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COMMENTARY

SIGNAL TRANSDUCTION MECHANISMS INVOLVING NITRIC OXIDE

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The interaction between nitric oxide (NO) synthesized in and released from one cell and the heme prosthetic group of cytosolic guanylate cyclase present in nearby target cells represents a wide-reaching signal transduction mechanism that couples diverse extracellular stimuli to the biosynthesis of cyclic GMP in target cells. Numerous mammalian cell types synthesize and release NO, and studies indicate that the principal action of NO is on nearby target cells. NO activates cytosolic guanylate cyclase, thereby elevating intracellular cyclic GMP levels in target cells, and cyclic GMP acts as an intracellular messenger to initiate cellular function. To accomplish this within the short life span of NO in biological fluids, NO must be synthesized, reach its target cells, and activate guanylate cyclase within seconds. This transcellular signalling mechanism represents a form of rapid intracellular communication that permits the simultaneous initiation of different but perhaps complementary cellular responses within a localized environment. The physiological and pathophysiological significance of the signal transduction mechanisms involving NO is discussed in this commentary.

Heme-dependent signal transduction

The heme-dependent signal transduction process involving NO represents a step that is intermediate in the pathway leading from stimulation of NO biosynthesis in the cells of origin to the cellular responses resulting from the action of NO in target cells. The NO diffuses out of its cells of origin and into its target cells, where the NO activates cytosolic guanylate cyclase.

The requirement of heme for the activation of cytosolic guanylate cyclase by NO and labile nitroso compounds was first demonstrated in crude enzyme fractions [1] and then in purified enzyme preparations from lung [2, 3], liver [4], and platelets [5]. Cytosolic guanylate cyclase was found to be a hemoprotein containing 1 mol of heme per mol of holoenzyme [3, 6, 7], and the heme group could be detached by procedures that do not denature the enzyme protein [3, 8]. This technique allowed for the unequivocal demonstration that heme is obligatory for guanylate cyclase activation by NO.

Protoporphyrin IX, the immediate precursor to heme in mammalian cells, activates guanylate cyclase by heme-independent mechanisms [9] and yet this enzyme activation is indistinguishable kinetically from that by which NO activates heme-containing guanylate cyclase [7]. Similarly, preformed NO–heme complex and protoporphyrin IX activates heme-deficient guanylate cyclase by indistinguishable mechanisms. Based on these and other observations, we forwarded the hypothesis that NO radical alters the conformation of heme by pulling the heme Fe^{2+} away from the enzyme protein, thereby breaking the axial ligand between Fe^{2+} and protein without altering the binding of the porphyrin ring [10]. Thus, the plane of NO–heme that binds to guanylate cyclase superficially resembles protoporphyrin IX (heme without Fe^{2+}). This configurational change at the porphyrin binding site of guanylate cyclase is depicted in Fig. 1, and accounts not only for the fact that NO–heme and protoporphyrin IX activate guanylate cyclase by virtually identical mechanisms but also for the requirement of heme for guanylate cyclase activation by NO. Guanylate cyclase activation is characterized by increased affinities of enzyme for MgGTP substrate and excess uncomplexed Mg^{2+}, and an increased V_{max} [7].

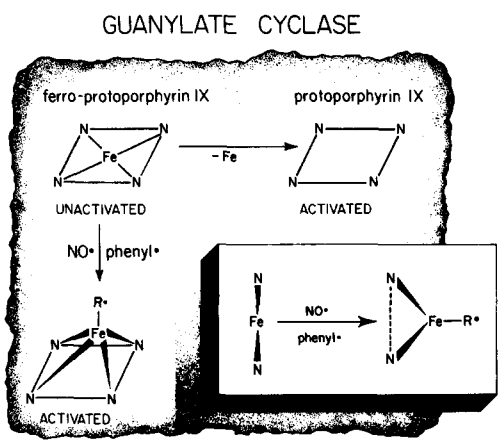

Fig. 1. Schematic illustration of the activation of cytosolic guanylate cyclase by NO or phenyl radical. The demetallation of ferro-protoporphyrin IX (heme) or its displacement by protoporphyrin IX yields the activated form of the enzyme. Heme reacts with NO or phenyl radical (R*) to form a modified porphyrin that resembles protoporphyrin IX. The inset is a side view of the displacement of iron from the planar porphyrin configuration. Reproduced, with permission, from *Adv Cyclic Nucleotide Protein Phosphorylation Res* **17**: 267–274, 1984. Copyright (1984) Raven Press, Ltd., New York. [Ref. 10].

Binding of the heme Fe^{2+} to enzyme protein maintains guanylate cyclase in a state of low catalytic activity. Replacement of Fe^{2+} with Zn^{2+} or Mn^{2+} abolishes guanylate cyclase activity, and removal of the metalloporphyrin restores enzymatic activity [11]. The signal transduction process is initiated by the binding of NO to heme and consequent disruption of the heme Fe^{2+} axial ligand, whereas the signal transduction process is terminated upon re-establishment of the heme Fe^{2+} axial ligand as the labile NO–heme complex decomposes with the liberation of NO_2^- and higher oxides of nitrogen. Thus, the heme prosthetic group of guanylate cyclase is of paramount importance in signal transduction.

Intracellular role of cyclic GMP

In at least two cell types, NO elicits its biological effects via the intracellular actions of cyclic GMP. NO causes relaxation of vascular smooth muscle and inhibition of platelet aggregation, and both cellular responses are mediated by cyclic GMP [12–14]. The mechanism by which intracellulir cyclic GMP promotes smooth muscle relaxation and inhibition of platelet function is still unknown, and these are timely areas for further investigation. One possibility is that cyclic GMP maintains a very low intracellular concentration of free calcium, perhaps by promoting its intracellular binding to calcium-binding proteins [15]. Another possible mechanism in vascular smooth muscle is that cyclic GMP activates a specific protein kinase, which results in the phosphorylation and inactivation of myosin light chain kinase, thereby resulting in dephosphorylation of myosin light chain and smooth muscle relaxation [16, 17]. More basic information on the precise physiological mechanisms involved in vascular smooth muscle contraction and platelet aggregation will likely be necessary before the mechanisms by which cyclic GMP causes relaxation and inhibition of platelet aggregation are elucidated.

The accessibility of target cell guanylate cyclase to NO is great in both magnitude and speed because of the physicochemical properties of NO, namely its small molecular size and high lipophilicity. NO can readily enter target cells and bind to the heme group of cytosolic guanylate cyclase. The onset of cyclic GMP accumulation in vascular smooth muscle after addition of NO is generally less than 5 sec and the onset of relaxation is about 10 sec in isolated preparations of bovine coronary and pulmonary artery [18, 19]. The onset times in response to endothelium-dependent relaxants (acetylcholine, bradykinin) generally lag 5 sec behind those of NO [20]. The difference in time reflects the time required for coupling of extracellular receptor occupancy to NO generation in endothelial cells.

Localized transcellular communication

The literature during the past two years reveals clearly the biosynthesis of NO in numerous cell types or tissues including vascular endothelium [21], macrophages [22, 23], neutrophils [24], hepatic Kupffer cells [25], adrenal tissue [26], and cerebellar tissue [27–29]. These observations are consistent with the much longer standing knowledge that cytosolic guanylate cyclase and cyclic GMP exist ubiquitously in mammalian cells. Until the discovery was made that mammalian cells could synthesize NO, a clear understanding of the reason for the widespread occurrence of the cyclic GMP system was unavailable. Now that the nearly ubiquitous formation of NO and cyclic GMP is appreciated, studies are focusing on the biological roles of these two messenger substances.

The assumption all along has been that the cellular effects of NO are mediated by intracellular cyclic GMP because of the very potent activating action of NO on cytosolic guanylate cyclase and because chemical agents that inhibit guanylate cyclase activation (methylene blue) also inhibit NO action, whereas inhibitors of cyclic GMP phosphodiesterase (M&B 22,948; 1-methyl-3-isobutylxanthine) enhance or potentiate NO action [14, 30]. But there is at least one biological action of NO that may not be mediated by cyclic GMP, and that is the cytotoxic action of macrophage-derived NO on target tumor cells and other tissues [31]. The latter action appears to be due to the nitrosation and consequent inactivation of iron- and iron–sulfur-containing enzymes in target cells.

The small molecular size, lipophilic nature, and chemical instability of NO make it well-suited for its probable role in local transcellular communication. These physicochemical properties also make it unnecessary to have special membrane transporters or enzyme systems for terminating the action of NO. Simple diffusion and a short half-life due to chemical instability and accelerated breakdown by oxygen and oxygen-derived radicals in biological fluids would account for the pharmacokinetic properties of NO.

Endothelium-derived NO is generated in response to extracellular chemical and mechanical stimuli. Chemical stimuli include the endogenous endothelium-dependent vasodilators, whereas mechanical stimuli include flow or shear forces. What couples such extracellular stimuli to NO biosynthesis is unknown, but calcium is thought to play an important role [32, 33]. The NO formed then diffuses out of the endothelial cell and into the adjacent or nearby vascular smooth muscle and into any platelets that may be adhering to the luminal surface of the endothelium. In both vascular smooth muscle cells and platelets NO activates guanylate cyclase, thereby causing intracellular cyclic GMP accumulation [13, 18]. In vascular smooth muscle the response is relaxation, and in platelets the response is inhibition of aggregation and adhesion. A physiological role for endothelium-derived NO in modulating local vascular smooth muscle tone and platelet function is consistent with the pharmacological actions of authentic NO discovered 10 years ago [12, 13]. It is conceivable that endothelium-derived NO could interact also with other nearby cell types including peripheral blood neutrophils, tissue macrophages, and brain tissues as these cells or tissues possess the capacity to generate large amounts of cyclic GMP [34–36]. Future studies should address this question.

The role of NO derived from cerebellum is unknown, but the current view is that NO biosynthesis is in some way coupled to the activation of cerebellar glutamate and kainate receptors [36]. The target cells for NO generated in neuronal or granule

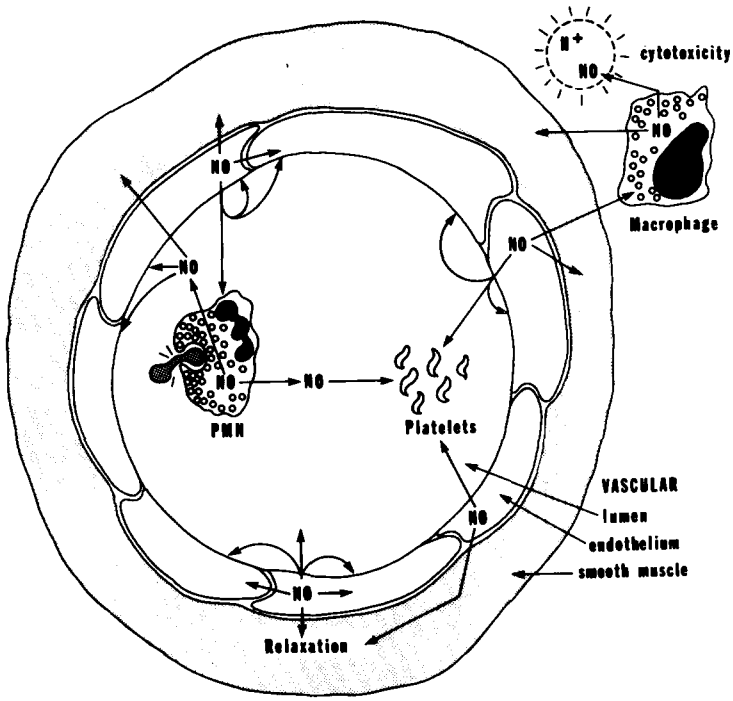

Fig. 2. Schematic illustration of the scope of transcellular signaling that is possible when NO is synthesized by and released from the vascular endothelium, circulating neutrophils, and tissue macrophages. NO triggers diverse but complementary cellular responses by stimulating different cells within a localized environment.

cells are believed to be the adjacent glial cells, presynaptic nerve endings, and perhaps the Purkinje fibers. It is conceivable that all central excitatory neurotransmitters stimulate NO formation and that the NO diffuses outward and communicates with nearby cells to modulate neurotransmission, perhaps via the intracellular action of cyclic GMP. Whether the cerebellum alone or also other brain regions generate NO is but one current problem under investigation. Moreover, a working hypothesis that the NO generated in brain tissue serves to maintain local blood flow by causing vasodilation and preventing thrombosis should be considered.

The principal action of the relatively large quantities of NO generated by immuno-activated macrophages appears to be a cytotoxic one on certain target cells or tissues [22, 31]. The mechanism of cytotoxic action is proposed to be NO-mediated nitrosation of key metabolic iron-containing enzymes or iron–sulfur proteins in the target cells [31, 37]. Such nitrosation reactions could easily provoke cellular killing of target cells. Why the cells of origin of NO, the macrophages, are not killed by the NO generated therein presents an interesting question. One possible explanation is that nitrosation reactions, involving NO cannot occur at pH values of neutrality and higher, whereas at the acidic pH associated with digestive vacuoles, lysosomes, damaged cells, and areas of cellular debris, the NO will rapidly form HONO (nitrous-acid), which is a strong nitrosating agent. Other actions of NO released from tissue macrophages are likely to be local vasodilation and attenuation of platelet function. Such varied but

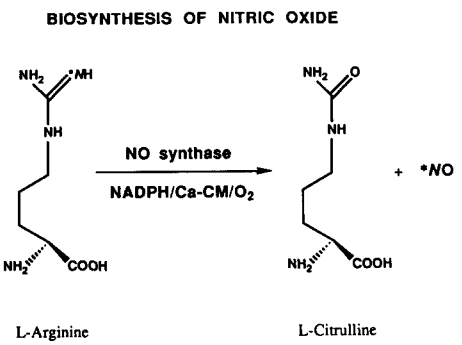

Fig. 3. Schematic representation of the biosynthesis of NO from L-arginine by the NO synthase system.

complementary actions of NO would constitute the best example of transcellular communication by an endogenous lipophilic messenger (Fig. 2).

Modulation of nitric oxide biosynthesis

The available experimental evidence is strong that endogenous NO derives from one of the two equivalent basic guanidino nitrogen atoms of L-arginine [21, 22, 38–40] via the catalytic action of a mono- or dioxygenase enzyme system termed NO synthase. Thus, L-arginine appears to be the principal precursor to NO, but an analog of L-arginine such as an L-arginine-containing peptide has not been ruled out. Studies with the macrophage and cerebellum have

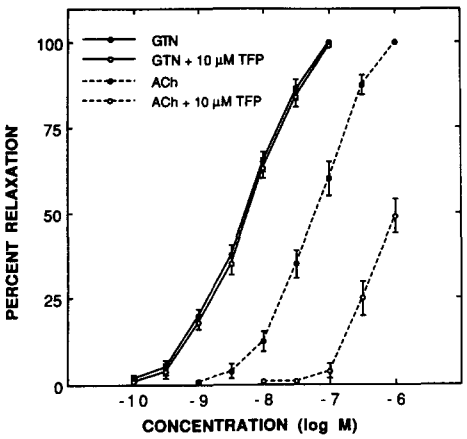

Fig. 4. Inhibition of endothelium-dependent but not endothelium-independent vascular smooth muscle relaxation by trifluoperazine. Isolated rings of endothelium-intact (acetylcholine; ACh) or endothelium-denuded (nitroglycerin; GTN) bovine pulmonary artery were precontracted by phenylephrine and challenged with ACh or GTN at cumulatively increasing concentrations. After obtaining control responses, rings were washed and allowed to equilibrate for 45 min before adding 10 μM trifluoperazone (TFP), Twenty minutes after addition of TFP, rings were precontracted and challenged with ACh or GTN. Data are the means ± SEM of eighteen rings from three separate experiments. Responses to ACh but not GTN were inhibited significantly ($P < 0.05$) by TFP (Student's paired t-test).

been more definitive than those with vascular endothelium in establishing a precursor role for L-arginine in the formation of NO [29, 39, 40]. Isozymes of NO synthase or an enzyme within the NO synthase system appear to exist in different cell types, as indicated by the strikingly different cofactor requirements. In the macrophage, L-arginine is converted to NO plus L-citrulline in an inducible cytosolic enzyme system that requires NADPH, tetrahydrobiopterin, and FAD plus a flavoprotein [39–41]. In cerebellum a partially purified enzyme has been identified that catalyzes a similar reaction but is a constitutive rather than inducible enzyme that requires NADPH, calcium, and calmodulin [29] (Fig. 3). The characteristics of NO synthase in vascular endothelium remain unreported.

Preliminary observations in this laboratory indicate that, like the cerebellar NO synthase system, the enzyme system in vascular endothelial cells may require calmodulin because the calmodulin antagonist trifluoperazine selectively inhibits endothelium-dependent but not endothelium-independent arterial smooth muscle relaxation and cyclic GMP formation (Fig. 4). We observed also that trifluoperazine inhibited the generation of NO and NO_2^- by perfused bovine pulmonary artery and cultured bovine aortic endothelial cells. Trifluoperazine also inhibited L-arginine-elicited NO formation and smooth muscle relaxation in arterial rings that had been depleted of endogenous L-arginine by incubation under tension for 24 hr. These observations are consistent with the knowledge that calcium is required for endothelium-dependent relaxation [32, 33]. More defnitive conclusions await studies with the isolated NO synthase system prepared from vascular endothelial cells.

N^G-Methyl-L-arginine was the first structural analog of L-arginine shown to be a competitive inhibitor of NO formation and endothelium-dependent relaxation [42]. This was followed by the findings that N^G-nitro-L-arginine [43] and N^G-amino-L-arginine [44] are about 100-fold more potent than N^G-methyl-L-arginine in inhibiting endothelium-dependent arterial relaxation. The inhibitory effects of these analogs can be readily overcome by addition of excess L-arginine but not D-arginine to the test system. Not only do these inhibitory L-arginine analogs antagonize endothelium-dependent relaxation but they also cause contraction of vascular smooth muscle that is accompanied by and attributed to a reduction in resting smooth muscle levels of cyclic GMP [44, 45]. These observations are consistent with the *in vivo* findings that N^G-methyl-L-arginine [46, 47] as well as N^G-amino-L-arginine (unpublished observations) cause a marked and sustained hypertension in anesthetized and conscious animals that can be reversed by infusion of L-arginine.

Summary and biological implications

In tracing the pathway by which NO elicits cellular responses, an extracellular signal must first interact with extracellular receptors located in the cells of origin of NO. Intracellular communication between the activated receptors and the NO synthase system must then occur to turn on the biosynthesis of NO. As the NO is generated, it diffuses outward and into nearby target cells (transcellular communication), where it binds to the heme prosthetic group of soluble guanylate cyclase and stimulates the intracellular accumulation of cyclic GMP. The cyclic GMP remains within the target cell to trigger a cascade of unknown reactions that constitutes the cellular response.

The biological implications of this intercellular communication process are profound. Generation of NO by one cell type can result in the functional stimulation of other cell types, thereby recruiting the local actions of diverse cells and providing different but perhaps complementary cellular responses. For example, endothelium-derived NO may function in response to or as an autacoid in initiating a local inflammatory reaction by maintaining local blood flow, minimizing the development of thrombosis, and recruiting the local actions of neutrophils and macrophages. The same could be suggested for NO released from neutrophils, macrophages, and hepatic Kupffer cells. The physiological or pathophysiological significance of NO formation in the cerebellum and perhaps other brain regions is unknown and, although NO may function to modulate central neurotransmission, an action on blood vessels and platelets cannot be dismissed.

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