. F. Murad, W. P. Arnold, C. K. Mittal, and J. M. Braughler. *Adv. Cyclic Nucleotide Res.* 11:175–204 (1979).
40. P. A. Craven and F. R. DeRubertis. *J. Biol. Chem.* 253:8433–8443 (1978).
41. C. A. Gruetter, D. Y. Gruetter, J. E. Lyon, P. J. Kadowitz, and L. J. Ignarro. *J. Pharmacol. Exp. Ther.* 219:181–186 (1981).
42. L. J. Ignarro, H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz, and C. A. Gruetter. *J. Pharmacol. Exp. Ther.* 218:739–749 (1981).
43. J. C. Edwards, L. J. Ignarro, K. S. Wood, A. L. Hyman, and P. J. Kadowitz. *J. Pharmacol. Exp. Ther.* 228:33–42 (1984).
44. B. T. Mellion, L. J. Ignarro, C. B. Myers, E. H. Ohlstein, B. A. Ballot, A. L. Hyman, and P. J. Kadowitz. *Mol. Pharmacol.* 23:653–664 (1983).
45. B. T. Mellion, P. M. Horwitz, A. L. Hyman, L. J. Ignarro, and P. J. Kadowitz. *Fed. Proc.* 41:1304 (1982).
46. D. J. Stuehr and M. A. Marletta. *Proc. Natl. Acad. Sci. USA* 82:7738–7742 (1985).
47. D. J. Stuehr and M. A. Marletta. *J. Immunol.* 139:518–525 (1987).
48. D. J. Stuehr and M. A. Marletta. *Cancer Res.* 47:5590–5594 (1987).
49. R. Iyengar, D. J. Stuehr, and M. A. Marletta. *Proc. Natl. Acad. Sci. USA* 84:6369–6373 (1987).
50. J. B. Hibbs, Z. Vavrin, and R. R. Taintor. *J. Immunol.* 138:550–565 (1987).
51. J. B. Hibbs, R. R. Taintor, and Z. Vavrin. *Science* 235:473–476 (1987).
52. J. B. Hibbs, R. R. Taintor, Z. Vavrin, and E. M. Rachlin. *Biochem. Biophys. Res. Commun.* 157:87–94 (1988).
53. U. Forstermann, U. Alheid, J. C. Frolich, and A. Mulsch. *Br. J. Pharmacol.* 93:569–578 (1988).
54. C. H. C. Twort and C. van Breemen. *Circ. Res.* 62:961–964 (1988).
55. T. L. Cornwell and T. M. Lincoln. *J. Biol. Chem.* 264:1146–1155 (1989).
56. L. J. Ignarro, K. S. Wood, B. Ballot, and M. S. Wolin. *J. Biol. Chem.* 259:5923–5931 (1984).
57. L. J. Ignarro, J. B. Adams, P. M. Horwitz, and K. S. Wood. *J. Biol. Chem.* 261:4997–5002 (1986).
58. R. Gerzer, E. Bohme, F. Hofmann, and G. Schultz. *FEBS Lett.* 132:71–74 (1981).
59. R. Gerzer, F. Hofmann, and G. Schultz. *Eur. J. Biochem.* 116:479–486 (1981).
60. E. H. Ohlstein, K. S. Wood, and L. J. Ignarro. *Arch. Biochem. Biophys.* 218:187–198 (1982).
61. L. J. Ignarro, K. S. Wood, and M. S. Wolin. *Adv. Cyclic Nucleotide Prot. Phosphorylat. Res.* 17:267–274 (1984).
62. M. S. Wolin, K. S. Wood, and L. J. Ignarro. *J. Biol. Chem.* 257:13312–13320 (1982).
63. L. J. Ignarro, B. Ballot, and K. S. Wood. *J. Biol. Chem.* 259:6201–6207 (1984).
64. L. J. Ignarro and P. J. Kadowitz. *Annu. Rev. Pharmacol. Toxicol.* 25:171–191 (1985).
65. S. C. Waldman and F. Murad. *Pharmacol. Rev.* 39:163–196 (1987).
66. L. J. Ignarro and C. A. Gruetter. *Biochim. Biophys. Acta* 631:221–231 (1980).
67. C. A. Gruetter, P. J. Kadowitz, and L. J. Ignarro. *Can. J. Physiol. Pharmacol.* 59:150–156 (1981).
68. P. Needleman and E. M. Johnson. *J. Pharmacol. Exp. Ther.* 184:709–715 (1973).
69. P. Needleman, B. Jakschik, and E. M. Johnson. *J. Pharmacol. Exp. Ther.* 187:324–331 (1973).
70. J. F. Brien, B. E. McLaughlin, T. H. Breedon, B. M. Bennett, K. Nakatsu, and G. S. Marks. *J. Pharmacol. Exp. Ther.* 237:608–614 (1986).
71. M. Feelisch and E. A. Noack. *Eur. J. Pharmacol.* 139:19–30 (1987).
72. M. Feelisch and E. A. Noack. *Eur. J. Pharmacol.* 142:465–469 (1987).
73. H.-L. Fung, S. Chong, E. Kowaluk, K. Hough, and M. Kakemi. *J. Pharmacol. Exp. Ther.* 245:524–530 (1988).
74. R. M. J. Palmer and S. Moncada. *Biochem. Biophys. Res. Commun.* 158:348–352 (1989).
75. I. J. Amber, J. B. Hibbs, R. R. Taintor, and Z. Vavrin. *J. Leuk. Biol.* 44:58–65 (1988).
76. G. Thomas and P. W. Ramwell. *Biochem. Biophys. Res. Commun.* 154:332–338 (1988).
77. G. Thomas and P. W. Ramwell. *Eur. J. Pharmacol.* 153:147–148 (1988).
78. G. Thomas, R. Mostaghim, and P. W. Ramwell. *Biochem. Biophys. Res. Commun.* 141:446–451 (1986).
79. L. J. Ignarro, M. E. Gold, G. M. Buga, R. E. Byrns, K. S. Wood, G. Chaudhuri, and G. Frank. *Circ. Res.* 64:315–329 (1989).
80. L. J. Ignarro. In R. Dulbecco (ed.), *Encyclopedia of Human Biology*, Academic Press, San Diego, (in press).