Nitric Oxide and Cyclic GMP Signaling

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Cyclic GMP (cGMP), along with cAMP, form a pair of classical second messengers with a long history of study, and there are many parallels and cross-interactions in their functions. Control of the formation of these two messengers is much more distinct, however. The control of cGMP production has become extremely interesting with the finding that another messenger molecule, nitric oxide (NO), is a physiological regulator. Research and interest in NO-related signaling and its connection to cGMP are increasing constantly. To satisfy these interests, a number of fine review articles have appeared which are more complete in scope than is possible here. This minireview will cover the general topic of NO- and cGMP-mediated signaling, while focusing on a few recent interesting findings. Additional review articles are used as references throughout.

Nitric Oxide

As vasorelaxants, organic nitrates such as nitroglycerin or isosorbide dinitrate and inorganic nitrocomplexes such as nitroprusside had been in use in the clinic for years before a mechanism of action was uncovered. These nitrovasodilator agents have in common the ability to serve as donors of NO, given the proper redox or metabolic conditions. Eventually, evidence was accumulated implicating endogenous production of NO in vasorelaxation and immune response (1–3). Now additional functions for NO have been catalogued, and we know the messenger is created in many cell types by the enzyme family NO synthases.

NO Synthases. Several distinct isozymes of nitric oxide synthase (NOS), which are the products of individual genes, all catalyze the formation of NO. The three main types of NOS and some of their properties are listed in Table I. As more effort is made to locate and characterize these enzymes, the list of isozymes may conceivably grow. NOS produces NO in a complex set of redox reaction steps, using molecular oxygen and the guanidino nitrogen of arginine as the substrates, NADPH as electron donor, and flavin adenine dinucleotide, flavin mononucleotide, heme, tetrahydrobiopterin, and Ca^{2+}/calmodulin as cofactors (4–7).

Type I NOS, also called neuronal NOS or brain constitutive NOS (bNOS), is an isozyme found in high concentration in some neuronal cells, both in the central nervous system and peripherally (7–9). The isozyme is activated by calmodulin when intracellular Ca^{2+} is elevated. NO produced by type I NOS is proposed to be a neurotransmitter in noncholinergic, nonadrenergic transmission (8, 10). Type II NOS is referred to as macrophage NOS or inducible NOS (iNOS). The enzyme is not present in resting cells, but is induced in a number of cell types, including macrophages, hepatocytes, smooth muscle cells, and many others by exposure to bacterial lipopolysaccharide in conjunction with cytokines (7, 9). This isozyme of NOS is nominally independent of activation by Ca^{2+}; more specifically, the enzyme is productively bound by calmodulin even resting intracellular Ca^{2+} (7, 11). Induction of type II NOS is probably part of the mechanism for mounting a cytotoxic response (9). Cells that express type II NOS synthesize much more NO than cells with activated type I or III NOS, and this higher amount of NO is believed to be essential to pathogen killing, but also may be cytotoxic to exposed cells of the host (7, 10). Type III NOS, also called endothelial constitutive NOS (eNOS), is the isoform commonly associated with production of endothelium-derived relaxing factor (4, 9, 12). Like type I NOS, this isozyme is activated by raised intracellular Ca^{2+} concentration and calmodulin binding to the enzyme. NOS type III is membrane associated, unlike the other types, and its regulation by other mechanisms such as phosphorylation is more closely established than for the other NOS isoforms (7).

Chemistry and Biochemistry of NO. *Chemistry.* Nitric oxide (NO) is a diatomic, free radical gas. NO is relatively nonreactive, for example in comparison with other biologically important radicals such as hydroxyl

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Table I. NO Synthase Isoforms

Isoform	Other name	Cell fraction	Regulation	M_r (kDa)	Location
NOS I	bNOS	Soluble	Ca^{2+}/calmodulin	155	Brain, neuroblastoma cells, peripheral neurons, pancreatic islets, etc.
NOS II	iNOS	Soluble	Expression induced by cytokines and endotoxin	125	Macrophages, hepatocytes, smooth muscle, etc.
NOS III	eNOS	Particulate	Ca^{2+}/calmodulin	135	Endothelial cells, kidney epithelial cells

or superoxide anion (13). NO radical should be considered one of a family of redox-related congeners, as NO^+ (nitrosonium) and NO^- (nitroxyl), which can be formed from NO by physiologically relevant redox mechanisms, have distinct preferred targets with which they interact (13, 14). Table II lists some of the preferred reactions and targets of the redox-related forms of NO.

Availability. The physical properties and chemistry of NO determine its biological fate and potential sites for interaction. NO is a highly diffusible compound; because of its low molecular weight and reasonable hydrophobicity and solubility, NO is theoretically able to reach anywhere within cells and tissues (11). Practical limits exist on the diffusion potential of NO, however, due to its many possible chemical interactions. The greatest factor limiting the availability of NO may be its rapid interaction with superoxide anion (13). Addition of superoxide dismutase to experimental systems has been found to preserve greatly the effectiveness of NO. Other well-characterized chemical reactions of NO including interaction with oxygen and self-association probably can be ignored in physiological systems because of the low NO concentration present in tissues (13). Two other major interactions can be discussed as a means of limiting NO availability to its intracellular sites of action, namely binding to iron centers in hemoglobin and other heme proteins, and to thiol groups in proteins. Hemoglobin binds NO with great avidity, which has been of utility of numerous experiments where hemoglobin is used as a sink for NO that diffuses outside the cell during the process under study. NO (especially at NO^+) also binds to thiols, among them the lone cysteine in serum albumin (14, 15). In animal blood NO is found mostly bound to albumin (14). This finding is interesting because NO in nitrosothiol form has a much longer half-life and is potentially delivered (via transfer of NO^+ equivalent) to targets spatially or temporally distant (14). In the case of endothelium-derived relaxing factor (EDRF), whether this agent is chemically equivalent to NO or a NO-containing compound such as a nitrosothiol is still not established.

Table II. Biochemical Interactions of NO and Related Species

NO species	Reactants	Product examples
$NO^{\bullet}$	Transition metals	Hemoglobin(Fe^{2+})-NO
	Oxygen	NO_{2-}
	Superoxide	$ONOO^-$
NO^+	Amines	Nitrosamines
	Thiols	Protein(cys)S-NO
	Aromatics	Ar-NO
NO^-	Dimerization	N_2O
	Thiols	R-S-NOH, RS-SR
	Metals	Hemoglobin(Fe^{3+})-NO
ONOOH, $ONOO^-$	DNA	Strand breaks, deaminated bases
	Thiols	R-S-OH, R-S-OOH, other oxidized species
	Tyrosine	Protein(tyr)-NO_2

Biochemical interactions. There are multiple sites of NO interaction with biochemical pathways, partly dependent on the NO species encountered (9, 13) (Table II). Nearly all cell components are possible targets, including proteins, carbohydrates, lipids, and nucleic acids, in addition to various small molecules. Most interactions of NO with enzymes cause inactivation, be it with heme groups, thiols, or other sites, with the notable exception of guanylyl cyclase, which is stimulated by NO (16, 17) (see Soluble Guanylyl Cyclase and Stimulation by NO below). NO can be cytotoxic to cells for a number of reasons. Metabolic dysfunction is caused by NO inhibition of the mitochondrial respiratory chain and enzymes such as dehydrogenases (9). NO and its oxidation products peroxynitrite (and peroxynitrous acid) can also indiscriminately damage cell constituents: DNA is subject to strand breakage and deamination of bases, lipids can be oxidized and peroxidized, and proteins can be oxidized at any of several groups (9, 13, 14).

NO interacts with metals, especially transition metals, in proteins; the major sits of interaction are the iron in heme groups and iron-sulfur centers. A number of mitochondrial enzymes, including complexes of the electron transport chain and the enzyme aconitase are inhibited by NO interaction with their Fe-S centers, which can effectively reduce mitochondrial respiration

in cells exposed to NO (9, 13). Thiols are subject to a variety of reactions with NO or peroxynitrite. Several oxidation states of protein cysteines can be created by these agents, and not all would be reversed by interaction with cellular reducing agents (13). One example of NO-induced thiol oxidation is viewed to be a component of a reversible, protective negative feedback mechanism, however (14). The vicinal thiols of the brain NMDA receptor are a redox modulatory site of the intrinsic ion channel in the receptor. Ca^{2+} that enters the neuron through the glutamate-bound, activated receptor channel causes NO synthesis in the cell. NO then presumably binds at one of the cysteines in the redox modulatory site, disulfide formation ensues, Ca^{2+} channel function is shut down, thereby stopping additional NO synthesis (14).

Cyclic GMP

Following by several years the discovery of cAMP, research into the newly discovered second messenger cGMP was very active, with measurement of cGMP formation in many tissues and tissue homogenates in response to numerous hormones and other agents. Molecular mechanisms of the second messenger formation were to be lacking for quite a while, however, until the enzymes that make cGMP were identified and characterized. Also lacking was a physiological effect for this messenger. A physiological function for cGMP was eventually found in experiments with preparations of smooth muscle, where addition of inorganic nitrogen compounds as well as classical vasorelaxants such as nitroglycerin or nitroprusside to these tissue samples both increased cGMP and caused relaxation (1–3, 18, 19).

Synthesis of cGMP. There are two major families of guanylyl cyclase, the particulate-associated enzymes, which are actually transmembrane receptors that contain guanylyl cyclase within their intracellular domains, and the soluble type that is activated by nitric oxide (20–23). With more details of the structure and function of these enzymes, however, this strict division may become blurred. For example, nitric oxide–stimulated guanylyl cyclase activity has been found associated with the membrane fraction of cells in some cases (2).

Gyanylyl cyclase receptors. This type of guanylyl cyclase has a unique and interesting structure: the polypeptide is a transmembrane receptor, containing an extracellular ligand binding domain, single transmembrane helix, and intracellular domain containing the enzyme activity (21, 23, 24). Within this subfamily of cyclases are a number of distinct members, for the most part each with its own specific ligand(s) (5, 23). Guanylyl cyclase–A binds atrial natriuretic peptide and brain natriuretic peptide, and is located primarily in the heart. Guanylyl cyclase–B is located in neural tissue, where it specifically binds the related natriuretic peptide CNP, which may function as a neurotransmitter. Guanylyl cyclase-C is the intestinal epithelial isotype; its natural ligand in guanylin, and it also is activated by the *Escherichia coli* heat stable enterotoxin, ST_a (25). Guanylyl cyclase in the retina may also be of the receptor type, with the peptide recoverin as its ligand (5).

The mechanism of activation of the receptor-type guanylyl cyclases is not entirely clear. As is the case with other single-transmembrane domain receptor types such as receptor-type tyrosine kinases, some sort of dimerization may be induced by ligand binding, which serves to activate the intracellular enzyme part of the receptor. As discussed below, dimerization is essential to enzyme activity in soluble guanylyl cyclases.

Soluble guanylyl cyclase and stimulation by NO. The structure of this family of cyclases is distinct from the receptor type, the active enzyme being an $\alpha\beta$ heterodimer of 76- and 80-kDa subunits (5, 24, 26). Although each subunit apparently contains catalytic and heme-binding domains, enzyme activity is absolutely dependent on the presence of both subunits (5, 24, 27). The enzyme possesses a basal cyclase activity that is preferentially dependent on Mn^{2+}, and activity is retained even in the absence of the heme prosthetic group (2). NO stimulates enzyme activity greatly, increasing k_{cat} by up to 200-fold (2). NO-activated by the more physiologically relevant Mg^{2+}, in addition to Mn^{2+} (2). Although the precise mechanism is not yet fully defined, NO appears to activate soluble guanylyl cyclase by interaction with the heme moiety (2, 5). Several studies have supplied convincing evidence supporting this hypothesis. In a reversible manner, removal and re-introduction of the heme group results in an enzyme that is nonresponsive or responsive to NO, respectively (5, 28). In addition, guanylyl cyclase that is mutated at the heme-coordination position His-105 of the β subunit was also found to be nonresponsive to NO, although basal activity comparable to native enzyme was retained (29). The primary complication to stating conclusively that NO activates soluble guanylyl cyclase by binding at the heme is the presence of regulatory vicinal thiols in the enzyme, which in other proteins are known to be regulated by NO and oxidative-related species (14, 30, 31). Thus, NO most likely activated the enzyme by binding to the heme group, but in addition NO itself or redox-related forms could interact at other sites to modify enzyme activity.

With the identification of additional isoforms of intracellular, heterodimeric guanylyl cyclase, its classification as a "soluble" enzyme may be too restrictive. A kidney-localized isoform of the B subunit, β_2, has an extension at the carboxyl end of the polypeptide that possesses the sequence leading to isopreny-

lation and carboxymethylation in other proteins (5), such as many members of the *ras* superfamily of low–molecular weight G proteins. These modifications, because of their highly hydrophobic nature, can cause membrane association of otherwise soluble guanylyl cyclase to intracellular membranes, possible the plasma membrane. It is unclear but conceivably the enzyme could be placed at the membrane for either localized production of cGMP or for regulatory interaction with other membrane components.

Protein Targets for cGMP. *Cyclic GMP–dependent protein kinase.* Regulation of cellular events by cGMP is accomplished by interaction of the nucleotide with several types of target proteins. One target is a cyclic nucleotide–regulated protein kinase, much analogous to the case with cAMP activation of cAMP-dependent protein kinase. cGMP-regulated protein kinases, or G-kinases, are either soluble or membrane enzymes (32). GKI is a soluble homodimer of 78-kDa subunits; two related types of GKI are comprised of monomers that arise from alternatively splicing of a single gene. GKI is most abundant in platelets, smooth muscle cells, and cerebellar Purkinje cells, but is present elsewhere also (5, 32). GKII is a membrane-associated monomer of 86 kDa that so far has only been located in the small intestine epithelial brush border membrane (32). Activation of G kinases is selective but not absolutely specific for cGMP, as cAMP is also able to bind and activate the enzymes; GKI has even less selectivity for cGMP after autophosphorylation (32). The various possible mechanisms for cross-talk between the two major cyclic nucleotide signaling pathways therefore includes control of G kinase as well as cyclic nucleotide degradation by the complex family of phosphodiesterases, and also the ability of activated guanylyl cyclase to synthesize cAMP (33).

cGMP-gated ion channels. A number of cation channels are gated by binding of cGMP. These include the cation channel of the retina and the apparently related olfactory epithelial cation channel (5, 32). These ion channels possess a single regulatory cGMP site that allows Ca^{2+} entry to the cell. Other tissues also contain cGMP-gated channels, for example, in the renal inner medullary collecting duct (5, 32). In the less defined cases, it is not always clear whether regulation of the ion channel by cGMP is through direct gating by cGMP binding or through phosphorylation by G-kinase.

Cyclic nucleotide phosphodiesterases. Cyclic nucleotide phosphodiesterases (PDE) comprise a large family of enzymes, that can be classified into five categories based on properties and distinct polypeptides (5, 31, 34). Briefly, the five families are described as follows: PDE I, Ca^{2+}/calmodulin-dependent; PDE II, cGMP-stimulated (cGX-PDE); PDE III, cGMP-inhibited PDE (cGI-PDE); PDE IV, cAMP-specific, low K_m; and PDE V, cGMP-specific. Because the different isozymes have different K_m and k_{cat} for hydrolysis of cAMP versus cGMP and different effects of the opposing nucleotide on activity, a variety of interactive controls can be envisioned between the cAMP and cGMP signal transduction pathways. Several examples of such interaction have appeared recently and are enumerated below (see Phosphoinositide Turnover).

Physiological Functions Regulated by cGMP. *Smooth muscle relaxation.* Relaxation of smooth muscle conceivably can be achieved by interfering with any of the steps leading to the contraction producing phosphorylation of myosin light chain. One step with which cGMP signaling is proposed to interfere is the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that is required for activation of myosin light chain (1, 5, 32). cGMP activation of GKI activation of GKI may be involved in lowering $[Ca^{2+}]_i$ by phosphorylating and activating Ca^{2+}-ATPase or components associated with the transporter (5, 32, 35).

Platelet inhibition. Platelet activation is stimulated by a large variety of Ca^{2+}-mobilizing agents including thrombin and ADP. NO from cells in neighboring tissue such as the endothelia, or from exogenous nitrovasodilators inhibits platelet activation, with a reduction in agonist-induced $[Ca^{2+}]_i$ as the probable mechanism (5, 32). Again, myosin light chain phosphorylation would then be inhibited, altering platelet morphology, and decreasing the ability to aggregate (32). Apparently, signaling by cGMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (5, 35) (also see Phosphoinositide Turnover below).

Other functions. In the lower intestine, cGMP is involved in ion secretion and readsorption (5, 25). cGMP production in the colon is stimulated by an endogenous peptide ligand, guanylin, and also by heat-stable enterotoxin (25). Elevated cGMP causes decreased Na^+ and Cl readsorption and activated Cl secretion in different portions of the colon. The effects may be mediated by the intestinal epithelia-specific GKII, or also by inhibition of cGI-PDE leading to increased cAMP that also stimulated ion fluxes out of the cells (5, 36).

In the cerebellum, NOS type I and components of the cGMP signaling system are localized to a subset of cells (5). Purkinji cells are enriched in soluble guanylyl cyclase and GKI, and a neuronal-specific GKI substrate protein is phosphorylated in these cells. The phosphoprotein reportedly affects neuronal signaling by inhibition of protein phosphatases 1 and 2A (5).

Interconnecting NO/cGMP with Other Pathways. *cAMP.* Signaling through cAMP and NO/cGMP pathways is interconnected at the level of con-

trol of the various isoforms of cyclic nucleotide PDE. In frog ventricular myocytes, Ca^{2+} current (I_{Ca}) through the L-type Ca^{2+} channel is regulated by cAMP-dependent kinase phosphorylation; these cells also contain NO-responsive guanylyl cyclase and several isoforms of cyclic nucleotide phosphodiesterases. A connection was made between the NO/cGMP pathway and cAMP effects by Méry *et al.* (37), who showed that low concentrations of NO donor compounds, such as SIN-1 at 0.1–10 n*M* augmented cAMP-stimulated I_{Ca}, due to inhibition of cGI-PDE by NO-induced cGMP produced in this case was sufficient to activate cGS-PDE resulting in lower cAMP in the cells. These results point out how the variety of phosphodiesterases, with different sensitivities, responses, and substrate selectivities, can serve to produce a variety of effects depending on the isoforms present and the NO/cGMP levels encountered.

Another example of this phenomena is in NO/cGMP-mediated vasorelaxation. Rat aortic smooth muscle rings exhibit cAMP-mediated relaxation that is not dependent on the endothelial cell layer and is not affected by blockers of NO signaling. cGMP was found to enhance cAMP-mediated relaxation, because of inhibition of cGI-PDE present in the smooth muscle cells (38). The endothelial cells themselves do not contain cGI-PDE but instead have PDE-IV, so that in endothelium-containing aortic rings specific inhibitors of this isotype such as rolipram or denbufylline cause relaxation that is blocked by inhibitors of NO synthesis (38). This system shows that the presence of different PDE isoforms in neighboring cell types can add a further level of control to systems in which NO, cGMP, and cAMP are involved in common pathways.

One other example is the inhibition of platelets, which is synergistic between agents that raise cAMP and NO compounds. Maurice and Haslam (39) provide evidence that NO-stimulated increases in cGMP potentiate cAMP-mediated platelet inhibition by inhibition of cGI-PDE. Together, these examples show that in a number of tissues, NO and cGMP signalling has active cross-talk with other important signaling pathways and second messengers.

Phosphoinositide turnover. Increased cGMP negatively affects agonist-induced phosphoinositide turnover and production of inositol 1,4,5-trisphosphate (IP_3), a second messenger involved in raising intracellular Ca^{2+} (35). Phosphoinositide turnover, in various systems, is under the control of guanine nucleotide-binding regulatory proteins (G proteins), tyrosine phosphorylation, or other as yet undefined mechanisms. The inhibitory effect of cGMP occurs at the level of G-protein activation or activated G-protein interaction with phospholipase C (PLC), but the exact mechanism is still not defined. In bovine aortic smooth muscle cell homogenates, the addition of cGMP decreases PLC activation caused by the agonist vasopressin or by the G-protein ligand GTPγS, but not by Ca^{2+} (40). The latter method of PLC activation bypasses the G-protein dependence, whereas the former two methods of PLC activation depend on activated G-protein interacting with PLC. ATP was required for the cGMP effect and the nonhydrolyzable ATP analogue adenylylimidodiphosphate did not substitute for ATP, suggesting that the cGMP effect was mediated by cGMP-dependent protein kinase.

A novel pathway: ADP-ribose. Another second messenger able to elevate intracellular Ca^{2+} is cyclic ADP-ribose, a messenger enzymatically derived from NAD that is the apparent physiological ligand for the ryanodine-receptor Ca^{2+} channel. Though in many cases cGMP acts to lower intracellular Ca^{2+} (35), in this case cGMP is involved in increasing the ion concentration. In sea urchin eggs and homogenates, cGMP activates ADP-ribosyl cyclase, the enzyme that produces cADP-ribose (41, 42).

Conclusions

NO and cGMP constitute an autocrine, paracrine, and possible endocrine signal transduction system. Cytosolic NO-responsive guanylyl cyclase can be stimulated by NO derived from its own cell, from similar or distinct neighboring cell types within a tissue, from a circulating pool of NO (as NO^+ equivalents coupled to plasma protein thiol groups), or from pharmacologic agents, the nitrovasodilators. NO and cGMP together comprise an especially wide-ranging signal transduction system when one considers (i) the many roles of cGMP in physiological regulation, including smooth muscle relaxation, visual transduction, intestinal ion transport, and platelet function; (ii) the many sources, biochemical interactions, and functions of NO; and (iii) the interactions of cGMP and its affected pathways with other signaling systems such as phosphoinosidites, eicosanoids, cAMP and Ca^{2+}.

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