In retrospect, basic research in the fields of NO and cyclic GMP during the past two decades appears to have followed a logical course beginning with the findings that NO and cyclic GMP are vascular smooth muscle relaxants, that nitroglycerin relaxes smooth muscle by metabolism to NO, progressing to the discovery that mammalian cells synthesize NO, and finally the revelation that NO is a neurotransmitter mediating vasodilation in specialized vascular beds. A great deal of basic and clinical research on the physiological and pathophysiological roles of NO in cardiovascular function has been conducted since the discovery that EDRF is NO. The new knowledge on NO should enable investigators in this field to develop novel and more effective therapeutic strategies for the prevention, diagnosis and treatment of numerous cardiovascular disorders. Since NO elicits a protective and beneficial action in many disease states, novel NO donor drugs for clinical use should prove to be very effective drugs for the treatment of essential hypertension, stroke, coronary artery disease, vascular complications of diabetes, impotency and other disorders involving the vascular system.

Key words: NO synthase; endothelium; vasodilatation; cyclic GMP; nitroglycerin; platelet aggregation; hypertension

INTRODUCTION

Nearly 25 years have gone by since we discovered the vascular smooth muscle relaxant properties of nitric oxide (NO). This observation was made by delivering a gaseous mixture of NO in nitrogen or argon by means of a gastight microliter syringe into an organ bath containing isolated precontracted strips of
bovine coronary artery (1). The pharmacological response was a marked but transient relaxation that was blocked by addition of hemoglobin or methylene blue. Moreover, NO activated soluble guanylyl cyclase isolated from bovine coronary artery. The closely similar pharmacological profile of action of NO and nitroglycerin led us to suspect that nitroglycerin causes vascular smooth muscle relaxation by mechanisms involving NO, perhaps by donating NO. Although the terms "NO donor drugs" or "nitrovasodilators" were not used in 1979, we were accumulating solid experimental evidence that nitroglycerin, other organic nitrate esters, some organic nitrite esters and nitroprusside all caused vasodilation by acting as NO donor agents on contact with tissues in aqueous solution. Struck by the extraordinarily high potency of nitroglycerin as a vascular smooth muscle relaxant both in vivo and in vitro, we suspected, as pharmacologists, that tissue receptors for nitroglycerin probably exist because there must be an endogenous nitroglycerin or similar NO donor or NO itself in mammalian tissues. Our original observations on the vasorelaxant properties of NO (1) were published one year before the discovery of endothelium-dependent vasorelaxation and endothelium-derived relaxing factor (2). The answer to the puzzle did not evolve for an additional 6-years.

While conducting experiments dealing with the vasorelaxant properties of NO, we discovered that NO also potently inhibits platelet aggregation (3). Indeed, the mechanism by which certain nitrovasodilators inhibit platelet aggregation was found to be identical to the mechanism by which these agents cause vascular smooth muscle relaxation, namely, through the action of NO (4). A series of S-nitrosothiols were synthesized and found to be potent inhibitors of platelet aggregation, and NO activated platelet-derived soluble guanylate cyclase. The objective of this chapter is to highlight the early research that led to our current understanding of the physiological and pathophysiological roles of NO in regulating vascular smooth muscle tone and blood flow.

**Mechanism of Action of Nitroglycerin**

Efforts in this laboratory, in the 1970s, were focused on elucidating the mechanism of vasodilator action of nitroglycerin and other nitrovasodilators. Evidence accumulated that these NO containing chemical agents either spontaneously released NO in aqueous solution or reacted with tissue thiols to generate chemically unstable intermediates, S-nitrosothiols, that subsequently decomposed with the liberation of NO (5-12). Based on these observations, the hypothesis was forwarded that lipophilic vasodilators like nitroglycerin, other organic nitrate esters and organic nitrite esters (isoamyl nitrite) permeate vascular smooth muscle cells and react with tissue thiols to form S-nitrosothiols, which then liberate NO, the common active vasodilator species (11). Several years earlier, NO was suspected to be the common species responsible for guanylyl cyclase activation by nitroglycerin and related agents (13).
The discovery that S-nitrosothiols were intermediates that served as NO donor agents in expressing the vasorelaxant action of many nitrovasodilators originated from experiments on the activation of soluble guanylate cyclase (5, 8-12). Thiols such as cysteine and glutathione markedly enhanced the activation of soluble guanylyl cyclase by nitrite and isoamyl nitrite. Cysteine was required for enzyme activation by nitroglycerin and other organic nitrate esters. Thiols were found to decrease the chemical stability of nitrites and nitrosothiols by liberating NO gas. The liberation of NO from these nitrovasodilators involved the formation of intermediate S-nitrosothiols, which turned out to be excellent NO donor agents. Like the direct vasorelaxant effect of NO, vasorelaxation elicited by S-nitrosothiois was accompanied by tissue cyclic GMP accumulation and both relaxation and cyclic GMP formation were inhibited by hemoglobin and methylene blue. In vivo experiments in anesthetized cats revealed that the profile of hemodynamic effects of the S-nitrosothiols was virtually identical to that of nitroglycerin and nitroprusside (11). On the basis of these observations, we proposed that nitrovasodilators elicit vasorelaxation by undergoing metabolic conversion in vascular smooth muscle cells to NO.

Elucidation of the mechanism of action of nitroglycerin led to a better understanding of the mechanism by which tolerance can develop to the vasodilator action of organic nitrate esters. An earlier hypothesis stated that tissue sulfhydryl groups (-SH) were required for expression of the vasodilator action of nitroglycerin and other organic nitrate esters (14, 15). The view was that repeated administration of relatively large doses of nitroglycerin led to the depletion or oxidation of tissue thiols, thereby leading to the gradual diminution of the action of nitroglycerin. Our studies on the mechanism of action of nitroglycerin are consistent with this earlier hypothesis on tolerance. Tissue thiols are required for the vasorelaxation effect of nitroglycerin because these thiols are required for activation of soluble guanylate cyclase by nitroglycerin. Since the vasorelaxant effect of nitroglycerin, like NO, is cyclic GMP-dependent, thiols are necessary for nitroglycerin to relax vascular smooth muscle. The likely explanation for the mechanism of tolerance development is that tissue thiols are required for chemical reaction with nitroglycerin to liberate NO from the intermediate S-nitrosothiols that are formed. Administration of sulfhydryl compounds often reverses or prevents tolerance to nitroglycerin in laboratory animals and in patients, just as sulfhydryl compounds facilitate the activation of guanylyl cyclase by nitroglycerin (16-22).

**Mechanism of Action of NO**

Activation of soluble guanylyl cyclase and increased synthesis of cyclic GMP in tissues were demonstrated before the vasorelaxant effect of NO was appreciated (23). The mechanism by which NO activates soluble guanylate cyclase was first suggested by Craven and DeRubertis (24). The hypothesis was
that soluble guanylyl cyclase contained heme iron, which was required for the binding and interaction with NO to cause enzyme activation. Using purified guanylyl cyclase, heme was found to be bound to the enzyme as a prosthetic group (25-32), and studies from this laboratory revealed the precise mechanism by which NO activates guanylyl cyclase (27, 32). In experiments designed to ascertain whether the iron in heme was required for binding of the protoporphyrin ring structure to guanylyl cyclase, enzyme that was rendered free of bound heme was reacted with protoporphyrin IX (heme without iron), passed through a gel filtration column to remove free unbound porphyrin and examined spectrophotometrically. Two important observations were made. Guanylyl cyclase was able to readily bind stoichiometric quantities of protoporphyrin IX and the porphyrin caused maximal enzyme activation. Heme-free guanylyl cyclase could not be activated by NO but was maximally activated by nM concentrations of protoporphyrin IX. Heme-containing or heme-reconstituted guanylyl cyclase was activated by both NO and protoporphyrin IX. In the latter case, the porphyrin displaced heme from the common binding site on guanylyl cyclase to cause enzyme activation.

Based on information available for hemoglobin and myoglobin, we forwarded the hypothesis that NO binds to the heme iron to form the nitrosyl-heme adduct of guanylyl cyclase. The heme bound to the enzyme was envisioned to bind similarly to the binding of heme in hemoglobin, namely, as a 5-coordinate complex with the 5th bond being the axial ligand between heme iron and histidine in the enzyme protein. On interaction of the heme iron with NO, the resulting NO-heme complex was believed to remain as a 5-coordinate complex, which means that the axial ligand must undergo cleavage and lead to the projection of heme iron away from the enzyme protein and out of plane of the porphyrin ring configuration (33). This conformational change at the porphyrin binding site of guanylyl cyclase was thought to modify the nearby catalytic site as well, perhaps by exposing the catalytic site to the surface where GTP substrate and magnesium must bind. This hypothesis would explain why NO causes a 100-fold increase in the Vmax as well as a 3-fold decrease in the Km for GTP (32).

Soluble guanylyl cyclase was also reported to contain bound copper although the reason for this was unknown (25). Based on our studies with S-nitrosothiols and recent reports that copper can potently liberate NO from S-nitrosothiols (34-36), we can forward a hypothesis at this time to suggest the role of copper in guanylyl cyclase. S-Nitrosothiols are well known to activate soluble guanylyl cyclase by heme-dependent mechanisms involving the action of NO (24, 32). The activation of guanylyl cyclase by S-nitrosothiols, like that of NO, is extremely rapid and may not be readily explainable on the basis of the slow release of NO from most S-nitrosothiols. Therefore, one role for copper may be to facilitate the release of NO from S-nitrosothiols, thereby facilitating the activation of guanylyl cyclase. This may be an important mechanism of
activation of guanylyl cyclase by endogenous S-nitrosothiols ranging from S-nitroso-amino acids to S-nitroso-proteins.

**Endogenous NO**

The realization that mammalian cells do indeed possess an endogenous nitroglycerin or NO came a little more than a decade ago when the elusive EDRF was identified as NO (37-39). Prior to that, experimental evidence was mounting in several laboratories that EDRF possessed pharmacological, biochemical and chemical properties that were similar to those for NO. For example, studies from this laboratory published in 1986 revealed that EDRF from artery and vein could activate soluble guanylyl cyclase (40). Additional experiments revealed that the mechanism of activation of guanylyl cyclase by EDRF was closely similar or identical to that for NO, thereby allowing us to first propose that EDRF is NO or a closely related nitroso compound (41). These observations explained why hemoglobin and myoglobin antagonize endothelium-dependent relaxation as well as the direct relaxant effect of EDRF. Subsequent studies showed that the activation of soluble guanylyl cyclase by EDRF, like NO, was heme-dependent (38). Other observations were also consistent with the view that EDRF was NO. For example, the short half-life of NO could be prolonged by the addition of superoxide dismutase to tissue baths or bioassay cascade superfusion systems, whereas superoxide anion generating agents decreased the half-life of NO (42, 43). We now understand the mechanisms of these effects, namely, the rapid reaction between NO and superoxide anion to generate peroxynitrite (44), which is orders of magnitude less potent than NO as a vasorelaxant. The finding in 1986 that EDRF inhibits platelet aggregation (45) was also consistent with the developing hypothesis that EDRF could be NO. Although these earlier studies had suggested that EDRF might be NO or a closely related nitroso species, the definitive studies on the chemical and biochemical characterization and identification of EDRF as NO came in 1987 (37, 39).

Following the discovery that EDRF is NO, many laboratories jumped into this field of cardiovascular research to study the physiological and pathophysiological roles of endothelium-dependent vasodilatation and endogenous NO and cyclic GMP in the regulation of systemic blood pressure, organ blood flow, hemostasis and cell proliferation. Since most of the pharmacological properties of nitroglycerin and other nitrovasodilators in laboratory animals and in humans were already well-appreciated, a systematic approach could be taken to better understand or appreciate the actions of endogenous NO.

**Regulation of Vascular Smooth Muscle Tone by NO**

It is now appreciated that EDRF or endothelium-derived NO is continuously generated from vascular endothelial cells in the absence of added endothelium-dependent vasodilators. One early clue for continuous or basal generation of NO
came from studies showing that vascular tissue cyclic GMP levels were higher in endothelium-intact than endothelium-denuded vascular preparations (46). Studies with hemoglobin and cyclic GMP phosphodiesterase inhibitors, both of which cause endothelium-dependent vasoconstriction, provided additional indirect evidence for basal NO generation. Direct evidence for basal generation of NO came from bioassay cascade experiments, which revealed the continuous formation and release of NO in effluents collected from perfused and/or superfused vascular preparations (46). Basal formation of NO varied considerably from one vessel type to another and across differing diameters in the same vessel segment. For example, greater basal formation of NO occurred in bovine pulmonary artery and vein with smaller rather than larger diameters (47). The smaller diameter endothelium-intact rings contained higher resting levels of smooth muscle cyclic GMP and were considerably more difficult than were larger diameter rings to maintain a steady level of tone when mounted and equilibrated under optimal resting tensions. Normal contractile responses to phenylephrine were restored by endothelium denudation or by addition of hemoglobin or methylene blue to tissue baths. In contrast, addition of cyclic GMP phosphodiesterase inhibitors to endothelium-denuded rings, which otherwise maintain constant tone, rapidly lost tone and in this way resembled endothelium-intact rings. Based on these original observations, we forwarded the hypothesis that the basal or continuous formation of arterial and venous NO may be important for the continuous modulation not only of vascular smooth muscle tone but also circulating platelet adhesion and aggregation (47). Later studies revealed that interference with the continuous production of endothelium-derived NO in animals by administration of NO synthase inhibitors caused a prompt and sustained increase in systemic blood pressure (48-50).

Despite the fact that the intrinsic stimulus for basal generation of NO was not appreciated in the early 1980s, later studies revealed that the shear stress or tangential shear force generated by flowing blood against the endothelial cell surface triggered the generation of NO in the endothelial cells. Several important early studies contributed to the development of this concept that flow-dependent vasodilation is endothelium-dependent (51-59). It now appears that shear forces trigger the opening of calcium channels on endothelial cells, thereby leading to the calcium-dependent activation of endothelial NO synthase and increase local production of NO (60, 62). However, both calcium-dependent and calcium-independent activation of endothelial NO synthase can occur and lead to NO-mediated vasorelaxation in response to shear stress (60, 61). In the case of calcium-dependent NO synthase activation, the increase in intracellular calcium concentration may be the result of tyrosine phosphorylation and activation of phospholipase C as well as protein phosphatases. Calcium-independent activation of NO synthase may involve tyrosine phosphorylation of endothelial NO synthase or the action of another regulatory protein.
The chemical and biological properties of NO endow this potent endogenous mediator with the capacity to act as a local modulator of blood flow and hemostasis. The vascular cell origin of NO is ideal for the local and immediate delivery of this lipophilic and labile vasodilator directly to the underlying smooth muscle as well as to the endothelial cell surface for interaction with nearby circulating platelets. The small size and lipophilic nature of NO are conducive to the rapid diffusion of NO through cell membranes to reach its target cells. The chemically labile property of NO allows for a truly local action, as does the high binding affinity of erythrocyte for NO. All of these properties of NO endow this biological mediator with the unique capacity to engage in cell-to-cell communication. In this manner, NO can recruit the functions of various cell types to elicit a concerted physiological or pathophysiological response, such as improved local blood flow to an injured tissue. Damage to the endothelial cell surface can interfere with the normal functions of NO in regulating local blood flow, as occurs in numerous pathophysiological disorders ranging from atherosclerosis to angioplasty techniques used to surgically treat patients with atherosclerosis. There may be several physiological control mechanisms that diminish NO production by inhibiting NO synthase or by downregulating the level of NO synthase protein. We first demonstrated that NO itself can act as a negative feedback modulator of NO synthase catalytic activity (62). This action could also be demonstrated in vitro using isolated vascular preparations or superfused vessels in a bioassay cascade (63) as well as in vivo in anaesthetized rabbits (64). The mechanism of this negative feedback effect was elucidated and determined to be an interaction between NO and the heme iron in NO synthase (65).

Physiological Role of NO in Erectile Function

In 1990, we first reported that the relaxation of isolated rabbit corpus cavernosum smooth muscle by electrical stimulation was accompanied by the production of NO and cyclic GMP and was prevented by treatment of tissues with NO synthase inhibitors, hemoglobin and methylene blue but not indomethacin (66). Relaxation of corpus cavernosum was mediated by nonadrenergic-noncholinergic (NANC) neurons and attributed to the generation and release of NO as the primary neurotransmitter. Based on these observations, we forwarded the hypothesis that mammalian penile erection is mediated by NO released from NANC neurons and that cyclic GMP serves as the signal transduction mechanism for smooth muscle relaxation. Additional studies from this laboratory revealed that exactly the same physiological mechanism for penile erection exists in human (67) and canine (68) corpus cavernosum and that electrical stimulation results in calcium-dependent activation of neuronal NO synthase present in corporal smooth muscle (66, 69). These original observations provided a rational basis for investigating the etiology and therapy of impotence. At least one form
of impotence may be attributed to a lesion or defect in the arginine-NO-cyclic GMP pathway, thereby resulting in diminished relaxation of corpus cavernosum smooth muscle in response to stimulation of the NANC nerves.

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