

# NITRIC OXIDE: A UNIQUE ENDOGENOUS SIGNALING MOLECULE IN VASCULAR BIOLOGY

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by

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## INTRODUCTION

My early research career in graduate school focused on trying to develop a better understanding of catecholamine mechanisms during the embryological development of the sympathetic nervous system. This research required a strong background not only in pharmacology but also in physiology and biochemistry. I recall reading some interesting papers on cyclic AMP, but I never addressed this topic in my early research. At that time, there was no such thing as cyclic GMP, and nitric oxide was considered to be no more than a pollutant in the atmosphere around us. After completing my postdoctoral training at the National Institutes of Health in Bethesda, I accepted an industrial research position to learn more about biochemical aspects of the inflammatory process in order to develop novel antiinflammatory drugs. Although my research did not yet involve cyclic AMP, I continued to read the literature on cyclic AMP and witnessed the discovery of cyclic GMP. I then decided to ascertain whether or not cyclic AMP and cyclic GMP played any modulatory roles in the inflammatory process. We found that cyclic AMP stabilized lysosomal membranes, whereas cyclic GMP promoted instability of lysosomal membranes. Moreover, cyclic AMP production was associated with the inhibition of human neutrophil function such as phagocytosis and lysosomal enzyme secretion, whereas cyclic GMP was associated with increased neutrophil function. In a different research project, it appeared that this new cyclic nucleotide, cyclic GMP, was involved in mediating the negative effects of acetylcholine in the heart, whereas cyclic AMP was already well known to elicit the opposite effects in the heart. A theme was clearly developing that cyclic AMP and cyclic GMP mediated opposing influences on cell function.

In the midst of our studies on cyclic AMP and cyclic GMP, I recall reading two interesting papers on cyclic GMP that were published by Ferid Murad and colleagues (1,2). These studies revealed that nitric oxide and nitro compounds that might release nitric oxide all activated cytosolic guanylate cyclase. Nitroglycerin was one of the nitro compounds studied. Nitric oxide and nitro compounds also stimulated cyclic GMP production in isolated tissues *in vitro* (3). These observations suggested that nitroglycerin might activate

guanylate cyclase and stimulate cyclic GMP formation by mechanisms involving nitric oxide. Additional studies revealed that nitric oxide might be involved in the nonvascular smooth muscle relaxant effects of nitroglycerin and other nitro compounds (4). These findings prompted me to conduct some experiments to ascertain whether nitroglycerin and related organic nitrate and nitrite esters could actually release nitric oxide gas in aqueous solution. After observing the generation of nitric oxide from a series of nitro compounds that were known to be smooth muscle relaxants, we thought that nitric oxide might also be responsible for the vasorelaxant effects of nitroglycerin and that cyclic GMP might be the intracellular second messenger mediating this effect of nitric oxide. Accordingly, a series of experiments was conducted to test the hypothesis forwarded by Ferid Murad that nitroglycerin, nitroprusside and nitroso compounds all cause relaxation of smooth muscle, including vascular smooth muscle, by liberating nitric oxide, which elicits its action by stimulating the production of cyclic GMP.

#### MECHANISM OF RELEASE OF NITRIC OXIDE FROM NITROGLYCERIN AND OTHER NITRO COMPOUNDS

The most important experiment that we conducted to test the hypothesis that nitric oxide is responsible for the vasorelaxant action of nitroglycerin was to determine whether nitric oxide itself causes vascular smooth muscle relaxation. In 1979, (5) we reported that nitric oxide gas is a potent relaxant of bovine coronary artery and activates guanylate cyclase isolated from this tissue (Figure 1). This observation confirmed what Ferid Murad had found and extended the hypothesis to include vascular smooth muscle. Therefore, nitroprusside, nitroglycerin and related nitro compounds appeared to relax vascular smooth muscle by liberating nitric oxide, which then stimulates cyclic GMP formation and results in vascular smooth muscle relaxation (Figure 2). The hypothesis that cyclic GMP is involved in smooth muscle relaxation was met with some controversy in the late 1970s because of the prevailing view that cyclic GMP and cyclic AMP mediate opposing biological actions. Cyclic AMP was known to mediate the smooth muscle relaxant effects of certain catecholamines and prostaglandins and cyclic GMP was thought to be involved in mediating smooth muscle contraction. However, the work conducted in several laboratories including those of Ferid Murad and myself indicated that cyclic GMP and cyclic AMP can mediate common biological actions in smooth muscle such as relaxation.

The next step in elucidating the precise mechanism of action of nitroglycerin was to ascertain how nitric oxide was released from the parent molecule in smooth muscle tissue. The spontaneous release of nitric oxide from nitroso compounds such as nitroprusside had been understood but nitroglycerin and other organic nitrate and nitrite esters are chemically stable compounds that would have to undergo enzymatic or chemical reactions to release nitric oxide. In early experiments, we found that thiols such as dithiothreitol enhanced the activation of guanylate cyclase by nitroprusside and N-nitroso

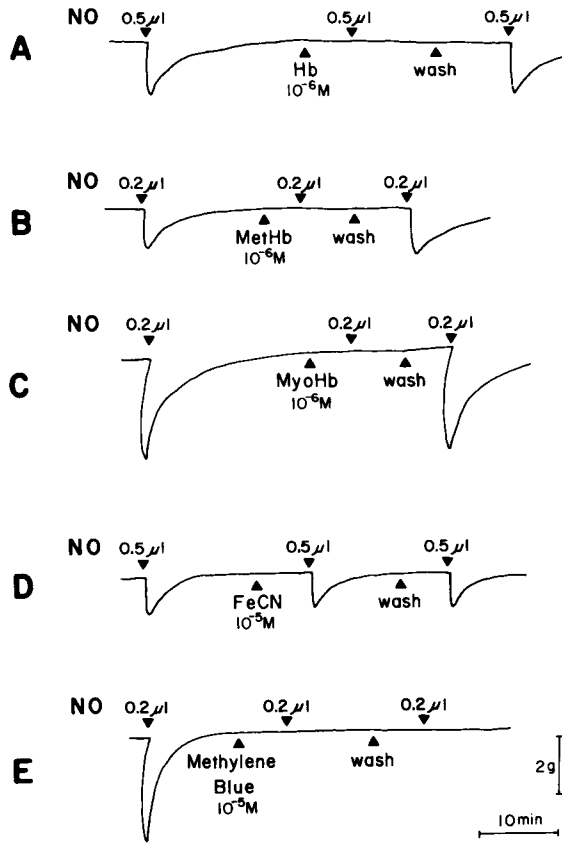


Figure 1. Relaxation of strips of bovine coronary artery by nitric oxide (NO). Strips were mounted, equilibrated and set up for recording changes in isometric force as described previously (5). Strips were submaximally precontracted by exposure to 30mM KCl. The concentrations of NO were estimated to be in the range of 0.01 to 0.05  $\mu$ M. Hemoglobin (Hb), methemoglobin (MetHb), myoglobin (MyoHb), potassium ferricyanide (FeCN) and methylene blue were added at the concentrations illustrated. Modified from Gruetter *et al.* (5) with permission.

compounds without affecting enzyme activation by nitric oxide itself (6–8). The mechanism of this enhancement was attributed to a reaction between thiol and nitroso compound to form an S-nitrosothiol which decomposed with the discharge of nitric oxide. A series of S-nitrosothiols were synthesized and found to be labile nitric oxide donor agents. In experiments using nitroglycerin, we found that nitroglycerin alone did not activate guanylate cyclase and that added dithiothreitol or glutathione failed to modify this lack of effect of nitroglycerin on guanylate cyclase. In the presence of cysteine, however, nitroglycerin caused a marked activation of guanylate cyclase (9). Sodium nitrite also failed to activate guanylate cyclase in the absence of added thiols but unlike nitroglycerin, a variety of thiols unmasked the enzyme activating action of sodium nitrite. Additional experiments revealed that cysteine reacts with nitroglycerin, whereas sodium nitrite reacts with any of a variety of thiols to generate the corresponding S-nitrosothiol (10). The S-nitrosothiols de-

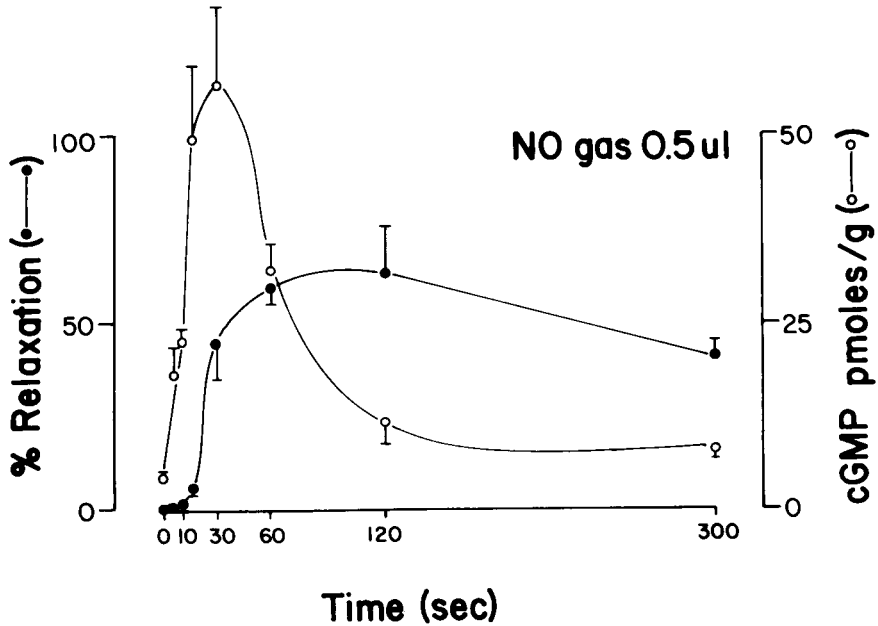


Figure 2. Time course of cyclic GMP accumulation in and relaxation of bovine coronary artery elicited by nitric oxide gas (NO). Strips were mounted, equilibrated and set up for recording changes in isometric force as described previously (5,36). Strips were submaximally precontracted by exposure to 30mM KCl. Relaxation and cyclic GMP (cGMP) levels were measured in the same arterial strips (10,36). Data are expressed as the mean  $\pm$  SEM using 16 arterial strips from 4 separate experiments. Modified from Gruetter *et al.* (36) with permission.

compose readily with the release of nitric oxide, which activates guanylate cyclase. These concepts are illustrated schematically in Figure 3. Examples of such S-nitrosothiols are S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CYSNO), each of which we synthesized and first examined their biological effects in mammalian systems (7-10). These S-nitrosothiols turned out to be useful nitric oxide donor agents that are now readily employed to test the effects of nitric oxide *in vitro* and *in vivo*.

#### INHIBITION OF PLATELET AGGREGATION BY NITRIC OXIDE

In reviewing the literature on nitroglycerin and nitroprusside, I came across a paper showing that nitroprusside inhibited human platelet aggregation (11). The obvious question raised was whether nitroprusside inhibited platelet aggregation by mechanisms involving nitric oxide and cyclic GMP just as had been shown for the smooth muscle relaxant effect of nitroprusside. Using a platelet-rich plasma fraction from human volunteers, we showed that nitric oxide was a potent inhibitor of platelet aggregation induced by ADP, thrombin, collagen and other aggregatory agents, and that the anti-platelet effect of nitric oxide was mediated by cyclic GMP (12). The anti-platelet action of nitroprusside appeared to involve the actions of S-nitrosothiols, which were

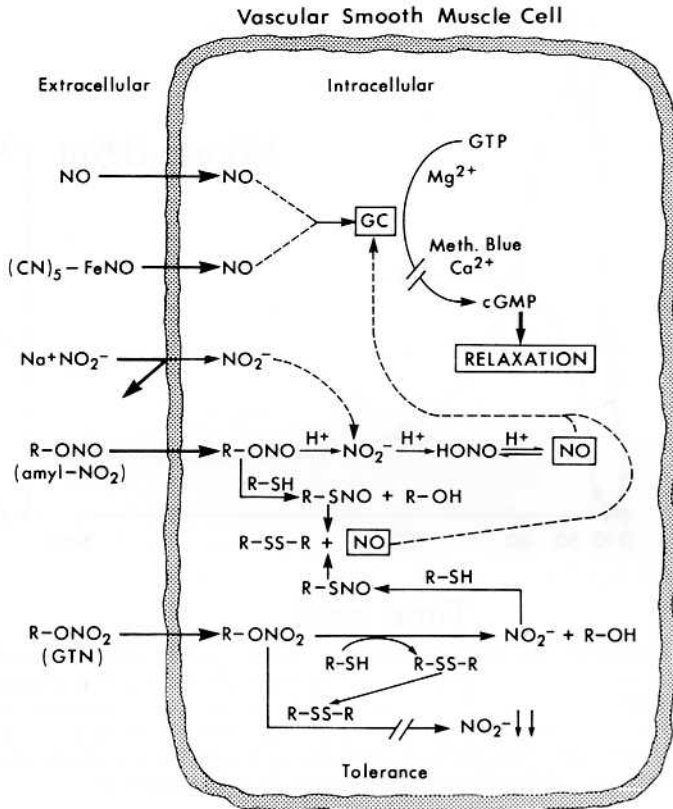


Figure 3. Schematic diagram of the proposed mechanisms by which organic nitrate and nitrite esters, inorganic nitrite and nitroso compounds relax vascular smooth muscle. Abbreviations: NO, nitric oxide; (CN)<sub>5</sub>-FeNO, nitroprusside; R-ONO, organic nitrite ester; amyl-NO<sub>2</sub>, amyl nitrite; R-ONO<sub>2</sub>, organic nitrate ester, GTN, nitroglycerin; GC, cytosolic guanylate cyclase; cGMP, cyclic GMP; Meth. Blue, methylene blue; R-SH, thiol; R-SNO, S-nitrosothiol. Nitroprusside liberates NO in solution, whereas organic nitrite and nitrate esters require chemical interaction with thiols to generate S-nitrosothiols, when then decompose with the liberation of NO. NO activates guanylate cyclase and the resulting cyclic GMP causes vascular smooth muscle relaxation. Tolerance to nitroglycerin develops as a result of depletion of thiols and therefore production of S-nitrosothiols and NO. This is a schematic representation of data published by Ignarro *et al.* (10).

shown to be potent inhibitors of platelet aggregation (13). Human platelets were rich in cytoplasmic guanylate cyclase and nitric oxide activated partially purified guanylate cyclase from platelets by heme-dependent mechanisms.

Thus, nitric oxide not only caused vascular smooth muscle relaxation but also inhibited platelet aggregation, and both biological responses were mediated by cyclic GMP. It is perhaps of interest that these two biological actions of nitric oxide were discovered before or at the time of the discovery of endothelium-dependent vascular smooth muscle relaxation by Robert Furchgott in 1980 (14). In an attempt to better understand the signal transduction mechanisms involved in the stimulation of cyclic GMP formation by nitric oxide, experiments were designed to elucidate the biochemical mechanism by which nitric oxide activates guanylate cyclase.

## MECHANISM OF ACTIVATION OF GUANYLATE CYCLASE BY NITRIC OXIDE

Some of the early mechanistic experiments on guanylate cyclase focused on the possible involvement of enzyme-bound thiols in the regulation of catalysis (15–18). We followed up on these studies and found that guanylate cyclase was very sensitive to oxygen, undergoing reversible inactivation with increasing oxygen tension (19). Sulfhydryl oxidizing agents produced identical effects which were prevented and reversed by addition of sulfhydryl reducing agents. Both basal enzymatic activity and activation of the enzyme by nitric oxide were affected similarly. Protection against enzyme inactivation was afforded by addition of either excess substrate (MgGTP) or thiol antioxidants. The thiol 2,3-dimercaprol, which possesses vicinal dithiols, but not dithiothreitol markedly inhibited guanylate cyclase activity, indicating that two closely juxtaposed -SH groups may be located at the active site. Additional studies suggested that enzyme -SH groups might bind to nitric oxide as part of the enzyme activation process. However, these studies were not definitive in terms of defining the mechanism of activation of guanylate cyclase by nitric oxide. After reading two papers published by Craven and DeRubertis (20,21), who suggested that heme may be required for guanylate cyclase activation by nitric oxide, we decided to purify cytosolic guanylate cyclase and look for enzyme-bound heme. Heme iron, when in the reduced ferrous state, has a high binding affinity for nitric oxide and this might explain why nitric oxide is such a potent activator of guanylate cyclase. Sure enough, cytosolic guanylate cyclase purified from bovine lung was found to contain one mole of heme bound per mole of enzyme dimer (22,23). Similar results were reported by another group (24).

We wanted to ascertain whether the heme bound to guanylate cyclase was indeed required for activation by nitric oxide and whether the heme played any role in the catalytic mechanism of the enzyme. Various procedures were attempted to remove the heme from guanylate cyclase without destroying the enzyme but this approach was unsuccessful at first. Accidentally, we found that passing relatively crude cytosolic fractions containing guanylate cyclase through ion exchange resins such as diethylaminoethyl cellulose at 48°C instead of 36°C (the cold room thermostat was not operating properly) caused the heme to detach from the enzyme without causing enzyme inactivation (23). That is, basal catalytic activity remained unaltered but guanylate cyclase was incapable of being activated by nitric oxide. Preformed nitrosyl-heme, however, markedly activated the heme-deficient enzyme. Addition of heme back to enzyme reaction mixtures completely restored activation of heme-deficient guanylate cyclase by nitric oxide. Eventually, we learned how to prepare heme-containing, heme-deficient and heme-reconstituted forms of guanylate cyclase from rat liver and bovine lung with relative ease (25). The interpretation of the data was clear (Figure 4). Cytosolic guanylate cyclase was a hemoprotein and the function of the heme prosthetic group was to bind to and react with nitric oxide to form the nitrosyl-heme complex and allow en-

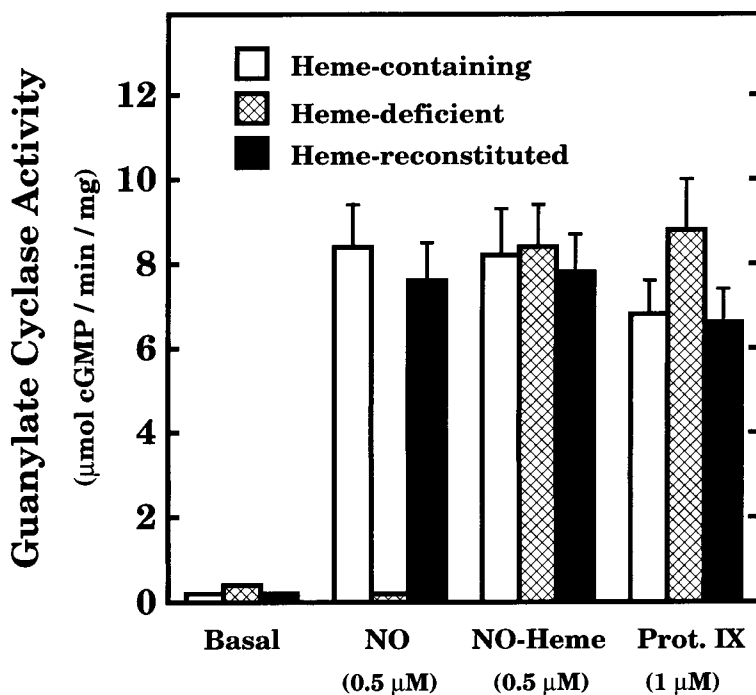


Figure 4. Heme-dependent activation of cytosolic guanylate cyclase by nitric oxide (NO). Guanylate cyclase was purified from bovine lung and assayed as described (22). The purified enzyme contained stoichiometric quantities of heme. Heme was removed from aliquots of native enzyme to yield heme-deficient guanylate cyclase, and the heme-deficient enzyme was reconstituted with stoichiometric quantities of heme as described (23,25). Enzyme reactions were conducted for 5 min under initial velocity conditions. NO-Heme, preformed nitrosyl-heme; Prot. IX, protoporphyrin IX. Data represent the mean  $\pm$  SEM of 12 determinations from 3 separate experiments.

zyme activation by nitric oxide. In addition, guanylate cyclase possessed at least two reactive -SH groups at or near the catalytic site which had to be in the reduced state for the full expression of catalytic activity and activation by nitric oxide (19).

The next step was to elucidate the mechanism by which nitrosyl-heme activates guanylate cyclase. This was a difficult question to answer because the structure of guanylate cyclase was unknown. After studying the published interactions between hemoglobin or myoglobin and nitric oxide, we came up with a hypothesis to explain how nitrosyl-heme activates guanylate cyclase. We envisioned that heme (iron-protoporphyrin IX) was bound to guanylate cyclase at a site that was adjacent to the catalytic site that binds the substrate MgGTP. As in other hemoproteins, the axial ligand (bond between heme iron and protein) was thought to involve a histidine residue in guanylate cyclase, thereby yielding a 5-coordinate complex (where the heme iron and each of the four pyrrole nitrogen atoms form ligands with the enzyme protein). Removal of the heme group does not influence basal catalytic ac-

tivity but abolishes enzyme activation by nitric oxide. We proposed that nitric oxide forms a covalent bond with the reduced heme iron (ferrous) to yield a 5-coordinate complex rather than a 6-coordinate complex. This means that the axial ligand would have to break and the resulting nitrosyl-heme complex would undergo a change in configuration while binding to the enzyme protein. This conformational change near the catalytic site could increase the affinity of guanylate cyclase for MgGTP and also increase the maximal velocity of catalysis (Figure 5). In order to test this hypothesis, protoporphyrin IX (heme without its iron) was tested to see if it could activate heme-deficient guanylate cyclase. The reasoning behind this experiment was that the axial

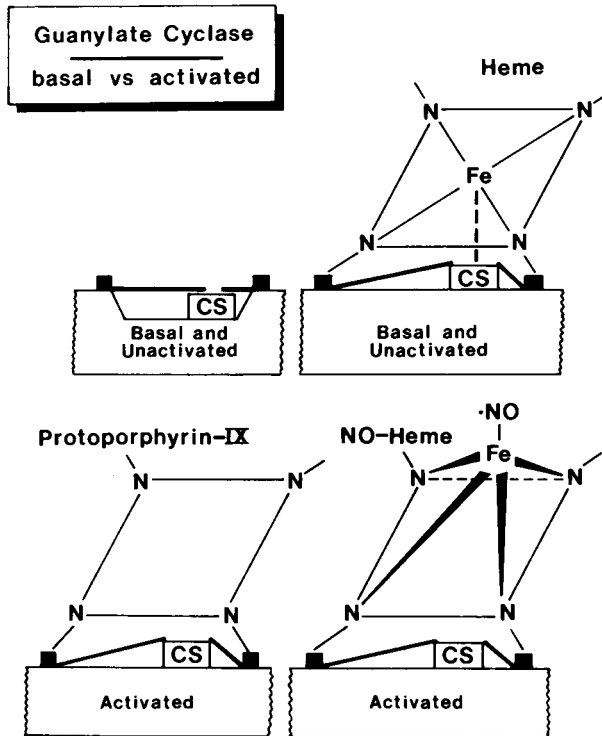


Figure 5. Schematic illustration of the mechanism of heme-dependent activation of cytosolic guanylate cyclase by nitric oxide (NO). This schematic is based on the knowledge that heme is required for the activation of guanylate cyclase by NO. In the basal or unactivated state and in the absence of the heme prosthetic group, the catalytic site (CS) in guanylate cyclase is only partially exposed so that substrate (MgGTP) availability is limited and cyclic GMP production is limited. In the presence of the heme prosthetic group (native enzyme) but in the absence of any NO (basal or unactivated state), catalytic activity is still minimal because of steric hindrance at the catalytic site. Enzyme-bound heme permits exposure of the catalytic site but the axial ligand (bond between heme iron (Fe) and enzyme protein near CS) limits access of substrate to CS, thereby limiting cyclic GMP production. NO binds to heme iron to form the nitrosyl-heme adduct as illustrated. This NO-heme complex remains as a 5-coordinate complex, thereby resulting in the breakage of the axial ligand and exposure of CS to enzyme substrate. The activated enzyme shows up to a 400-fold increase in  $V_{max}$  and a 3-fold decrease in the  $K_m$  for MgGTP. Addition of protoporphyrin IX to heme-deficient guanylate cyclase mimics the effect of NO-heme because protoporphyrin IX binding allows exposure of CS without any steric hindrance due to the absence of heme iron.



ligand between heme iron and guanylate cyclase might create steric hindrance to the binding of MgGTP to the catalytic site, whereas protoporphyrin IX should bind to the heme binding site but create no steric hindrance because of the absence of iron, and the result should be enzyme activation by protoporphyrin IX. That is exactly what we observed. Protoporphyrin IX was a potent activator of guanylate cyclase (22) and it mimicked the activating effect of nitrosyl-heme in that enzyme activation by protoporphyrin IX was heme-independent, like that for preformed nitrosyl-heme (26). A kinetic analysis revealed that protoporphyrin IX and nitrosyl-heme activated guanylate cyclase by kinetically indistinguishable mechanisms (26).

These observations revealed that guanylate cyclase possessed a porphyrin binding site that was responsible for enzyme activation by nitric oxide. More elaborate studies were later conducted by others (27–31) to verify our original hypothesis (32). Revelation of the crystal structure of cytosolic guanylate cyclase will undoubtedly explain the more precise mechanisms by which nitric oxide activates guanylate cyclase.

#### IDENTIFICATION OF ENDOTHELIUM-DERIVED RELAXING FACTOR AS NITRIC OXIDE

Most of the research discussed above was conducted before or shortly after the discovery of endothelium-dependent vasorelaxation by Robert Furchgott in 1980 (14). The knowledge that nitroglycerin was such a potent vasorelaxant and the more recent findings that nitric oxide was a potent vasorelaxant and inhibitor of platelet aggregation led to the question of why do mammals have receptors for such potent pharmacological compounds. This thought suggested that mammals might possess either an endogenous nitro compound that releases nitric oxide or nitric oxide itself. The early studies on endothelium-derived relaxing factor (EDRF) were conducted without any knowledge about its chemical structure. We started to study endothelium-dependent vasorelaxation in 1983, because we wanted to determine whether cyclic GMP was involved, and not because we thought that EDRF might be nitric oxide. Indeed, a separate project was also underway to find the endogenous nitric oxide. Just as we completed our experiments on EDRF and cyclic GMP, Ferid Murad's group published a paper showing that endothelium-dependent vasorelaxation by acetylcholine and other agents was associated with cyclic GMP production in vascular smooth muscle (33). We observed similar results and also found that methylene blue, an inhibitor of guanylate cyclase activity, prevented both the cyclic GMP accumulating effect and vasorelaxant effect of acetylcholine (34). At first, we entertained the idea that acetylcholine might be stimulating the formation of a metabolite of arachidonic acid, which then somehow activated guanylate cyclase to elevate cyclic GMP levels. This view was supported by studies from this and other laboratories showing that agents that interfere with the formation of arachidonic acid and certain arachidonic acid metabolites also interfere with acetylcholine-mediated vasorelaxation (33–35). But I was not satisfied with this

view because experiments in my laboratory failed to reveal that anything other than nitric oxide could activate guanylate cyclase. After reviewing our data again, we suddenly realized that our finding that methylene blue prevented both the increase in cyclic GMP and vasorelaxation in response to acetylcholine was similar to our earlier finding on the prevention of both cyclic GMP formation and vasorelaxation in response to nitric oxide (36). This indicated that the vasorelaxant action of acetylcholine was pharmacologically similar to that for nitric oxide. This concept is indicated in Figure 6, which was published in 1984 (34). We were cautious, however, and did not suggest in writing that EDRF might be nitric oxide. Later, after other laboratories showed that EDRF was a very unstable molecule (37,38), we realized that the data from several laboratories were consistent with our view that EDRF and nitric oxide were closely similar. The two distinct projects in my laboratory, one focusing on EDRF/cyclic GMP and the other focusing on endogenous nitric oxide, converged but we were not yet ready to propose that EDRF was nitric oxide.

A series of experiments was designed to test our yet unpublished hypothesis that EDRF might be nitric oxide. The first experiment was to determine whether EDRF released from artery and vein could activate guanylate cyclase and thereby account for elevated tissue cyclic GMP levels in response to acetylcholine or bradykinin, and this study was published in 1986 (39). Isolated arterial and venous rings prepared from bovine intrapulmonary vessels were incubated in reaction mixtures containing cytosolic guanylate cyclase purified from bovine lung. Addition of acetylcholine to reaction mixtures containing

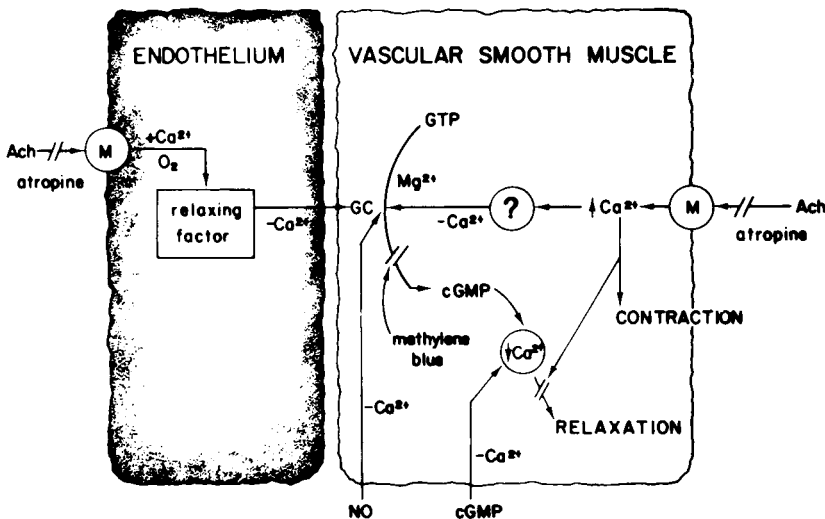


Figure 6. Schematic diagram of the similarities in the mechanisms of vascular smooth muscle relaxation elicited by acetylcholine and nitric oxide (NO). Abbreviations: Ach, acetylcholine; M, muscarinic receptor; GC, cytosolic guanylate cyclase; cGMP, cyclic GMP;  $-Ca^{2+}$ , calcium-independent mechanism;  $+Ca^{2+}$ , calcium-dependent mechanism. Acetylcholine and NO cause vasorelaxation by mechanisms involving cyclic GMP formation. Blockade of cyclic GMP formation by methylene blue inhibits vasorelaxation by both acetylcholine and NO, as illustrated. Modified from Ignarro *et al.* (34) with permission.

arterial rings resulted in the activation of guanylate cyclase, and bradykinin elicited the same effect in reaction mixtures containing either arterial or venous rings. Activation of guanylate cyclase by acetylcholine and bradykinin was dependent on the presence of an intact endothelial layer in arterial and venous rings, and the response was blocked by methylene blue and enhanced by antioxidants. These experiments revealed that EDRF from artery and vein activates guanylate cyclase by mechanisms that can be inhibited by methylene blue and enhanced by antioxidants. Clearly, the data were pointing toward the possibility that EDRF is nitric oxide or a chemically related substance such as a labile nitroso compound. The next experiment was to determine whether the activation of guanylate cyclase by EDRF was heme-dependent as is enzyme activation by nitric oxide. Experiments similar to those described above were repeated using guanylate cyclase that had been purified in both the heme-containing and heme-deficient forms. Sure enough, guanylate cyclase activation by EDRF, like enzyme activation by nitric oxide, was heme-dependent (Figure 7). At the end of the first experiment of this series, we

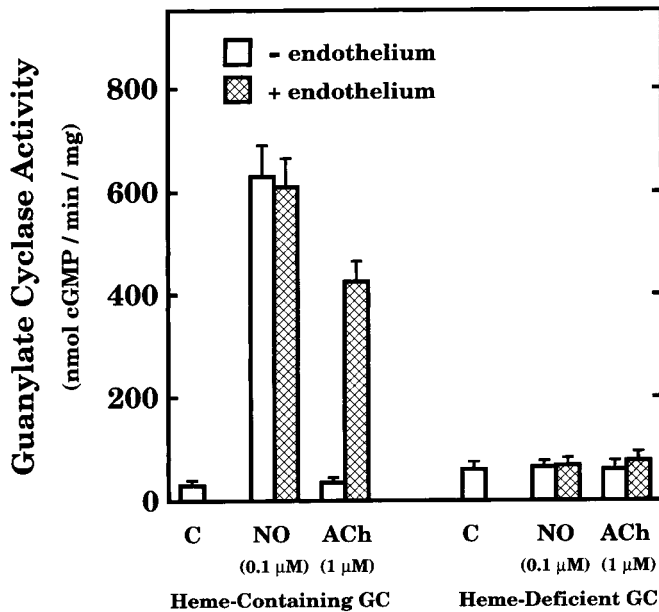


Figure 7. Endothelium-dependent and heme-dependent activation of cytosolic guanylate cyclase by endothelium-derived relaxing factor (EDRF) released from arterial rings. These experiments were based on techniques described previously (39). Bovine intrapulmonary arterial rings were prepared with and without intact endothelium and mounted in tissue baths for monitoring vasorelaxation. Acetylcholine (ACh) and nitric oxide (NO) were added to tissue baths to monitor relaxant responses. ACh elicited endothelium-dependent, whereas NO elicited endothelium-independent vasorelaxation. After the first round of additions to the tissue baths, the rings were rapidly detached and lowered into reaction vessels containing either heme-containing guanylate cyclase or heme-deficient guanylate cyclase, to which were added acetylcholine or NO. As illustrated, acetylcholine and NO caused heme-dependent activation of guanylate cyclase, but only NO caused endothelium-independent activation of guanylate cyclase. The conclusion was that acetylcholine causes the formation of NO from the vascular endothelium, and the NO causes heme-dependent activation of guanylate cyclase. Data represent the mean  $\pm$  SEM of 12 determinations from 6 separate experiments.

knew that we had it. EDRF must be nitric oxide. But I wanted to conduct more definitive experiments before going out on a limb and advocating that EDRF is nitric oxide. A bioassay cascade apparatus was set up in order to study the chemical and pharmacological properties of EDRF released from artery and vein, and to compare these with the properties of nitric oxide. These experiments revealed the very close similarity of EDRF and nitric oxide, and were presented at several conferences in 1986 and early 1987 (40–42) before their publication in 1987 (43,44).

There was one experiment in particular that convinced me beyond any shadow of a doubt that EDRF was nitric oxide. We took advantage of a previous experiment in my laboratory on guanylate cyclase, in which we demonstrated that guanylate cyclase was a hemoprotein, like hemoglobin, that reacted with nitric oxide to form the nitrosyl-heme adduct by monitoring the spectral shift in the Soret region (23). This classical chemical analysis provides proof of the presence of heme iron, and established guanylate cyclase as a hemoprotein. Accordingly, we conducted similar experiments with EDRF released from cultured bovine aortic endothelial cells and showed that EDRF caused a spectral shift in hemoglobin that was identical to that obtained for authentic nitric oxide (43) (Figure 8). Suddenly, all previous data from our laboratory and several other laboratories made complete sense and were consistent with the view that EDRF was nitric oxide. EDRF is a small, labile and lipophilic molecule that is inactivated by oxygen and oxygen-derived radicals, activates cytosolic guanylate cyclase by heme-dependent mechanisms, causes smooth muscle relaxation and inhibition of platelet aggregation and adhesion by cyclic GMP-dependent mechanisms, and whose actions are blocked by hemoproteins or methylene blue but enhanced by antioxidants or oxygen radical scavengers.

At the time we were conducting some of the above experiments, other laboratories were conducting experiments that turned out to be consistent with the view that EDRF was nitric oxide. For example, hemoglobin was reported to interfere with acetylcholine-elicited, endothelium-dependent vasorelaxation (45,46), superoxide anion was shown to decrease, whereas superoxide dismutase increased the chemical stability of EDRF (37,38) and EDRF inhibited platelet aggregation (47). All of these effects on or actions of EDRF were identical to those for nitric oxide. Despite this obvious analogy, no one wanted to go out on a limb and propose that EDRF was nitric oxide, except for Robert Furchgott and myself, when we independently proposed this hypothesis at the same international conference in Rochester, Minnesota in June of 1986 (40).

#### THE PHYSIOLOGICAL SIGNIFICANCE OF NITRIC OXIDE IN VASCULAR BIOLOGY

Soon after the discovery that EDRF is nitric oxide, evidence was presented that vascular endothelial cells can produce nitric oxide and citrulline from arginine (48). This finding was remarkably similar to the observation in cyto-

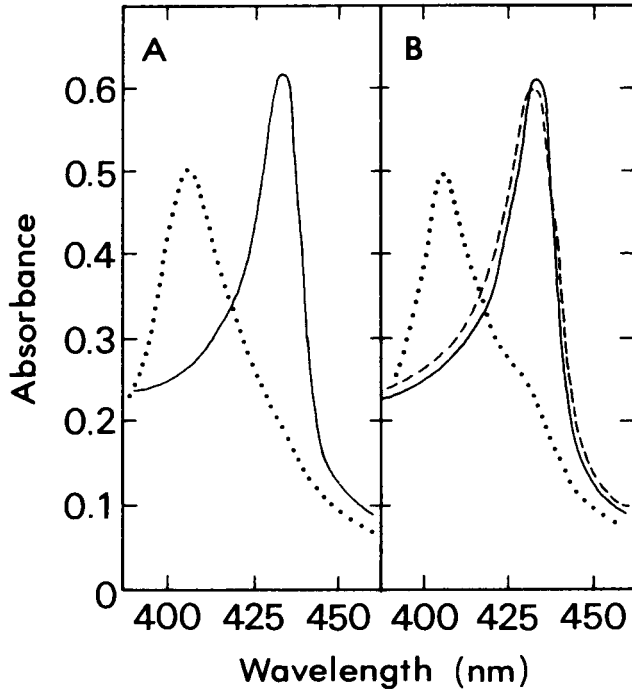


Figure 8. Identification of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) by spectrophotometric analysis of the reaction between deoxyhemoglobin and NO or EDRF. The EDRF was generated from freshly isolated bovine aortic endothelial cells stimulated by the calcium ionophore, A23187. Experiments were performed in quartz cuvettes as described previously (43). Panel A: solid curve signifies 5  $\mu\text{M}$  deoxyhemoglobin alone; dotted curve signifies deoxyhemoglobin gassed with NO. Panel B: solid curve signifies 5  $\mu\text{M}$  deoxyhemoglobin alone; dashed curve signifies deoxyhemoglobin after reaction with  $10^6$  endothelial cells; dotted curve signifies deoxyhemoglobin after reaction with endothelial cells in the presence of 1  $\mu\text{M}$  A23187. Data illustrated are from one experiment representative of 3 separate experiments. Modified from Ignarro *et al.* (43) with permission.

kine-activated macrophages that nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ) are produced from arginine (49–51). Indeed, these studies revealed that a structural analog of arginine,  $\text{N}^G$ -methylarginine, inhibited the formation of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  from arginine in macrophages and also inhibited the production of nitric oxide from arginine in endothelial cells. Confirmation of these original observations and unequivocal demonstration of an enzyme that catalyzes the conversion of arginine to nitric oxide plus citrulline was made by Bredt and Snyder (52). The enzyme was termed nitric oxide synthase and this study started an avalanche of research leading to the isoform characterization, purification, elucidation of catalytic mechanisms and structure-function characterization of nitric oxide synthase (53–61).

During this period of time, incredible progress was also being made on the physiological and pathophysiological properties of endogenous nitric oxide. Vascular endothelial cells synthesize nitric oxide continuously for the purpose of causing vasodilation to limit any increases in systemic blood pressure (62,63). This conclusion was drawn from experiments in which  $\text{N}^G$ -methyl-

arginine, a competitive inhibitor of nitric oxide synthase, caused a sustained increase in systemic blood pressure in animals after intravenous injection. The hypertensive response was completely reversed by intravenous injection of excess L-arginine, the substrate for nitric oxide synthase. The continuous generation of endothelium-derived nitric oxide appears to occur as a consequence of continuous blood flow or shear stress (64), which can trigger the calcium-dependent activation of endothelial nitric oxide synthase (65). Endothelium-derived nitric oxide also functions to inhibit platelet aggregation and adhesion (66), and to slow or prevent proliferation of the underlying vascular smooth muscle (67). Research continues on the elucidation of the protective role of nitric oxide in reperfusion or reoxygenation injury, restenosis, and atherosclerosis. The protective effects of nitric oxide may stem from its capacity to improve local blood flow, inhibit thrombosis or cell adhesion, interfere with cell proliferation, and inhibit key enzymes involved in mediating cellular modification or destruction.

The neurotransmitter function of nitric oxide was first shown in the brain (68). These observations were based on the findings that glutamate and nitric oxide stimulate the formation of cyclic GMP in the brain, and that arginine can also elevate cyclic GMP levels under certain conditions. The function of nitric oxide as a neurotransmitter in the central nervous system remains unknown but one possible hypothesis has been that nitric oxide modulates long-term potentiation in the control of learning and memory (66). The bad side of nitric oxide is that excessive production of nitric oxide in the brain stimulated by glutamate can lead to serious tissue injury in specialized regions of the brain (69). Moreover, excessive production of nitric oxide in close proximity to oligodendrocytes can lead to inhibition of myelin formation and perhaps multiple sclerosis (70). Evidence has amassed that nitric oxide is an important transmitter of the nonadrenergic-noncholinergic (NANC) neurons that innervate many smooth muscle tissues such as the airways, gastro-intestinal tract and genito-urinary tract (66).

We explored the possibility that nitric oxide might be the principal neurotransmitter that mediates penile erection. The reason for pursuing this project was that before 1990 the physiological mechanism by which nerve stimulation promotes erectile function was unknown. The common belief was that excitation of the parasympathetic nervous system caused acetylcholine-mediated erectile responses. The erectile response consists of neuronally mediated relaxation of both vascular and nonvascular smooth muscle associated with the cavernous or trabecular or sinusoidal arrangement of arterioles in the corpus cavernosum. The problem that this hypothesis presented to pharmacologists and physiologists is that the cholinergic neurotransmitter, acetylcholine, cannot relax nonvascular smooth muscle and can relax vascular smooth muscle only by endothelium-dependent mechanisms. It was viewed highly unlikely that acetylcholine, a quaternary ammonium compound (highly charged) would reach the vascular endothelial cells from the neuronal synapses at the vascular smooth muscle cells. However, excitation of any nitric oxide-containing NANC nerves that innervate the nonvascular and vas-

cular smooth muscle in the corpus cavernosum would be expected to promote extensive smooth muscle relaxation and consequent penile erection. At the time of our first experiment, it was not known whether or not erectile tissue in mammals was innervated by NANC nerves, and we were not equipped to conduct such histochemical experiments. Instead, we used the classical tissue bath approach in which strips of corpus cavernosum isolated from rabbit and human penis were mounted in bath chambers, precontracted and then electrically stimulated in order to cause smooth muscle relaxation.

Our first experiment revealed that NANC stimulation of isolated strips of rabbit corpus cavernosum resulted in smooth muscle relaxation that was blocked by inhibitors of nitric oxide synthase, hemoglobin and methylene blue (71). Nitric oxide in the form of S-nitrosothiols also caused smooth muscle relaxation, and electrical stimulation of the tissue resulted in elevated levels of both nitrite ( $\text{NO}_2^-$ ) and cyclic GMP (71). The conclusion was clear. Nitric oxide is the neurotransmitter in NANC nerves that innervate the corpus cavernosum and that nerve stimulation results in nitric oxide-mediated vascular and nonvascular smooth muscle relaxation. The action of the nitric oxide neurotransmitter is, in turn, mediated by cyclic GMP. Therefore, the signal transduction pathway involving nitric oxide as a neurotransmitter appears to be the same as that for endothelium-derived nitric oxide. Additional experiments with human corpus cavernosum yielded virtually identical data as the experiments using rabbit tissue (72,73). Figure 9 illustrates the typical marked but transient smooth muscle relaxation responses elicited by electrical stimulation of corpus cavernosum from both rabbit and human. We were struck with the very transient nature of the responses and that these electrically-induced responses could be mimicked by addition of authentic nitric oxide gas. Perhaps the most convincing experiment indicating that electrical stimulation triggers erectile function via mechanisms involving nitric oxide was the finding that  $\text{N}^G$ -nitroarginine, a competitive inhibitor of nitric oxide synthase, abolished the electrical responses, whereas excess enzyme substrate, L-arginine, overcame the inhibitory effect of  $\text{N}^G$ -nitroarginine (Figure 10). Our early studies also revealed that inhibition of the degradation of cyclic GMP through the use of phosphodiesterase inhibitors caused an enhancement of both electrically-induced smooth muscle relaxation and nitric oxide-elicited smooth muscle relaxation (72-74). The data for enhancement of nitric oxide-elicited relaxation of corpus cavernosum by the cyclic GMP phosphodiesterase inhibitor, M&B 22,948 (zaprinast), is illustrated in Figure 11. These observations indicated clearly that relaxation of corpus cavernosum smooth muscle by NANC nerve excitation or addition of nitric oxide is mediated by cyclic GMP.

These observations shed a great deal of light on the physiological mechanism of penile erection by revealing a critical signal transduction pathway that could be modulated by drugs. For example, drugs that interfere with the formation or action of nitric oxide or cyclic GMP could also interfere with the erectile response, and might be useful for treating priapism. But much more importantly, drugs that enhance the formation or action of nitric oxide or

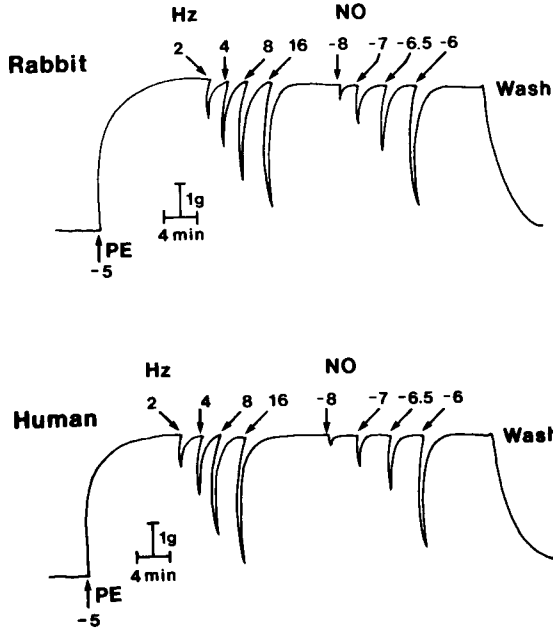


Figure 9. Relaxation of corpus cavernosum smooth muscle by nitric oxide (NO) closely resembles relaxation elicited by electrical field stimulation of NANC nerves. Strips of corpus cavernosum from rabbit and human penis were prepared, mounted in tissue baths and isometric tension recordings obtained (71,73). Smooth muscle was precontracted by addition of phenylephrine (PE) as shown. Electrical field stimulation was conducted at 2, 4, 8 and 16 hertz (Hz) as shown. Concentrations of NO and PE are expressed as Molar (ie.  $10^{-8}$  M). Tracings are representative of 12-18 strips from 4 rabbits or 4 humans.

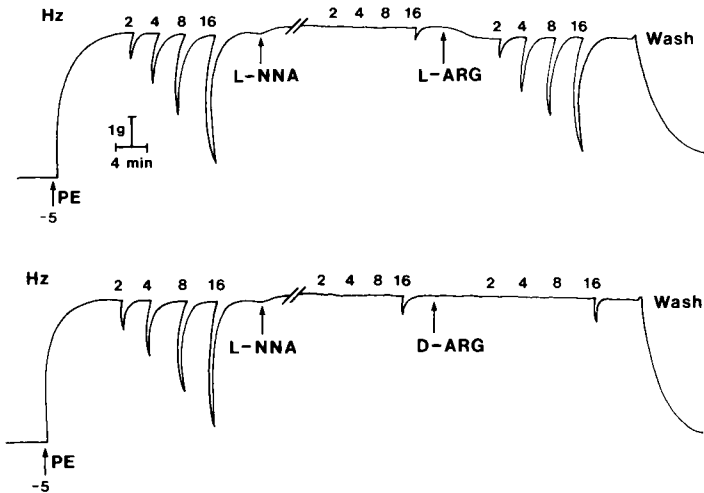


Figure 10. Relaxation of human corpus cavernosum smooth muscle by electrical field stimulation is blocked by nitric oxide (NO) synthase inhibitors. Strips of corpus cavernosum from human penis were prepared, mounted in tissue baths and isometric tension recordings obtained (71,73). Smooth muscle was precontracted by addition of phenylephrine (PE) as shown. Electrical field stimulation was conducted at 2, 4, 8 and 16 hertz (Hz) as shown.  $N^G$ -Nitroarginine (L-NNA) and arginine (L-ARG or D-ARG) were tested at  $30 \mu\text{M}$  and  $300 \mu\text{M}$ , respectively. Tracings are representative of 12-18 strips from 4 humans.



cyclic GMP could also enhance the erectile response, and might be effective in the treatment of impotence, the most prevalent medical disorder in men. In 1998, the drug sildenafil (Viagra<sup>R</sup>) was marketed for the effective treatment of impotence. Sildenafil is a relatively selective inhibitor of one of the phosphodiesterase isoforms that degrades cyclic GMP and is present in relatively large quantities in the corpus cavernosum. Therefore, sildenafil allows intracellular cyclic GMP in corpus cavernosum smooth muscle to accumulate to high levels when ever there is a nitric oxide signal coming from the NANC nerves. Penile erection resulting from any form of excitation of the NANC nerves innervating the corpus cavernosum, whether mental or physical, would be enhanced by sildenafil, but the drug alone would not be expected to cause an erectile response in the absence of any NANC nerve excitation.

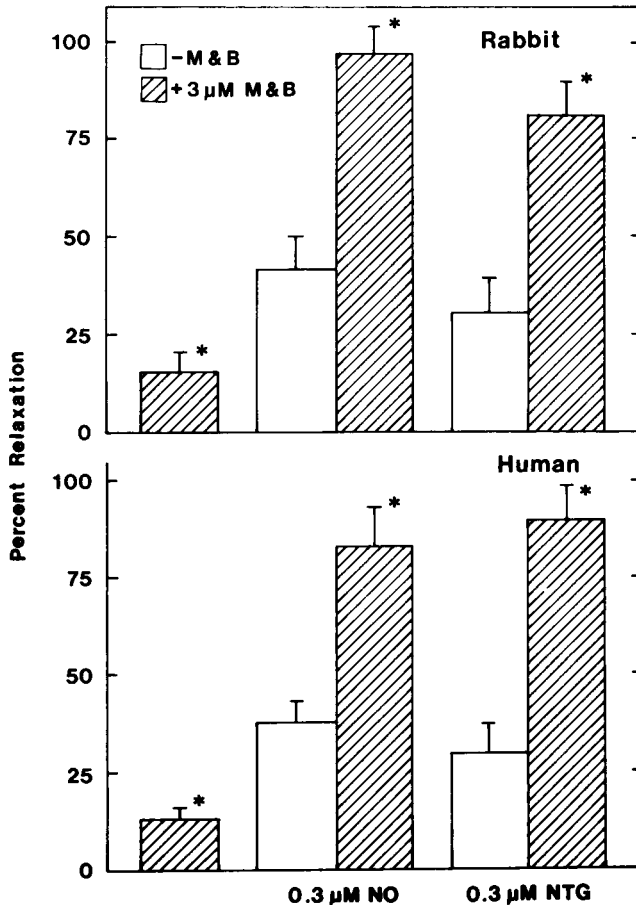


Figure 11. Enhancement of nitric oxide (NO)-elicited and nitroglycerin (NTG)-elicited relaxation of rabbit and human corpus cavernosum smooth muscle by M&B 22,948 (M&B). Strips of corpus cavernosum from rabbit and human penis were prepared, mounted in tissue baths and isometric tension recordings obtained (71,73). Smooth muscle was precontracted by addition of 10 μM phenylephrine. Each point represents the mean ± SEM of 9-12 strips from 4 rabbits or 4 humans. \* All values in presence of M&B 22,948 are significantly different ( $p < 0.001$ ) from corresponding values obtained in absence of M&B 22,948.

Sildenafil appears to be very effective in treating various types and degrees of impotence. Our improved understanding of the physiology of erectile function will enable scientists to unravel the pathophysiology and causes of impotence, thereby further opening the doors to develop even more promising drugs for the treatment of impotence.

#### FUTURE DIRECTIONS FOR NITRIC OXIDE RESEARCH IN VASCULAR BIOLOGY

The knowledge that vascular endothelium-derived nitric oxide and neuro-nally-generated nitric oxide promote the relaxation of vascular and nonvascular smooth muscle associated with the vascular system will undoubtedly afford scientists and clinicians the opportunity to make great advances in the diagnosis, treatment and prevention of life-threatening cardiovascular disorders such as coronary artery disease, essential hypertension, stroke, vascular complications of diabetes and related diseases. Already, it appears that nitric oxide plays many critical physiological roles in the regulation and protection of numerous tissue and organ functions. Defects or lesions in the nitric oxide-cyclic GMP pathway can probably account for much of the observed pathophysiology of cardiovascular disease. The development of a better understanding of basic physiological mechanisms involving the nitric oxide-cyclic GMP pathway should lead to the development of novel therapeutic strategies to reduce the morbidity and mortality attributed to cardiovascular disease.

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