Brief Review

Nitric Oxide
A Novel Signal Transduction Mechanism for Transcellular Communication

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Nitric oxide first captured the interest of biologists when this inorganic molecule was found to activate cytosolic guanylate cyclase and stimulate cyclic guanosine monophosphate (GMP) formation in mammalian cells. Further studies led to the finding that nitric oxide causes vascular smooth muscle relaxation and inhibition of platelet aggregation by mechanisms involving cyclic GMP and that several clinically used nitrovasodilators owe their biological actions to nitric oxide. Nitric oxide possesses physicochemical and pharmacological properties that make it an ideal candidate for a short-term regulator or modulator of vascular smooth muscle tone and platelet function. Nitric oxide is synthesized by various mammalian tissues including vascular endothelium, macrophages, neutrophils, hepatic Kupffer cells, adrenal tissue, cerebellum, and other tissues. Nitric oxide is synthesized from endogenous L-arginine by a nitric oxide synthase system that possesses different cofactor requirements in different cell types. The nitric oxide formed diffuses out of its cells of origin and into nearby target cells, where it binds to the heme group of cytosolic guanylate cyclase and thereby causes enzyme activation. This interaction represents a novel and widespread signal transduction mechanism that links extracellular stimuli to the biosynthesis of cyclic GMP in nearby target cells. The small molecular size and lipophilic nature of nitric oxide enable communication with nearby cells containing cytosolic guanylate cyclase. The extent of transcellular communication is limited by the short half-life of nitric oxide, thereby ensuring a localized response. Labile nitric oxide–generating molecules such as S-nitrosothiols may be involved as precursors or effectors. Further research will provide a deeper understanding of the biology of nitric oxide and the nature of associated pathophysiological states. *(Hypertension 1990;16:477-483)*

Nitric oxide is a unique molecule as far as endogenously formed regulatory substances are concerned. Nitric oxide is a very small lipophilic molecule that can rapidly diffuse through biological membrane barriers and thereby reach the intracellular compartments of nearby cells of diverse function. Interestingly, much like oxygen, nitric oxide is actually a gas that is sparingly soluble in aqueous medium and functions biologically as a molecule in solution. The ultrashort half-life of nitric oxide (probably less than 5 seconds in biological tissues) limits the availability of newly synthesized nitric oxide to adjacent or nearby cells. Under physiological conditions of neutral pH, nitric oxide is not very reactive, but its paramagnetic property is responsible for its remarkably high binding affinity for heme iron. Thus, nitric oxide reacts with numerous hemoproteins (reduced Fe²⁺ state) to generate their nitrosyl-heme adducts, and these are relatively more stable than free nitric oxide.

The most important hemoprotein interaction involving nitric oxide is that of the reaction between cytosolic guanylate cyclase and nitric oxide to yield the nitrosyl-heme-enzyme ternary complex, which represents the activated state of guanylate cyclase. The binding of nitric oxide to the heme group of guanylate cyclase causes an immediate and profound increase in catalytic activity, resulting in a 50-fold to 200-fold increase in the velocity of conversion of magnesium guanosine 5′-triphosphate (MgGTP) substrate to guanosine 3′, 5′-monophosphate (cyclic GMP). This interaction between nitric oxide and the heme group of guanylate cyclase represents a novel and widespread signal transduction mechanism that links extracellular stimuli to the biosynthesis of the second messenger cyclic GMP in adjacent cells. The importance of nitric oxide in signaling transcellular communication takes on new meaning in view of the findings that not only vascular endothelial cells but...
also many other cell types can synthesize nitric oxide from L-arginine or an analogue of L-arginine. Nitric oxide generated in one cell may thereby recruit the functions of adjacent cells, some of which may be different cell types from the original cell type that responded to an extracellular stimulus by generating nitric oxide. In this manner, one cell type can signal different and perhaps complementary cellular responses within a localized environment.

Signal Transduction Mechanisms Involving Nitric Oxide

The requirement of reduced iron (Fe²⁺) in the form of heme for the activation of cytosolic guanylate cyclase by nitric oxide was first appreciated by Craven and DeRubertis in 1978. These investigators found that their partially purified hepatic guanylate cyclase preparations were devoid of heme and could not be activated by nitric oxide or nitroso compounds until free heme or a reduced hemoprotein was added back to enzyme reaction mixtures. The activation of guanylate cyclase by nitric oxide in the presence of heme was accounted for by the reaction between nitric oxide and heme to form nitrosyl-heme (NO-heme), as preformed NO-heme complex markedly activated guanylate cyclase. These observations were later confirmed with guanylate cyclase purified from bovine lung, rat liver, and human platelets. Soluble guanylate cyclase was found to be a hemoprotein containing 1 mol heme/mol holoenzyme and was markedly activated by nitric oxide and nitroso compounds in the absence of added heme. Heme can be readily detached from enzyme protein without causing denaturation, and this heme-deficient enzyme form cannot be activated by nitric oxide unless heme or a suitable hemoprotein is added back to enzyme reaction mixtures. The heme-deficient enzyme can be readily reconstituted with heme, and this enzyme form behaves indistinguishably from the native heme-containing enzyme.

Nitric oxide and S-nitrosothiols mimic one another in the mechanism by which they cause heme-dependent activation of cytosolic guanylate cyclase. The S-nitrosothiols are chemically unstable and may activate guanylate cyclase after spontaneous degradation accompanied by liberation of nitric oxide. In aqueous solution above pH 7 in an oxygen-free atmosphere, the liberated nitric oxide can be recovered from the atmospheric compartment above the solution. Alternatively, the S-nitrosothiols may directly activate guanylate cyclase without obligatory conversion to nitric oxide, although this has not been demonstrated unequivocally.

In a study designed to ascertain the characteristics of binding of heme to guanylate cyclase, we discovered that the iron of heme was not required for binding as protoporphyrin IX bound tightly to heme-deficient guanylate cyclase. Much to our surprise at that time, we observed that protoporphyrin IX caused a pronounced activation of guanylate cyclase that was indistinguishable in kinetic mechanisms from that by which nitric oxide activated heme-containing guanylate cyclase or preformed NO-heme activated heme-deficient enzyme. We postulated that the paramagnetic species nitric oxide alters the conformation of heme by pulling the iron away from the enzyme and out-of-plane relative to the planar porphyrin ring configuration. This configurational change at the porphyrin binding site of guanylate cyclase stresses and breaks the axial ligand thereby allowing the iron, but not the porphyrin ring, to detach from the enzyme protein. Thus, the plane of NO-heme that remains bound to guanylate cyclase superficially resembles protoporphyrin IX (heme without its iron). The binding of protoporphyrin IX to heme-deficient guanylate cyclase causes pronounced enzyme activation. Indeed, heme-containing guanylate cyclase is also markedly activated by protoporphyrin IX because the apparent binding affinity of enzyme for protoporphyrin IX is so high (Kₐ=1.4 nM) that heme is displaced from the porphyrin binding site and replaced with protoporphyrin IX. The introduction of a divalent metal into the coordination complex of protoporphyrin IX abolishes the capacity for enzyme activation and can actually cause enzyme inhibition. Thus, heme (ferroporphyrin IX) inhibits the basal catalytic activity of heme-deficient guanylate cyclase. Similarly, zinc- and manganese-protoporphyrin IX inhibit basal enzymatic activity and are potent competitive inhibitors of protoporphyrin IX-induced and NO-heme-induced enzyme activation. Guanylate cyclase activation is characterized by increased affinities of enzyme for MgGTP substrate and excess uncomplexed Mg²⁺, as well as an increased Vₘₐₓ.

To be consistent with this hypothetical model of guanylate cyclase activation, any direct enzyme activation by an S-nitrosothiol (a diamagnetic species) would require that the nitroso moiety be cleaved in a reaction with heme iron to yield the paramagnetic NO-heme adduct of guanylate cyclase. We have observed that the NO-heme adducts of guanylate cyclase and hemoglobin can be isolated from reaction mixtures of hemoprotein plus S-nitroso-L-cysteine after gel filtration to remove any residual low molecular weight S-nitrosothiol, but these observations do not rule out the possibility that free nitric oxide is an intermediate in these reactions. The "on" signal for guanylate cyclase activation by nitric oxide is represented by the binding of nitric oxide to heme and the accompanying detachment of the heme Fe²⁺ axial ligand from the enzyme protein. The "off" signal is represented by breakdown of the unstable NO-heme complex with liberation of nitric oxide and reestablishment of the heme Fe²⁺ axial ligand.

Transcellular Communication Involving Nitric Oxide

Nitric oxide is a small lipophilic and chemically unstable molecule that is very well-suited for what appears to be its probable biological role. Nitric oxide readily permeates biological membranes as can
be easily demonstrated experimentally by its release from cells and subsequent interaction with cytosolic guanylate cyclase located in other cells. Moreover, in a series of unpublished observations we repeatedly noted that application of a small drop of aqueous nitric oxide, S-nitroso-L-cysteine, or even sodium nitroprusside solution under the tongue of anesthetized cats caused a discernible decrease in systolic blood pressure within 40 seconds of application. These physiological properties of nitric oxide may allow it to diffuse out of one cell and into one or more adjacent cells. This diffusion may occur rapidly, before the nitric oxide undergoes spontaneous oxidation to NO$_3^-$ and higher oxides of nitrogen.

Studies conducted thus far suggest that nitric oxide synthesized in a given cell elicits its principal biological response, not in the cells of origin, but rather in nearby target cells. These observations are consistent with the physical properties of nitric oxide discussed earlier. Thus, the nitric oxide synthesized by vascular endothelial cells rapidly diffuses out of these cells of origin and into the underlying smooth muscle cells to cause relaxation and into nearby adhering platelets in the lumen of the blood vessel to inhibit platelet adhesion and aggregation. In addition to its principal action on target cells, the nitric oxide synthesized within endothelial cells is capable of elevating cyclic GMP in these cells of origin, and it has been suggested that cyclic GMP may modulate the formation of endothelium-derived nitric oxide.

Endothelium-derived nitric oxide can therefore activate cytosolic guanylate cyclase present in diverse cell types located in close proximity to its cell of origin. In this manner the nitric oxide generated by vascular endothelial cells communicates with nearby cells, calling on them to act in a complementary manner to increase local blood flow and diminish or prevent thrombosis. The biological half-life of authentic nitric oxide at concentrations of 10–100 nM in physiological solutions of high oxygen tension is 3–5 seconds. Although oxygen tensions in tissues are not as high as those in aqueous solutions gassed with 95% oxygen, the ubiquitous formation of superoxide anion in tissues may ensure a short half-life of nitric oxide. We have observed that authentic nitric oxide perfused in solution at concentrations of 10–100 nM through intact blood vessels or isolated hearts has a biological half-life of less than 1 second. The short half-life of nitric oxide, together with its capacity to bind hemoglobin, myoglobin, and other reduced hemoproteins, ensures that a highly localized action will prevail.

Endothelium-derived nitric oxide may interact with other target cells in addition to vascular smooth muscle and platelets. For example, back in the mid-1970s we showed that human neutrophils and macrophages contained guanylate cyclase and that intracellular cyclic GMP levels could be elevated markedly in response to various immune reactants. The increase in cyclic GMP levels was associated closely with the initiation of lysosomal enzyme release and phagocytosis in the case of neutrophils. Thus, it is conceivable that nitric oxide–elicited cyclic GMP accumulation could turn on the cytotoxic functions of such cells. Also plausible is the possibility that endothelium-derived nitric oxide could trigger the pathophysiological functions of circulating neutrophils and tissue macrophages. In this manner the formation of nitric oxide by a single cell type can signal and thereby recruit the complementary actions of numerous cell types for the purpose of initiating a complete pathophysiological response. Such a response could represent a local inflammatory reaction to tissue injury.

It is of great interest that nitric oxide is synthesized not only by vascular endothelial cells but also by macrophages, neutrophils, hepatic Kupffer cells, and brain tissue. All of these cell types appear to use a similar biochemical pathway involving the enzymatic conversion of L-arginine or a close structural analogue to nitric oxide plus L-citrulline. The biological function of macrophage- and neutrophil-derived nitric oxide may be a cytotoxic one in causing functional disruption or death of foreign target cells. The role of brain cell–derived nitric oxide is unknown, although nitric oxide formation is associated with activation of cerebellar glutamate and kainate receptors, and the target sites for nitric oxide appear to be, not its neuronal cells of origin, but rather the adjacent glial cells and presynaptic nerve terminals. It is also conceivable that nitric oxide synthesized by all of these cell types diffuses out and into adjacent vascular smooth muscle cells and platelets to improve local blood flow and ensure a nonthrombogenic vascular endothelial surface.

Thus, nitric oxide may play a widespread pathophysiological role in local transcellular communication by acting as a short-lived, potent activator of cytosolic guanylate cyclase. This concept is illustrated schematically in Figure 1. The biological significance of this rapid communication may be to signal the initiation of several localized complementary cellular responses, each contributing toward a more complete response. In the attainment of this end, nitric oxide would maintain local blood flow and prevent thrombosis in the coronary, cerebral, pulmonary, renal, and gastrointestinal mucosal circulatory beds. Other plausible functions of nitric oxide synthesized in critical organs such as the heart, brain, kidney, and lung could be to ensure patency of the associated extensive arteriolar networks to maintain flow to these organs. In this capacity nitric oxide may function as an autoregulatory molecule. One way to test this hypothesis is to examine the influence on vascular resistance in isolated vascular beds of L-arginine analogues that inhibit the formation of nitric oxide from L-arginine. One such analogue is N\(^2\)-methyl-L-arginine, which causes an increase in systemic blood pressure after intravenous administration of high doses. A much more potent L-arginine analogue would be more useful to study the role of nitric oxide in maintaining local blood flow. If nitric oxide is...
Biosynthesis of Nitric Oxide and the Influence of L-Arginine Analogues

Although one can understand and appreciate those physiochemical and pharmacological properties of nitric oxide that endow this molecule with the capacity to serve as a unique modulator of local blood flow, thrombosis, and function of macrophages and neutrophils, it is important for interested investigators to focus their attention on factors affecting the biosynthesis of nitric oxide. It follows that agents that promote or inhibit the conversion of L-arginine to nitric oxide should enhance or inhibit, respectively, endothelium-dependent relaxation and local blood flow. Failure to obtain such data would force the question of the biological significance of endothelium-derived nitric oxide.

The precursor to endothelium-derived nitric oxide appears to be L-arginine, but an analogue of L-arginine has not been ruled out. The demonstration that radiolabeled L-arginine is converted to nitric oxide does not prove that L-arginine is the endogenous substrate precursor. L-Arginine may merely be capable of being converted to nitric oxide by an enzyme system that normally uses a structural analogue of L-arginine. Studies with the macrophage have been more definitive in establishing a precursor role for L-arginine in the enzymatic synthesis of nitric oxide, NO$_2^-$, and NO$_3^-$. In the macrophage, several steps are involved in the conversion of L-arginine to nitric oxide by an inducible cytosolic enzyme system that is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin and stimulated by Mg$^{2+}$. There appears also to be a requirement for flavin adenine dinucleotide and a flavoprotein (D. Stuehr, personal communication, April 11, 1990). This reaction is characterized by the oxidation of one of the two equivalent basic guanidino nitrogen atoms of L-arginine and its subsequent cleavage to yield nitric oxide plus L-citrulline. An enzyme that catalyzes the conversion of L-arginine to nitric oxide plus L-citrulline in cerebellum has been purified and partially characterized. This enzyme is different from that found in macrophages in that it requires calcium, calmodulin, and NADPH and is constitutive in that enzymatic activity is expressed immediately on addition of calcium and the required cofactors. Whether a similar reaction to that in cerebellum for the biosynthesis of nitric oxide occurs also in the vascular endothelium remains to be determined.

While developing methods of studying the enzymatic formation of nitric oxide by mammalian cells, we found that pulmonary arterial rings and cultured aortic endothelial cells contain high concentrations of L-arginine and that incubation of arterial rings in Krebs bicarbonate medium for 24 hours causes a sharp decline in endogenous levels of L-arginine, which is associated with the development of complete refractoriness to endothelium-dependent relaxation. Arginine-depleted arterial rings but not control rings (mounted and tested within 2 hours of isolation) relaxed up to 100% in response to 0.1 mM L-arginine, and L-arginine restored the responsiveness to endothelium-dependent relaxants.
ation of arginine-depleted arterial rings. Relaxation is accompanied by increases in tissue levels of cyclic GMP and all responses are markedly inhibited by hemoglobin or methylene blue. Certain dipeptide analogues of L-arginine, such as L-arginyl-L-alanine, were more active than L-arginine.

Substitution on one or both of the two basic amino nitrogen atoms of L-arginine results in analogues that competitively antagonize endothelium-dependent relaxation. We have synthesized numerous analogues of L-arginine and found that many analogues inhibit endothelium-dependent relaxation, lower basal levels of cyclic GMP, and cause endothelium-dependent contraction. Substitution on one of the guanidino nitrogen atoms of L-arginine with an allyl, ethyl, succinyl, nitro, or amino group conferred pronounced inhibitory activity. The most potent analogue was N°-amino-L-arginine, which was approximately 100-fold more potent than N°-methyl-L-arginine. The biotransformation of L-arginine to nitric oxide as well as the inhibitory effect of N°-substituted analogues of L-arginine is illustrated schematically in Figure 1.

The likely mechanism of action of these N°-substituted analogues of L-arginine is competitive inhibition of the conversion of endogenous L-arginine or an arginine-containing substance to nitric oxide. Certainly, experiments using cell-free nitric oxide synthase systems prepared from macrophages and cerebellum revealed such a mechanism of action. Although comparable experiments with endothelium-derived nitric oxide synthase are lacking, other studies suggest indirectly that the N°-substituted analogues of L-arginine antagonize endothelium-dependent relaxation by inhibiting nitric oxide formation rather than by releasing a smooth muscle contractile factor. For example, the N°-methyl, N°-nitro, and N°-amino analogues of L-arginine lower basal levels of cyclic GMP and cause contractions of isolated arterial rings, both of which are reversed by addition of L-arginine but not D-arginine and are unaffected by indomethacin.

Limited information on the modulation of nitric oxide formation by such analogues is possible when using only vascular smooth muscle tone as an end point of measurement. These experiments are complicated by the real possibility that added L-arginine and analogues may compete for common membrane transporters in being taken up into the cells. More meaningful studies necessitate monitoring the enzymatic conversion of L-arginine or analogues to nitric oxide plus L-citrulline in broken cell preparations. Only in this manner will it be possible to comprehend fully what the L-arginine analogues are really doing.

**Role of S-Nitrosothiols as Precursors to Nitric Oxide**

There is good chemical evidence that nitric oxide alone cannot account entirely for the endothelium-derived relaxing factor (EDRF)–elicited relaxant responses to endothelium-dependent relaxants. Therefore, the question arises as to what additional chemical substance does in fact account for the full relaxation if it is not nitric oxide. Indirect but convincing evidence indicates that this additional substance, if it does exist, must be a labile nitroso compound. The evidence is: 1) the endothelium-derived substance released by acetylcholine, Bradykinin, or A23187 from intact perfused artery or vein and from cultured aortic endothelial cells has a half-life of less than 5 seconds; 2) this released substance activates cytosolic guanylate cyclase and elevates cyclic GMP levels in target smooth muscle cells; 3) the biological actions of this substance released by acetylcholine and other endothelium-dependent relaxants can be antagonized or abolished by hemoglobin or methylene blue. Clearly then, although nitric oxide may not account chemically for the entire relaxant response to endothelium-dependent relaxants, nitric oxide does account for relaxation pharmacologically as the above effects are identical to those that are produced against authentic nitric oxide. One possible explanation for all of these observations is that nitric oxide is synthesized and released under basal or resting conditions, whereas endothelium-dependent relaxants trigger the release of a nitroso compound.

To account for the extensive evidence documenting the properties of EDRF, this labile nitroso compound would either have to permeate cell membranes directly or liberate free nitric oxide extracellularly, as the active species must gain ready entry into vascular smooth muscle cells and platelets to activate guanylate cyclase and elevate cyclic GMP levels. There is preliminary evidence that such a nitroso substance may function intact and that it may be more potent than nitric oxide itself. We have observed that S-nitroso-L-cysteine and nitric oxide are equipotent in relaxing vascular smooth muscle, inhibiting platelet aggregation, and activating guanylate cyclase. The existence of a nitroso compound that is more potent than nitric oxide in causing either vascular smooth muscle relaxation of guanylate cyclase activation has yet to be demonstrated.

Investigators are beginning to appreciate that the chemical techniques presently used to assay nitric oxide are not entirely selective for nitric oxide, in that labile nitroso precursors that spontaneously liberate nitric oxide would obviously be detected as well. The discrepancy, however, is that in some laboratories at least one procedure, the nitric oxide chemiluminescence technique, is unable to detect sufficient increases in the quantity of nitric oxide released from endothelial cells to account for the relaxant effects of endothelium-dependent relaxants. That is, basally released nitric oxide can be demonstrated but the additional nitric oxide release caused by endothelium-dependent relaxants is insufficient to account for the smooth muscle relaxation elicited. Although it is possible that a nitroso compound is released that...
liberates nitric oxide after permeating target cells, no evidence for this possibility exists.

A hypothesis that may be worth testing is that the vascular endothelium releases nitric oxide and an S-nitrosothiol.47 Such a hypothesis is consistent with most of the observations made on EDRF thus far.15,48 The biological actions of S-nitrosothiols are indistinguishable from and attributed to the actions of nitric oxide.9 There are some differences in the chemical properties between S-nitrosothiols and nitric oxide that probably account for the discrepancies observed between EDRF and nitric oxide. Nitric oxide is a paramagnetic species, whereas EDRF and S-nitrosothiols are diamagnetic (Reference 49 and L.J. Ignarro and K.S. Wood, unpublished observations). Moreover, the sensitivity of certain chemical procedures involving chemiluminescence, diazotization, and nitrosation for pure nitric oxide greatly exceeds that for EDRF and S-nitrosothiols (L.J. Ignarro and K.S. Wood, unpublished observations). An extension of this hypothesis is that a portion of the nitric oxide that is continuously synthesized from L-arginine leaks out of the endothelial cell, whereas a portion of the nitric oxide reacts with an intracellular thiol to form an S-nitrosothiol. The S-nitrosothiol may be stored within the acidic environment of lysosomes, where it spontaneously decompose by a homolytic state cleavage reaction with the liberation of nitric oxide and formation of the corresponding disulfide. The S-nitrosothiol could be released from the endothelial cell by the process of exocytosis. Thus, endothelium-dependent relaxants could trigger, not the de novo synthesis of nitric oxide from L-arginine, but rather calcium-dependent secretion of a preformed S-nitrosothiol packaged and stabilized within the acidic environment of lysosomes or secretory granules. This concept is illustrated schematically in Figure 2.

In conclusion, nitric oxide is turning out to be an important endogenously formed molecule that possesses numerous cellular functions. One of the most unique characteristics of nitric oxide is that it possesses the physicochemical properties to permeate numerous cell types within a localized environment and bind to the heme group of cytosolic guanylate cyclase. This represents a novel transduction mechanism by which an extracellular signal gains access to an intracellular enzyme and causes enzyme activation to stimulate the intracellular accumulation of cyclic GMP. This signal transduction mechanism is highly conducive to a biological role of nitric oxide in transcellular communication to recruit the different but complementary actions of nearby cells.

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