

SPECIAL ARTICLE

SHATTUCK LECTURE

Nitric Oxide and Cyclic GMP in Cell Signaling and Drug Development

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AS AN M.D.–PH.D. STUDENT, I WAS FORTUNATE TO HAVE WORKED IN THE laboratories of Earl Sutherland and Theodore Rall shortly after their discovery of cyclic adenosine monophosphate (AMP) in 1957 as a second messenger that mediates the glycogenolytic effects of epinephrine and glucagon.¹ My assignment was to examine the effects of catecholamines on cyclic AMP synthesis and determine whether these effects were mediated through the beta or alpha adrenergic receptor. This was actually a straightforward and simple student assignment.² I also found that choline esters such as acetylcholine, acting by transduction at the muscarinic receptor, inhibited adenylyl cyclase activity.² This experience set the stage for my long-term continued interest in cellular signaling and the role of intracellular second messengers in mediating the effects of various hormones and drugs.

A few years later, cyclic guanosine monophosphate (GMP) was discovered in urine.³ Its identification as a natural product encouraged several laboratories to look for three entities. All three were enzymes — guanylyl cyclase, which effects the synthesis of cyclic GMP; phosphodiesterase, which hydrolyzes cyclic GMP; and a protein kinase that is selectively activated by cyclic GMP. In the late 1960s and early 1970s, all three enzymes were described.⁴ In fact, these enzymes are each members of large families of isoforms. Some of us in the field suspected that cyclic GMP could be yet another intracellular “second” messenger that mediated the effects of some hormones (Fig. 1).

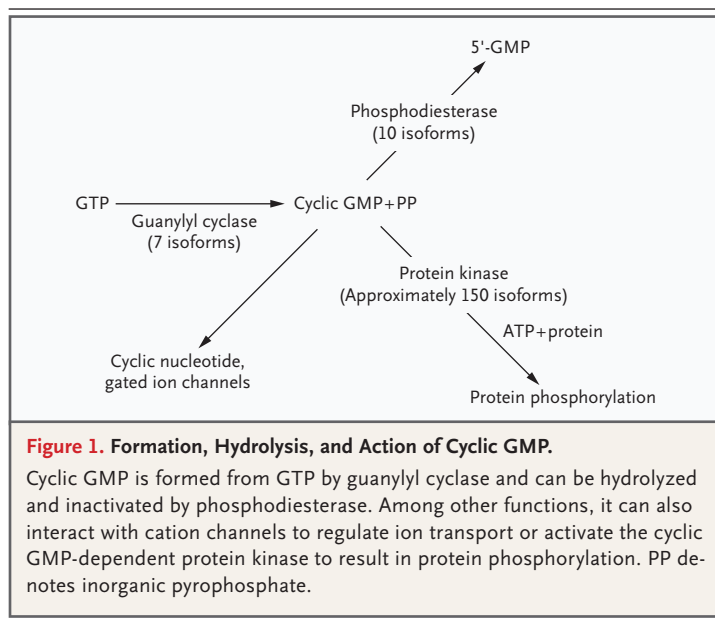
As I completed my training at the National Institutes of Health in 1970 to accept a faculty position at the University of Virginia, I decided to move most of my research interests from cyclic AMP to cyclic GMP. I wanted to address two questions. The first was to determine how the binding of hormone ligands to their receptors regulated guanylyl cyclase. Specifically, I asked, “What are the molecular coupling events?” I thought that understanding the coupling of receptors to guanylyl cyclase might permit the use of agents or drugs to enhance or inhibit hormonal effects in some clinical disorders. Acetylcholine and some eicosanoids were found to increase cyclic GMP in cardiac and vascular tissue preparations, but the mechanisms of the molecular coupling of their receptors to guanylyl cyclase were unknown. Second, no one knew what physiological effects or functions cyclic GMP might have if it were indeed an intracellular messenger. I hoped to define its role as a messenger in regulating some biochemical and physiological functions. The field of research on cyclic GMP was less crowded than that of cyclic AMP research, and as a new young faculty member, I found this appealing.

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GUANYLYL CYCLASE ACTIVITY

My colleagues and I began our studies by examining guanylyl cyclase activity in various tissue homogenates and surprisingly found activity in both high-speed super-



nanat and particulate fractions. The kinetic properties of the activity differed in the two types of tissue preparations, and the most striking feature was the cooperative catalytic kinetics of the particulate activity with regard to the substrate guanosine triphosphate (GTP). In contrast, the soluble guanylyl cyclase (sGC) activity demonstrated typical Michaelis–Menten kinetics; this finding suggested that the sGC activity represented a single catalytic GTP site.^{5,6} Although we suspected that the enzyme had different isoforms, we could not rule out spurious artifactual data because our crude preparations also contained nucleotidases, phosphatases, and phosphodiesterases that were competing for the substrate or product. To address this question definitively would require the purification, characterization, cloning, expression, and recharacterization of the enzyme, which took us about a dozen years.

If indeed there were different isoforms of guanylyl cyclase in different intracellular compartments, perhaps they would be regulated by different groups of hormones and drugs to create discrete intracellular compartments of cyclic GMP with different functions. Quite quickly, the project showed considerable potential and stirred a great deal of excitement in our laboratory. To take a shortcut and temporarily avoid purification of the enzyme, we added a variety of substances to our incubations in order to inhibit contaminating phosphatases, nucleotidases, and phosphodiesterases; this

step allowed us to redefine the kinetic behavior of the enzyme.⁷

The shortcut led us to a second surprising and serendipitous observation. Some of these substances, including sodium azide, sodium nitrite, and hydroxylamine, activated the enzyme.⁷ Although several hormones increased cyclic GMP in intact tissues, as noted above, they had no effect in cell-free preparations. We thought these new activators of guanylyl cyclase could help us reconstitute hormonal activation in homogenates and determine their molecular mechanism of coupling. This intuition proved to be correct.

Azide activation was tissue-specific, required oxygen, was enhanced with some thiols, such as cysteine, and took several minutes before reaching the maximum rate of reaction. We suspected that azide was converted in our incubations to an activator of guanylyl cyclase. We did simple reconstitution experiments by mixing supernatant fractions that did or did not respond to azide. These experiments demonstrated that heart extracts possessed an inhibitor of azide activation. The substance was heat labile, could not be dialyzed, and was presumably a protein. After much work, we discovered that hemoglobin and myoglobin were the inhibitors.⁸ When extracts of liver and cerebral cortex were mixed, the azide effect was enhanced or potentiated, because liver possessed a factor that converted azide to an activator of both preparations. When purified, this factor was found to be catalase.⁹

Azide, nitrite, and hydroxylamine also increased cyclic GMP levels in various tissues, including tracheal smooth-muscle preparations.^{10,11} The increase in cyclic GMP was associated with smooth-muscle relaxation; the data demonstrated straightforward dose–response relationships. Nitroglycerin, a drug used clinically since the 1870s for angina pectoris, also activated sGC; increased cyclic GMP levels in various tissues, including tracheal smooth muscle; and caused smooth-muscle relaxation.¹² Sodium nitroprusside, another smooth-muscle relaxant, had similar effects.^{12–14}

My colleagues and I had a growing list of sGC activators that also acted as relaxants with tracheal, gastrointestinal, and vascular smooth muscle.^{13,14} We coined the term “nitrovasodilators” for these prodrugs that we believed were converted to nitric oxide because chemically generated nitric oxide activated all of the sGC preparations we tested.^{15,16} Considering the high affinity of nitric

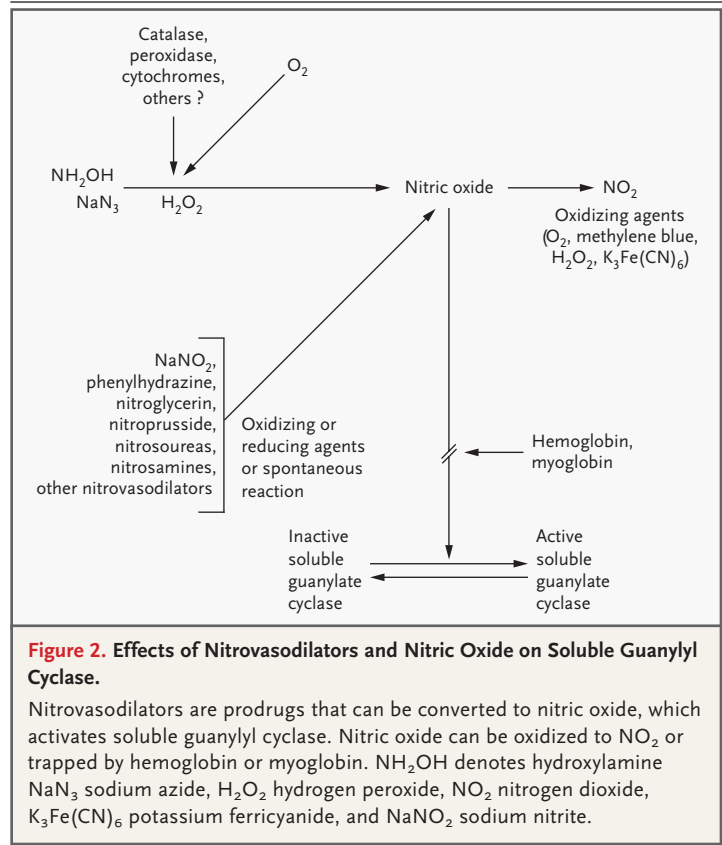
oxide for heme, this also explained the inhibitory effects of hemoglobin and myoglobin (Fig. 2), which presumably trapped or scavenged the nitric oxide generated in our incubations.

We recognized that we had defined the mechanism of action of these nitric oxide prodrugs. We also proposed that nitric oxide could function as an intracellular messenger for hormones and drugs that could increase its production from an endogenous precursor.^{13,14} The idea that we could activate an enzyme with a free radical (nitric oxide) and then propose that this free radical was an endogenous messenger molecule was viewed very skeptically by the scientific community. Because activation of purified sGC occurred at nanomolar concentrations and because assays for nitric oxide and its oxidation products, nitrite and nitrate, were insensitive, it took another 7 to 8 years before the hypothesis was definitively confirmed and accepted owing to the development of new technologies and assays for nitric oxide.

ENDOTHELIAL ACTIVITY

Whereas nitric oxide is probably an ancient simple molecule that may have participated as a messenger in evolution, before 1980 it was largely viewed as a pollutant released from smokestacks and present in automobile exhaust and cigarette smoke.¹⁷ However, this view of nitric oxide changed in the 1980s. To understand the events as they unfolded, we need to examine a classic set of experiments performed by Robert Furchgott. In these experiments, he identified an unknown and labile activity released from endothelial cells when they were stimulated with certain substances. His work showed that the endothelium released a labile factor, which he termed “endothelial derived relaxant factor” (EDRF), that caused vascular relaxation when vascular smooth-muscle preparations were stimulated with vasodilators such as acetylcholine, histamine, and bradykinin.¹⁸

I heard Furchgott present his data at a seminar in 1980 before its publication in *Nature*.¹⁸ On the basis of his observations, I suspected that EDRF was a reactive free radical that might be analogous to nitric oxide in increasing cyclic GMP levels in smooth muscle. I further suspected that EDRF would turn out to be a nitric oxide complex or adduct and began to call EDRF the “endogenous nitrovasodilator.”¹⁹ We found that EDRF increased cyclic GMP levels,^{20,21} activated cyclic GMP-depen-



dent protein kinase, and phosphorylated the same vascular smooth-muscle proteins as did nitrovasodilators (Fig. 3).^{22,23}

Other agents causing vascular relaxation, such as phosphodiesterase inhibitors, calcium antagonists, and adenylyl cyclase activators, do so by increasing cyclic nucleotide levels or lowering intracellular calcium. Subsequently, Furchgott and Ignarro et al. proposed that EDRF was nitric oxide.^{24,25} In fact, as originally described by Furchgott and Zarwanski,¹⁸ EDRF is likely to be a group of substances such as nitric oxide, nitric oxide complexes and adducts, and endothelial-dependent hyperpolarizing factors.²⁶ These relaxants function at very low (probably nanomolar) concentrations, and the present technology does not allow us to identify them definitively and resolve this important question.

NITRIC OXIDE SYNTHASES

Degucci and his colleagues, who were also interested in guanylyl cyclase in neuroblastoma and brain preparations, found that these preparations

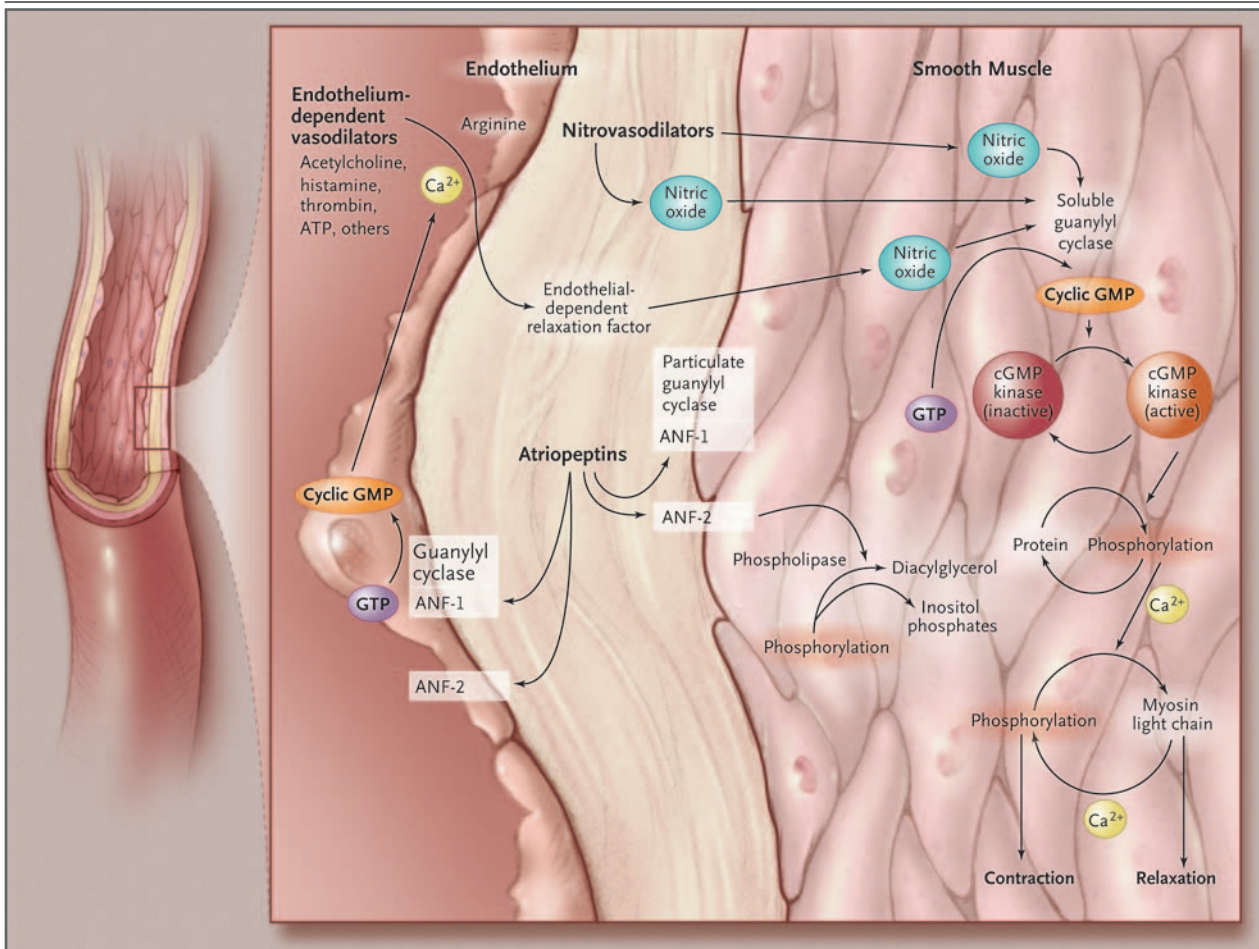


Figure 3. Endothelium-Dependent Relaxation of Smooth Muscle.

Endothelium-dependent vasodilators or nitrovasodilators produce nitric oxide, which activates soluble guanylyl cyclase to form cyclic GMP (cGMP). cGMP activates cGMP-dependent protein kinase, resulting in protein phosphorylation, decreased cytosolic calcium levels, myosin light chain dephosphorylation, and relaxation. Atrial natriuretic factors are atriopeptins whose receptors, ANF-1 and ANF-2, are coupled to particulate guanylyl cyclase activation or phospholipid metabolism.

were activated by L-arginine, which could be inhibited by agents we knew to be inhibitors of nitric oxide activation.²⁷ Subsequently, Hibbs et al. found that the cytotoxic properties of macrophages in cocultures with tumor cells could be enhanced with L-arginine and inhibited with L-N-methyl-arginine.²⁸ The cytotoxicity was associated with the accumulation of nitrite in the conditioned medium.

These important studies set the stage to identify a pathway of L-arginine metabolism that could produce nitric oxide and nitrite. Within several years, a number of scientists, including Snyder, Marletta, Stuehr, Nathan, Moncada, and my colleagues and I, had described various isoforms of

nitric oxide synthase in numerous tissues, including brain, macrophages, and endothelial cells. L-arginine was the enzyme's substrate and L-N-methyl-arginine was an inhibitor of nitric oxide synthase; these observations explained the findings of Degucci and Hibbs and their colleagues.

The three isoforms of nitric oxide synthase that were characterized, purified, and cloned, in chronological order, were neuronal nitric oxide synthase, or NOS-1, inducible nitric oxide synthase, or NOS-2, and endothelial nitric oxide synthase, or NOS-3 (Table 1). These isoforms are encoded by three different genes on different chromosomes and have about 50 to 60% homology with each other and the cytochrome P-450 enzymes. They

are found in numerous mammalian tissues and in lower forms, have a heme prosthetic group, and are active as homodimers. They use a complicated array of cofactors and cosubstrates, including oxygen, NADPH, flavin mononucleotide, flavin adenine dinucleotide, calmodulin, and tetrahydrobiopterin (BH4). They oxidize the guanidino nitrogen of L-arginine to L-hydroxy-arginine and then to nitric oxide and citrulline. Although crystal structures of expressed fragments of the nitric oxide synthases have helped define the role of some of the cofactors, the holoenzymes have not been crystallized, and this represents an important challenge.

Other inhibitors of the nitric oxide synthases have also been described and have been invaluable in both in vitro and in vivo studies to define the physiological role of nitric oxide. Unfortunately, only a few inhibitors are partially selective for one or another isoform of nitric oxide synthase, and none are specific for an isoform.

The nitric oxide synthases are found in various cellular compartments, can be modified and regulated post-translationally with phosphorylation or acylation with myristate or palmitate, and can have associations with other soluble or cytoskeletal proteins. For example, NOS-3 is associated with the caveolae of endothelial cells in association with caveolin. The association of calmodulin or phosphorylation can result in the mobilization of NOS-3 from the membrane and activation of the enzyme.

Investigators have been successful in preparing mice with targeted deletion of each of the nitric oxide synthases; studies in the resulting null mice have helped delineate the physiological role of each enzyme. Indeed, mice that are missing two of the three enzymes have also been produced. These useful models have confirmed the role of nitric oxide in various tissues as well as provided models for drug development.

ACTIVATION OF sGC

The receptor for nitric oxide is sGC; to date at least seven different isoforms of guanylyl cyclase have been identified. The soluble, or cytosolic, isoform of sGC is a heterodimer with an α subunit (approximately 80 kD) and a β subunit (approximately 70 kD).²⁹ Although there are two different α subunits (α_1 and α_2) and two different β subunits (β_1 and β_2), most tissues possess an $\alpha_1\beta_1$ sGC. Few tissues possess $\alpha_1\beta_2$, and the other pos-

Table 1. The Three Nitric Oxide Synthase Isoforms.

Isoform	Description
NOS-1 (155 kD)	Type: neuronal or brain Found in central and peripheral neurons, nonadrenergic and noncholinergic neurons, islets, endometrium, skeletal muscle, and other sites
NOS-2 (125 kD)	Type: inducible Found in macrophages, liver, smooth muscle, endothelium, heart, and other sites Regulated by lipopolysaccharide, cytokines, and glucocorticoids
NOS-3 (125 kD)	Type: endothelial Found in endothelium, brain, heart, and other sites Subject to acylation and phosphorylation

sible combinations have not been described in mammalian tissues. The genomic structures and promoters of the α_1 and β_1 sGC subunits are also known, and transcriptional regulation of sGC expression has been described by several laboratories.³⁰⁻³²

At the molecular functional level, there is a ferrous heme prosthetic group liganded to the histidine 105 residue of the β subunit. When nitric oxide binds to the ferrous heme iron, the association of the iron to histidine 105 is disrupted and the inhibition of the catalytic activity of sGC by the heme is overcome.³³ The redundant catalytic domain near the C-terminal of the α and β subunits is activated, resulting in a marked increase in the V_{\max} (by a factor of 200 to 400) and a decrease in the GTP K_m . Carbon monoxide can also bind to the heme iron with only modest activation of sGC (by a factor of two to four), and the concentrations of carbon monoxide required for activation are much higher (micromolar) than those needed for nitric oxide activation (nanomolar). Compounds that interact with heme or with heme-independent mechanisms can also activate or inhibit sGC. Some of these compounds are being investigated in clinical trials for the treatment of cardiovascular disorders such as angina and hypertension. There are many other possible indications for such agents, including pulmonary hypertension, Raynaud's disease, vasospasm, and some clotting disorders.

There are six, or perhaps more, particulate isoforms of guanylyl cyclase. These are receptor cyclases that are transmembrane proteins with an extracellular peptide receptor domain, a short transmembrane domain, and intracellular kinase-

like and catalytic domains.^{34,35} All of the soluble and particulate guanylyl cyclases and adenylyl cyclases have considerable homology in their catalytic domains, suggesting they were probably derived from a common ancestral gene. This undoubtedly explains why activated sGC can synthesize cyclic AMP as well as cyclic GMP.^{36,37} A single amino acid substitution in adenylyl cyclase can convert it to an activated form of guanylyl cyclase.

STEADY-STATE LEVELS OF CYCLIC GMP

The steady-state levels of cyclic GMP as a second messenger — or any other second messenger or metabolite, for that matter — are a function of its rate of production and the rate of its removal or degradation. Thus, the rate of cyclic GMP synthesis and accumulation by one or another guanylyl cyclase isoform is obviously influenced by the rate of production, the compartmentation, the availability of the activating ligands (e.g., nitric oxide and atriopeptins), the presence of other allosteric regulators, and the availability of the GTP substrate and Mg^{2+} cofactor. As with other intracellular messengers, the levels of cyclic GMP are regulated by its release (transport or extrusion) from the cell and the rate of its degradation and inactivation by phosphodiesterase.

At present there are 10 or 11 cyclic nucleotide phosphodiesterases that hydrolyze the phosphodiester bond of cyclic GMP or cyclic AMP, converting them to 5'-GMP or 5'-AMP, respectively, and thereby inactivating them. Some isoforms selectively hydrolyze cyclic GMP or cyclic AMP, and some hydrolyze both cyclic nucleotides. There are a variety of ways to regulate the activity of this complex family of isoforms. For example, the methylxanthine phosphodiesterase inhibitors, such as caffeine, theophylline, and theobromine, have been used for decades as bronchodilators in asthma and other pulmonary diseases, as vasodilators, and as diuretics.^{38,39} They are often present in coffees, teas, elixirs, traditional Chinese medicines, and other preparations.

A number of new compounds are more or less selective for some of the phosphodiesterases, and some of these compounds are on the market for the treatment of erectile dysfunction and are used off-label for other indications. In the penis, these agents increase the accumulation of

cyclic GMP in the corpus cavernosum vessels; nitric oxide is released as a neurotransmitter from the nitrinergic nerves, innervating these vessels. Some of these agents are used to decrease right to left shunting and hypoxemia in premature infants with pulmonary hypertension and patients with certain forms of congenital heart disease. The agents can also be used to inhibit platelet aggregation and to treat vasospasm. However, some of these agents are often abused or used without supervision by a physician; such use can result in serious drug interactions, hypotension, and arrhythmias in patients who are also using nitrovasodilators (e.g., nitroglycerin for angina), various stimulants or weight-loss preparations, or dietary methylxanthines.

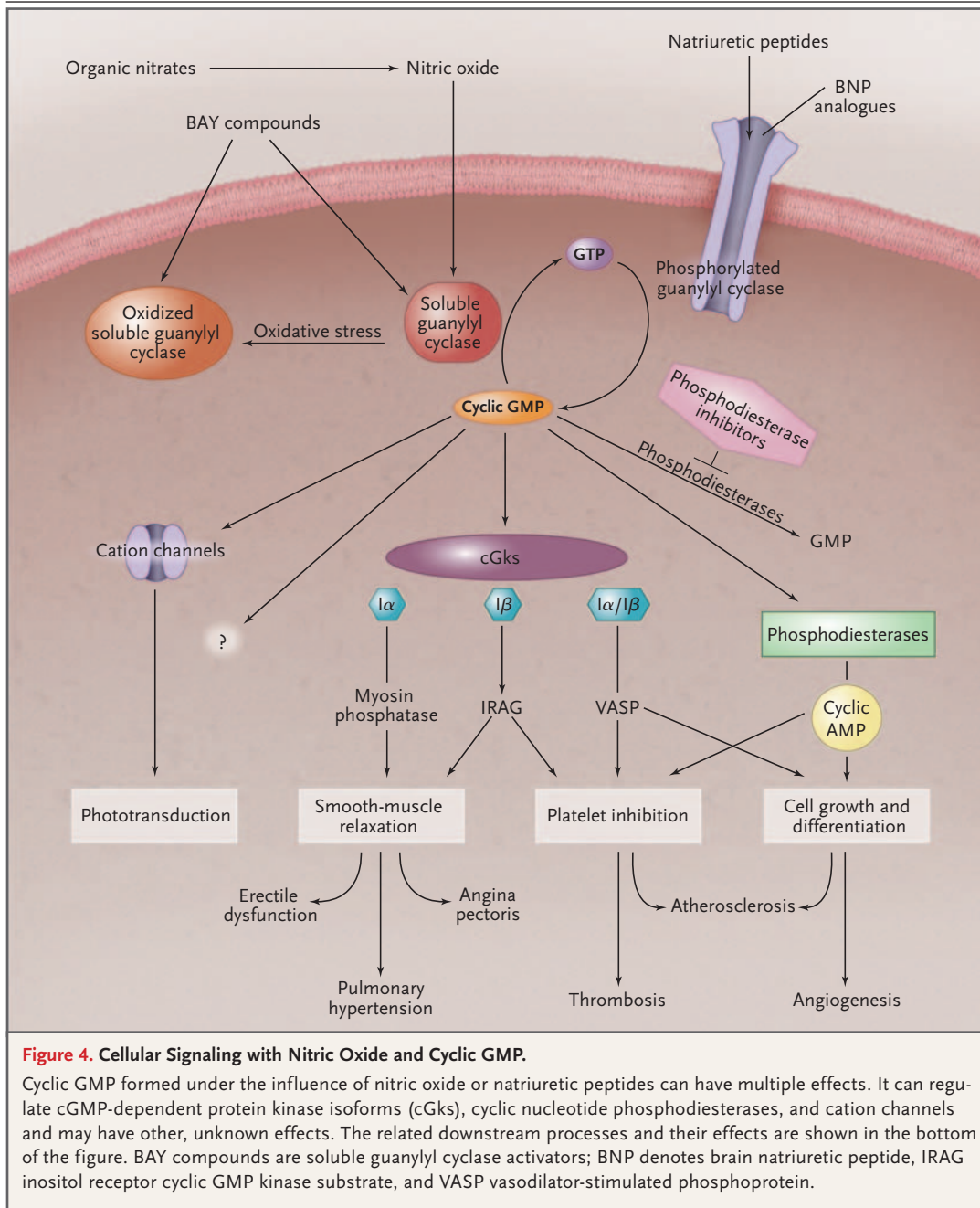
The phosphodiesterases, as well as ligands and their receptors, nitric oxide synthases, guanylyl cyclases, and protein kinases, have become important molecular targets for drug development. Such efforts should result in numerous new agents for future medical use.

CYCLIC GMP SIGNALING IN DRUG DEVELOPMENT

Cyclic GMP can regulate cation channels, cyclic GMP-dependent protein kinases, and some isoforms of phosphodiesterase (Fig. 4). Each of the molecular sites in these pathways can become an obvious target for new drugs used as single agents and can also provide the basis for rational approaches to combination therapies. It should come as no surprise that more than 75,000 publications addressing these pathways have appeared since the initial findings on nitric oxide that my colleagues and I reported in 1977.^{15,16} Numerous biotechnology companies have been founded on the basis of various aspects of nitric oxide, natriuretic peptides, and cyclic GMP biology; most large multinational pharmaceutical companies have projects in these areas as well.

Let me address one area in which nitric oxide is a major player in clinical disorders as an example of the potential clinical applications of this area in cellular signaling and therapeutics. Patients who have hypertension, diabetes, or atherosclerosis, patients who smoke, and perhaps those who are obese often have endothelial dysfunction in that their blood vessels produce insufficient amounts of nitric oxide.

One of several pathways for the processing and



degradation of proteins is the methylation of arginine residues in the proteins to produce symmetric and asymmetric dimethyl L-arginine. Asymmetric dimethyl L-arginine is a competitive inhibitor of the nitric oxide synthases, competing with L-arginine at the catalytic site. Dimethylarginine is metabolized in a pathway that requires reduced cofactors. Similarly, as discussed earlier, the nitric oxide synthases require the reduced cofactors

NADPH and BH₄ to produce nitric oxide. If the cofactors are deficient or oxidized, nitric oxide synthase produces superoxide anion instead of nitric oxide. The diseases discussed above are associated with oxidative stress, production of reactive oxygen species such as hydrogen peroxide, superoxide anion, and the hydroxyl radical that can oxidize the required cofactors. Thus, nitric oxide synthase produces superoxide anion instead of ni-

tric oxide, and asymmetric dimethyl L-arginine accumulates, further inhibiting nitric oxide synthase. In addition, the reactive oxygen species — particularly superoxide anion — interacts with nitric oxide to produce the reactive species peroxynitrite. The net effect is decreased nitric oxide formation, accelerated nitric oxide removal, and production of a toxic species, peroxynitrite. Blood vessels do not dilate properly, blood pressure increases further, the inflammatory process with atherosclerosis is accelerated, and the cardiovascular system is further compromised.

Some studies in animals and some early clinical studies suggest that dietary supplementation with L-arginine and various antioxidants could be used as rational supplements to other therapies for these disorders. Further studies will be of considerable interest, since such supplements are not accepted uniformly and rigorous clinical data are limited. Although the results of biochemical studies and studies in animals provide strong support for the use of such supplements, well-designed, controlled clinical trials are in the early stages.

SUMMARY

Our understanding of the nitric oxide and cyclic GMP signaling pathways has advanced considerably in the past three decades. Although there has been substantial progress in this area, numerous

questions remain unanswered. Nevertheless, these pathways have provided us with numerous novel targets through which many approaches to a vast array of disorders will be discovered and developed. These advances will continue to require the collaborations of biochemists, molecular biologists, cell biologists, physiologists, pharmacologists, medicinal chemists, and clinical scientists. As a clinical pharmacologist, drug discoverer, and drug developer, I believe it is possible to develop highly selective and specific new therapies without unwanted side effects. The more we know about the biochemical regulation of various macromolecular players and isoforms of all of the participants in the signaling cascade, the more likely we will be to succeed. Various formulations, selective delivery systems, and the pharmacokinetic properties of the agents can also assist the process. I expect to see many new agents coming from the field of nitric oxide–cyclic GMP research over the next one to two decades that will effectively contribute to our therapeutic armamentarium.

Dr. Murad reports having received consulting fees from Tripos, Encysive Pharmaceuticals, Arginox Pharmaceuticals, and Zhen-Ao and owning stock and options in IRX Therapeutics, PLX Technology, NiOxx, Lumen Therapeutics, BioCardia, Tissue Regeneration, Encysive Pharmaceuticals, Applied Neurosolutions, Lexicon Genetics, Merck, Abbott, Alkermes, Arena Pharmaceuticals, Genzyme, Geron, Johnson & Johnson, Eli Lilly, Monsanto, Pfizer, Regeneron Pharmaceuticals, Schering-Plough, and Teva Pharmaceutical Industries. Most of these companies do not have nitric oxide programs.

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