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Nitric Oxide-Regulated Soluble Guanylate Cyclase

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Nitric oxide plays significant roles in many physiological processes, and in most of these cases it works through activation of soluble guanylate cyclase and elevation of cyclic GMP concentration. Soluble guanylate cyclase is a heterodimeric protein made up of one α and one β subunit. The beta subunit has one molecule of heme attached to it through α histidine moiety. NO activates the enzyme by binding to the heme iron and weakening its bond to the histidine. The enzyme is deactivated upon dissociation of NO. Prolonged exposure to high concentrations of NO oxidizes heme or cysteines of the enzyme leading to inactivation and inability to respond upon subsequent treatment with NO. In some cells, exposure to NO results in rapid desensitization of the enzyme, thus preventing undesirable consequences of continued, unabated cyclic GMP production.

Nitric oxide (NO), which for long had the reputation of being an air pollutant that contributed, along with its oxidation products, to smog and respiratory illnesses[1-3], is now known to be produced in nearly every tissue in the human body. It is found to be involved in the regulation of blood flow, in the control of respiration, in neurotransmission, in the defense against infections and in a host of other vital physiological processes[4,5]. Ironically, it is now recognized as the most effective treatment of term and near-term (>34 weeks) neonates with hypoxic respiratory failure associated with pulmonary hypertension, where it improves oxygenation and reduces the need for the highly invasive procedure of drawing the blood, oxygenating and then pumping it back[6] (http://www.pulmonaryreviews.com/may00/pr_may00_nitricoxide.html), (http://www.fda.gov/cder/da/da1299.htm).

Glyceryl trinitrate (GTN, nitroglycerine), synthesized by Ascanio Sobrero in 1847 and immediately found to be useful as an explosive, was soon discovered to be highly effective in treating chest pain (angina pectoris) and in lowering blood pressure[7]. One hundred and fifty plus years later, it was realized that nitroglycerine and some other nitrogen bearing compounds such as nitroprusside, azide and hydroxylamine behave much like NO gas in dilating blood vessels. It is generally accepted now that these nitro compounds including GTN become effective vasodilators upon biotransformation to NO[8,9].

In the nineteen eighties it became clear that not only do blood vessels dilate in the presence of externally supplied NO, but also produce a vasodilator substance endogenously in response to neurotransmitters like acetylcholine and bradykinin[10,11]. This substance was termed endothelium-derived relaxing factor (EDRF) because it appeared to be produced in the endothelial cells: vessels denuded of endothelial cells did not dilate in response to acetylcholine. EDRF was eventually identified as NO in the eighties[12-14], and since then NO is found to be synthesized enzymatically in many tissues including brain[15-17].

The vasodilator action of NO is mediated by cyclic GMP (guanosine-3',5'-monophosphate), a nucleotide produced from GTP in a reaction catalyzed by an enzyme called guanylate (or guanylyl) cyclase, and hydrolyzed by an enzyme called cyclic GMP phosphodiesterase. In addition to mediating NO's action in relaxing blood vessels, cyclic GMP is a mediator or second messenger in many other physiological processes including platelet disaggregation[18], ol-

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faction[19], fluid secretion[20] and memory[21,22]. Cyclic GMP influences signal transduction pathways through one or more of the following reactions: it opens non-specific cation channels influencing cell polarity; regulates cyclic AMP phosphodiesterase thus altering the cellular concentration of cyclic AMP; and activates protein kinases which phosphorylate specific proteins thereby increasing or decreasing their biological activity[23]. Factors that severely alter cellular concentration of cyclic GMP by affecting the activity of either cyclase or phosphodiesterase could lead to pathological conditions such as blindness[24], hypertension[25] and impotence[26].

Cyclic GMP is not the sole mediator of all known effects of NO. For example, the cytotoxic effects of high concentrations of NO produced by activated macrophages are probably due to peroxynitrite[27]. Likewise, not all cyclic GMP is produced by NO-activated guanylate cyclase. Two types of guanylate cyclases are known: a membrane form activated by peptide hormones or intracellular calcium-binding proteins, and a soluble form (sGC) activated principally by NO. Membrane forms of guanylate cyclase are involved in vision[28], fluid secretion in kidneys and intestine[29], and olfaction[30] among others, while sGCs are involved in light adaptation of retina[31], modulation of neurotransmitter release[32] regulation of the tone of blood vessels[33], platelet aggregation[18] and sodium excretion in the kidneys[20,34] among others[29].

The awareness that NO influences numerous signal transduction pathways through activation of sGC and elevation of cyclic GMP concentration has generated a lot of interest in this enzyme over the last two decades. Structural features of sGC that permit activation by NO, the kinetics of activation and deactivation, and its adaptation to NO rich or poor environments have all received considerable attention. This paper will briefly review the discoveries in these areas.

Subunit structure:

Soluble guanylate cyclase is a heterodimer, made up of two different subunits termed α and β. The α subunit in bovine lung has 691 amino acids and a molecular mass of 77,500 Da[35]. The β subunit has 619 amino acids and a molecular mass of 70,500 Da[36]. Both α and β subunits must be expressed simultaneously to form a catalytically active enzyme that can be stimulated by nitric oxide[37]. Homodimers of α or β not only fail to be activated by NO, but also do not have basal catalytic activity[38]. With the discovery of isoforms of α and β subunits, the original α and β subunits are now referred to as α1 and β1. Of the reported isoforms only α2 and β2 are definitively identified[39] and studied. While α1β1 is the most studied and the most abundant form, α2β1 is also catalytically active with properties nearly identical to those of α1β1[40]. α1β2 is reported to be much less activated by NO, and higher expression of β2 (and thus, formation of more α1β2) and a correspondingly lower expression of β1 (reduced formation of α1β1) is thought to cause hypertension in rats[41].

Soluble guanylate cyclase is a hemoprotein: it contains one molecule of heme (Fe-protoporphyrin IX) per heterodimer (α1β1)[42-43]. Heme is bound to the β1 subunit at histidine 105[44,45]. The presence of bound heme is essential for the activation of the enzyme by NO[46]. Interestingly, there is no histidine at or near position 105 in the β2 subunit which is known to form an enzyme (α1β2) with little sensitivity to NO[41,47].

Catalytic domain:

Soluble guanylate cyclases catalyze the formation of cyclic GMP from GTP. In this aspect, they are functionally identical to membrane guanylate cyclases though the regulators of the two types of cyclases are different. Adenylate cyclases, enzymes that catalyze the formation of cyclic AMP from ATP, are close cousins of guanylate cyclases. It is therefore not surprising that all the above cyclases contain domains of about 250 amino acids that have considerable sequence homology. These homologous domains, found in the C-terminal portion of the proteins, are thought to be responsible for the catalytic activity of the enzymes[48-49]. In the case of sGC, both α and β subunits have this catalytic domain and both are required for catalytic activity. In the case of membrane guanylate cyclases which have a single subunit, binding of an activator brings about dimerization of the enzyme thus bringing two homologous domains together[51-52]. Adenylate cyclase is also a single subunit protein but contains two cyclase homology domains[49,53]. The conclusion from these observations is that in all these enzymes, two catalytic domains, either intra- or inter-molecular, are essential for the formation of a catalytically functional enzyme.

Deletion of 64 amino acids from the N-terminus of the β1 subunit and 131 amino acids from the N-terminus of α1 subunit does not prevent formation of a dimer with basal catalytic activity. However, the mutant enzyme is not activated by NO indicating that these N-terminal regions are involved in the activation by NO[54]. In the C-terminal portion, mutation of aspartic acid residues at the 513 and 529 positions of α1 subunit results in the formation of an enzyme

that is catalytically inactive and not activated by NO indicating that these residues are probably located in the active site of the enzyme[47,54].

Activation:

Crude preparations of soluble guanylate cyclase are activated by azide, nitrite, hydroxylamine, nitroglycerine, nitroprusside and NO. It is now understood that NO is the activator and that the other nitro-compounds produce NO or NO-derivatives when incubated with tissues or tissue homogenates[8]. Purified soluble guanylate cyclase is activated 300 to 700-fold by NO[43,43]. There are variations between reports in the extent of activation, which is probably due to partial loss of heme during purification.

Preparations that lost their heme entirely during purification are not activated by NO. Such heme-deficient preparations show higher basal activity which is inhibited by the addition of heme. Heme-reconstituted enzyme is once again capable of activation by NO[56]. When reconstituted with protoporphyrin IX (heme minus iron), heme-deficient enzyme demonstrates as much activity as the enzyme reconstituted with NO-bound heme[57]. This led to the hypothesis that activation of soluble guanylate cyclase by NO involves removal or displacement of iron from the protein-bound heme[58].

Kinetic studies by Marletta and his colleagues suggest that NO first forms a 6-coordinate complex with the histidine-bound heme of the cyclase, i.e., the iron in the heme forms four bonds with nitrogens of the protoporphyrin IX ring, the fifth with histidine 105, and the sixth with NO. In a subsequent step, the iron-histidine bond is broken leading to the formation of a 5-coordinate nitrosyl-heme complex and the activated state of the enzyme[59-61]. The formation of the 5-coordinate complex is thought to be at least three orders of magnitude slower than the first step, and dependent on NO concentration suggesting that NO might bind at a second site in addition to histidine 105 and influence the kinetics of activation. The need for a second NO-binding site is, however, questioned[62]. With or without a second site, activation of cyclase by NO at 37° is suggested to occur in less than 0.1 sec[63].

Carbon monoxide also activates sGC[64,65]. However, unlike NO which activates cyclase several hundred-fold, CO activates it only about 4-fold[64,66] thus casting a doubt on its validity as a physiological stimulator of the enzyme. However, studies on the activation by CO have been helpful in understanding the mechanism of activation. It is suggested that CO binds heme in sGC but fails to break off the histidine-Fe bond, thus leaving the heme iron in a 6-coordinate complex[67]. If breaking off the histidine-Fe bond is essential for activation of the enzyme, how does CO stimulate it even 4-fold? May be it is not the breakage of the histidine-bond, but the "geometry of the porphyrin plane" that influences activation. In basal sGC the heme might be slightly pulled towards histidine, a condition marked by low (basal) activity. In CO-sGC the heme could be slightly pulled away from histidine resulting in low level activation, whereas in NO-sGC the heme is probably pulled further away causing explosive activation[68].

An interesting turn in the activation saga came with the discovery that YC-1 (3-(5¢-hydroxymethyl-2¢-furyl)-1-benzyl indazole) activates sGC[69] as well as turns CO into a potent activator of sGC: in the presence of YC-1, CO stimulates sGC to the same level as NO[70]. How does YC-1 bring about this change? Does YC-1 break the histidine-Fe bond in CO-sGC? Does it increase the affinity of CO for the enzyme? Stone and Marletta[67] reported that YC-1 had no effect on the binding of CO to cyclase (k_{on} = 3.6 x 10^4 $M^{-1}s^{-1}$) or its dissociation from cyclase (k_{off} = 3.5 s^{-1}). However, Friebe and Koesling noted that YC-1 potentiated the activation of cyclase by reducing the dissociation of the ligand, either CO or NO[71]. In addition, Kharitonov and colleagues observed that YC-1 increases the affinity of CO for cyclase[72]. While effects of YC-1 on the affinity of CO for and dissociation from cyclase could explain its potentiation of activation by CO, the question whether Fe-histidine bond has to be broken for a high level of cyclase activation remains to be definitively resolved.

Sharma and colleagues suggest that weakening the Fe-histidine bond might be sufficient to fully activate cyclase[73]. Breaking the bond may be overkill when weakening it is sufficient. This would explain why CO-sGC remains in a 6-coordinate iron complex and yet has high activity in the presence of YC-1. This hypothesis seems to be gaining ground since Marletta's laboratory has also concluded recently that a six coordinate sGC-NO complex can have substantial activity[74] (fig. 1).

Deactivation and Inactivation:

NO-activated enzyme returns to the basal or unactivated state by the removal or dissociation of NO. To study the kinetics of deactivation, NO dissociated from NO-sGC complex has to be trapped so that it would not activate cyclase again. The most commonly used trap in such studies is oxyhemoglobin which has a high affinity for NO[75].

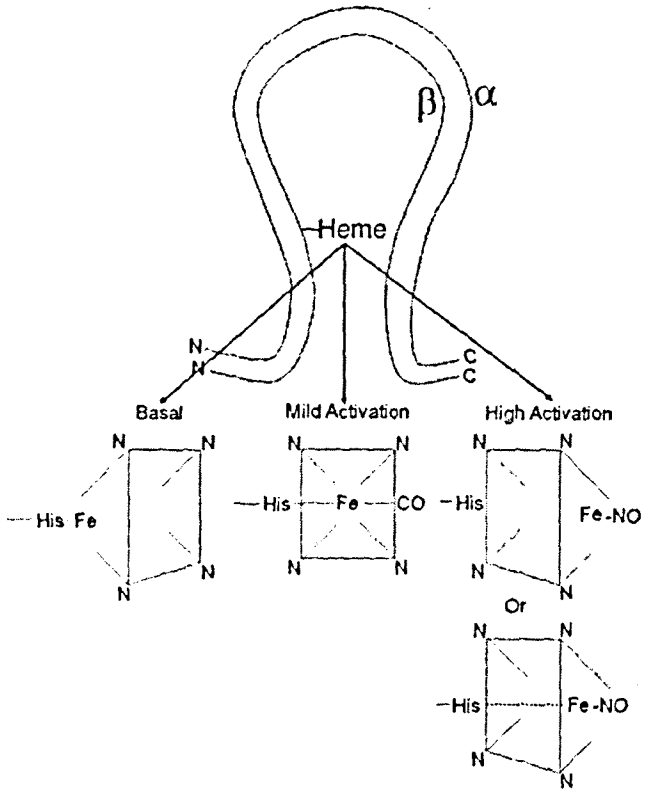

Fig. 1. Schematic representation of soluble guanylate cyclase.

The scheme shown here is based on references 68, 73 and 74. sGC has two subunits: α1 and β1. A heme moiety is attached to histine (-His) 105 of the β1 subunit. In the unstimulated (basal) state, heme iron is pulled towards –His and the enzyme activity is low. When activated by CO, the ligand pulls heme Fe slightly away from –His and this results in a mild activation. When activated by NO, the bond between –His and NO is severely weakened or broken and the enzyme is highly activated.

In the absence of a trap, the half-life of NO-sGC is about 90 min at 37°, and accelerates to about 5 s or less in its presence[43,75-78]. Cyclase which is deactivated rapidly in the presence of oxyhemoglobin can be fully reactivated with nitric oxide showing that the deactivation is reversible[77]. These observations reveal that in tissues that have oxyhemoglobin, in other words, all vascularized tissues, activated cyclase is deactivated very rapidly, thus keeping it in a NO-sensitive state.

Inactivation of the enzyme occurs when NO-sGC is modified in a fashion that would not permit reactivation with NO. The effects of oxidizing agents, including that of the popular inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) fall in this category[79]. Oxidation and loss of heme inactivates the enzyme which can be reactivated by NO only after supplementing the apoprotein with heme[79-81]. Oxidation of cysteine residues in the protein that play a role in heme binding may also inactivate the enzyme[82].

Down regulation, desensitization and hypersensitization:

In the early days of industrial production of nitroglycerine as an explosive, it was observed that workers who came into contact with the chemical suffered from headaches and dizziness, but soon became tolerant, apparently due to desensitization to the chemical[7]. The molecular mechanism of such desensitization remained unknown until recently and still remains incompletely understood.

It is reasonable to assume that one or more of the downstream elements in NO-regulated pathways become less available or less active when a tissue is exposed for a prolonged period of time to NO or chemicals like GTN that produce NO, thus leading to desensitization. The question is: which elements and how are they affected?

Before we discuss this further it might be added that the terms desensitization, down regulation and inactivation are unfortunately used interchangeably in recent literature. Down regulation should refer only to decreased production or enhanced degradation of a response element in the face of prolonged stimulation. Transient inactivation of existing responder molecules, thus reducing the magnitude of the observed activation, might be called desensitization. When the inactivation is irreversible or can be reversed only upon reconstitution with a prosthetic group or reduction with thiols, it is helpful not to refer to it as desensitization.

In the nineteen eighties it was observed that isolated blood vessels exposed for 1 to 2 h to 0.1 to 1.0 mM concentrations of GTN not only relaxed poorly on subsequent treatment with GTN, but also showed reduced activation of sGC[83,84]. This influence on sGC was noted not only in crude tissue extracts of desensitized tissues, but also on the enzyme purified from such extracts suggesting that the reduced activation is due to a stable modification of the enzyme[83]. Oxidation of critical cysteines in the protein might have been responsible for this effect[85] because treatment with dithiothreitol restored the response to GTN[86]. Pretreated smooth muscle cells recovered their sensitivity to GTN over several hours[87,88] probably through reduction of thiols on the cyclase. This type of modification of cyclase might be considered inactivation rather than desensitization because it

depends upon treatment with high (usually 0.1 to 1.0 mM) concentrations of NO-producing chemicals, hours of exposure to them, and the reversal is slow.

In cultured rat medullary interstitial cells and pulmonary artery smooth muscle cells long-term treatment with the NO donor sodium nitroprusside reduced the amount and stability of mRNAs of both α1 and β1 subunits of sGC as well as the proteins translated off them[89,90]. This can be considered typical down regulation, and apparently it is one of the methods used by cells to respond to prolonged NO exposure.

Prolonged exposure to a NO-releasing compound, SNAP ((±)-*S*-nitroso-N-acetylpenicillamine), reduced the expression of a cyclic GMP-dependent protein kinase in rat aortic smooth muscle cells indicating that elements downstream of cyclic GMP production are also subject to down regulation upon prolonged treatment with NO[91].

In contrast to down regulation in the face of persistent stimulation by NO, cyclase activity becomes hypersensitive to NO when the tissue is briefly, but completely deprived of NO. For example, in blood vessels, NO is produced in endothelial cells in response to stimulation by acetylcholine and it diffuses to smooth muscle cells where it activates sGC and causes relaxation. Denuding the vessels of endothelium or incubating them with inhibitors of NO synthesis for 15 min potentiated the vasoconstrictor effects of phenylephrine, decreased the amount of cyclic GMP, and highly sensitized the soluble cyclase to NO[92]. In NO-deprived vascular rings, it took 20 times less GTN to activate cyclase. These observations suggest that a normal vascular tone is maintained by a balance between the vasoconstrictor (norepinephrine) and vasorelaxant (NO), and that when deprived of the relaxant, the cyclase, and thus the tissue, become supersensitive. As would be expected from the above study, cyclase becomes supersensitive to NO in mice in which endothelial NO synthase is knocked out[93]. The mechanism of supersensitivity remains unknown. However, since supersensitivity develops within minutes of treatment with NOS inhibitors, it is unlikely to be due to excessive production of soluble cyclase. In fact, Brandes *et al.* observed that α1 and β1 subunits of cyclase were not expressed more in endothelial NOS knockout mice than in the wild-type [94]. They hypothesized that continuous exposure to NO, as in the wild-type animals, oxidizes heme on the cyclase and makes it unresponsive to NO, while deprivation of NO or inhibition of NO synthesis helps restore it to the reduced state and once again available for activation by NO. In short, the pool of activatable enzyme is higher in NOS knockout animals and in NOS-inhibited tissues.

In a recent paper, Bellamy and colleagues reported that when exposed to 1 μM DEA/NO (complex of diethylamine with nitric oxide), a NO donor, sGC was desensitized within seconds in platelets and in cerebellar astrocytes. Cells desensitized for 2 min recovered sensitivity slowly, but fully, in 10 min[78]. The low concentration of activator required for desensitization in this study, the rapid onset of desensitization and slower resensitization are characteristics associated with neurotransmitter receptors and reinforce the idea that the relationship between NO and cyclase is similar to that of a neurotransmitter and its receptor[95]. In addition, these properties distinguish the observation of Bellamy *et al.* from down regulation of cyclase reported in other studies mentioned above where much higher concentrations of NO and longer periods of exposure were required.

The molecular mechanism of rapid desensitization remains to be determined. In analogy with hormone and neurotransmitter receptors, phosphorylation of one or both of the subunits of NO-activated cyclase is a potential mechanism[96-99]. In fact, Zwiller and colleagues reported phosphorylation of sGC in purified preparations as well as in intact PC12 cells[100,101]. However, this phosphorylation, catalyzed by protein kinase C (PKC), increased cyclase activity, and it is not clear if NO has any influence on it. It is, therefore, unlikely that PKC-mediated phosphorylation is involved in desensitization of cyclase. Dephosphorylation through a tyrosine phosphatase has been shown to inhibit cyclase activity in PC12 cells[102]. The kinetics of dephosphorylation is, however, not determined. In studies on bovine chromaffin cells, Ferrero *et al.* observed that prior elevation of intracellular cyclic GMP reduced the activation of cyclase by subsequent treatment with the NO donor SNP, and that the effect is brought about by dephosphorylation of the β1 subunit of cyclase by a phosphatase activated by a cyclic GMP-dependent kinase[103]. This could potentially be a mechanism of desensitization of cyclase, but it remains to be demonstrated that the kinetics of dephosphorylation is compatible with the rate of desensitization observed by Bellamy *et al*[78]. Cyclic GMP-dependent phosphorylation of soluble cyclase, not dephosphorylation, was shown to inhibit the enzyme in smooth muscle cells. Though the magnitude of inhibition was modest and the kinetics of inhibition is unknown, this too is a potential mechanism for desensitization[104] (Table 1).

In summary, sGC is the intracellular receptor for NO.

How quickly the enzyme is activated by NO, how long the enzyme remains activated, and what happens to the enzyme when exposed to NO for a prolonged period of time are all important questions that have a bearing on the involvement of NO in various physiological processes. At this time it appears that that the activation is very rapid (less than a tenth of a second) and deactivation is slower (half-time of about 20 s). The high concentrations of NO and the long periods of treatment required to inactivate sGC in blood vessels suggests that the enzyme is probably not subject to desensitization in smooth muscle. The rapid desensitization and resensitization observed in brain astrocytes sug-

TABLE 1. SOLUBLE GUANYLATE CYCLASE AT A GLANCE.

		Reference
Protein Composition		
Subunit types	α1, α2, β1, β2	39
Number of subunits	2	38
Composition	α1β1, α2β1, α1β2	38
Prosthetic group	Heme	42
Activators	NO, CO	8, 64
Activity/Activation		
K_m For GTP	55 μM (10°)	68
V_{max} of basal enzyme	221 nmol / min / mg protein (37°)	66
V_{max} of CO stimulated enzyme	996 nmol / min / mg protein (37°)	66
V_{max} of NO stimulated enzyme	28,200 nmol / min / mg protein (37°)	66
K_{on} for NO	$1.4 \times 10^8 M^{-1}.s^{-1}$ (4°) – step 1	63
	$2.4 \times 10^5 M^{-1}.s^{-1}$ (4°) – step 2	63
K_{on} for CO	$8.1 \times 10^4 M^{-1}.s^{-1}$ (23°)	73
K_{off} for NO	0.04 s^{-1} (20°)	76
K_{off} for CO	10.3 s^{-1} (23°)	73
Modification		
Inactivation	Oxidation of sulfhydryl groups	82
	Oxidation of heme	79, 80, 81
Deactivation	Dissociation of NO	76, 77
Down regulation	Decreased synthesis of α, β subunits	89, 90
	Destabilization of mRNA	90
Desensitization	Unknown	78
Hypersensitization	Enzyme entirely in reduced form	92
Phosphorylation by PKC	Increases activity	100, 101
Phosphorylation by PKG	Decreases activity	104
Dephosphorylation by tyrosine phosphatase	Decreases activity	102
Dephosphorylation by protein phosphatse2A	Decreases activity	103

gests that defense mechanisms do exist in some cells against exposure to NO for undesirably long periods, though the mechanism of such defense remains unknown.

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