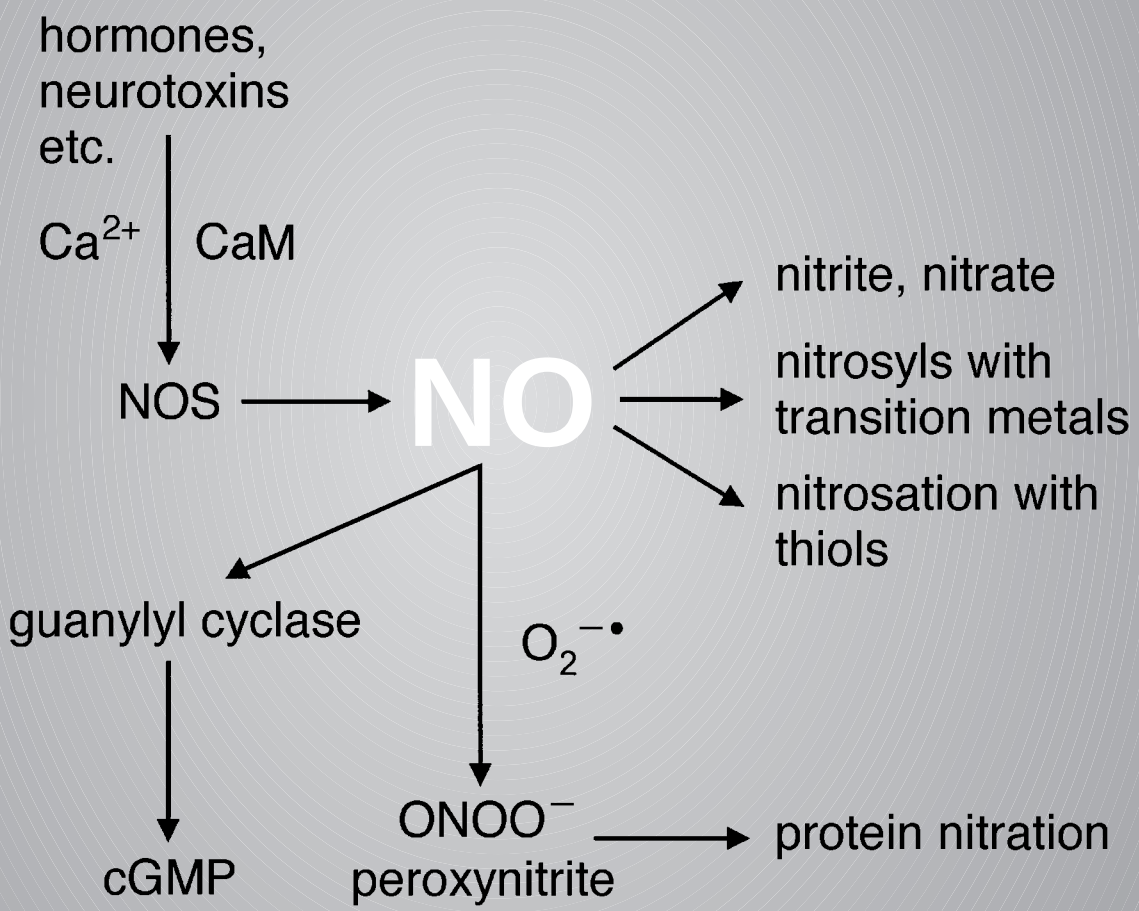
Formation and fate of nitric oxide



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Discovery of Some of the Biological Effects of Nitric Oxide and Its Role in Cell Signaling (Nobel Lecture)**

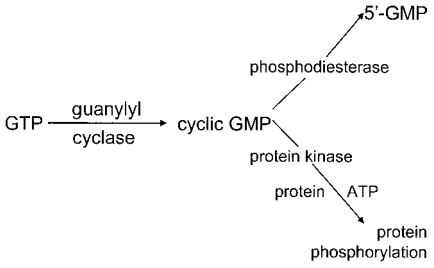
Ferid Murad*

The role of nitric oxide in cellular signaling in the past 22 years has become one of the most rapidly growing areas in biology with more than 20000 publications to date. Nitric oxide is a gas and free radical with an unshared electron that can regulate an ever-growing list of biological processes. In many instances nitric oxide mediates its biological effects by activating guanylyl cyclase and increasing cyclic GMP synthesis from GTP. However, the list of effects of nitric oxide that are independent of cyclic GMP is also growing at a rapid rate. For example, nitric oxide can interact with transition metals such as iron, thiol groups, other free radicals, oxygen, superoxide anion, unsaturated fatty acids, and other molecules. Some of these reactions result in the oxidation of nitric oxide to nitrite and nitrate to terminate its effect, while other reactions can lead to altered protein structure, function, and/or catalytic capacity. These diverse effects of nitric oxide that are either cyclic GMP dependent or independent can alter and regulate important physiological and biochemical events in cell regulation and function. Nitric oxide can function as an intracellular messenger, an autacoid, a paracrine substance, a neurotransmitter, or a hormone that can be carried to distant sites for effects. Thus, it is a unique simple molecule with an array of signaling functions. However, as with any messenger molecule, there can be too little or too much of the substance, and pathological events result. Some of the methods to regulate either nitric oxide formation, metabolism, or function have been in clinical use for more than a century, as with the use of organic nitrates and nitroglycerin in angina pectoris that was initiated in the 1870s. Current and future research with nitric oxide and cyclic GMP will undoubtedly expand the clinicians' therapeutic armamentarium to manage a number of important diseases by perturbing nitric oxide and cyclic GMP formation and metabolism. Such promise and expectations have obviously fueled the interests in these signaling molecules for a growing list of potential therapeutic applications.

Keywords: enzymes · nitric oxide · Nobel lecture · nucleotides · signal transduction

Introduction

As a combined degree student in the laboratories of Earl Sutherland and Theodore Rall shortly after the discovery of cyclic AMP, I was taught by them to enjoy science and I quickly became convinced of the important role of second messengers in hormonal signaling systems. After additional clinical training, I went to the National Institutes of Health (NIH) in 1967 to work with Martha Vaughan, and cyclic GMP began to emerge as another potential second messenger. Sutherland's laboratory and Aurbach's laboratory independently described the enzyme guanylyl cyclase that catalyzed the formation of cyclic GMP from GTP.[1, 2] Also, a new isoform of cyclic nucleotide phosphodiesterase that preferred to hydrolyze and inactivate cyclic GMP[3] and a subsequent novel cyclic GMP-dependent protein kinase, presumably its molecular target,[4] were described (Scheme 1).



Scheme 1. Enzymes involved in cyclic GMP synthesis, metabolism, and function.

[*] Prof. Dr. F. Murad
Department of Integrative Biology, Pharmacology and Physiology
University of Texas-Houston Medical School
P.O. Box 20708, Houston, TX 77225 (USA)
Fax: (+1) 713-500-7444
E-mail: fmurad@girch1.med.uth.tmc.edu

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The cyclic AMP area was becoming very popular and crowded due to new and simpler assay systems and more and more hormones mediating their effects through this intracellular messenger. Thus, cyclic GMP became more appealing to me as I planned to launch an independent academic career when I moved to the University of Virginia in 1970. In our earliest studies, we did numerous descriptive experiments where we added one agent or another to tissues or cells to correlate cyclic GMP accumulation with the function of the preparation. While these early studies seemed necessary at this time and were productive, we gained little insight into the true regulation of cyclic GMP synthesis and its functions. We wanted to address two simple, and obviously, naïve questions: 1) how do hormones, neurotransmitters, and various ligands regulate guanylyl cyclase activity and cyclic GMP synthesis (i.e., what are the molecular coupling events between hormone binding to its appropriate receptor and guanylyl cyclase activation)? and 2) what are some biological functions resulting from increased cyclic GMP levels?

Possible Isoforms of Guanylyl Cyclase

After two to three years of what some of my trainees called "dumping experiments", where we added various hormones and agents to preparations and measured cyclic GMP accumulation, we decided that there must be a better fundamental approach to our questions. About 1973 Hiroshi Kimura, a fellow with me, and I turned our attention to an examination of guanylyl cyclase in cell-free systems. We quickly learned that there was enzyme activity in both high-speed supernatant and particulate fractions of most tissue homogenates. Furthermore, the activities were quite different (Table 1).[5–7] While ATP inhibited both activities, the soluble activity was more sensitive to ATP inhibition. Calcium ion could either activate or inhibit activity dependent upon its concentration, but the soluble and particulate activates had different sensitivities to these effects. The most striking difference was that the soluble activity gave linear kinetics (double reciprocal plots) with regard to the substrate GTP, while the particulate activity gave curvilinear double reciprocal plots indicating cooperativity with respect to GTP, with presumably multiple GTP binding sights. The Hill coefficient of the soluble enzyme was 1.0, while the particulate activity had a Hill coefficient of about 1.7.[5–7]

Table 1. Guanylyl cyclase in cytosolic and particulate fractions of homogenates with different properties.

	Soluble	Particulate
Ca^{2+}	stimulates	inhibits
ATP, IC_{50}	0.4 mM	>1 mM
GTP, h[a]	1.0	1.74
detergent	stimulates 50–100%	stimulates >300%

[a] h = Hill coeffecient.

From our subsequent work with the characterization and purification of the enzyme and the cloning of the cDNAs for guanylyl cyclase we were able to demonstrate definitively that several isoforms and separate gene products were present in tissues.[8–11] Similar studies have since been done by other laboratories, most notably in the laboratory of David Garbers.[12, 13] Today we know that numerous soluble and particulate isoforms can exist. This topic is beyond the scope of this review, and interested readers are referred to other reviews.[8–13]

Effects of Azide, Hydroxylamine, and Nitrite

Our initial reaction to these early data was that we were dealing with two different isoforms of guanylyl cyclase, one in the cytosolic compartment and one in the membranous compartment or organelles. However, we could not exclude that such differences in the activities were due to artifacts and spurious results with crude high-speed soluble and particulate preparations. We thought that contaminating phosphatases, nucleotidases, and perhaps cyclic nucleotide phophodiesterases might also explain our observations. At the time we had no desire or plans to purify the protein(s). A simpler approach was to add azide, pyrophosphate, hydroxylamine, phosphodiesterase inhibitors, fluoride, etc. individually or in combination to preserve the substrate or product and recharacterize the crude soluble and particulate guanylyl cyclase. Quite surprisingly, we found that azide, hydroxylamine, and nitrite activated many, but not all preparations of guanylyl cy-

Ferid Murad was born in 1936 in Whiting, Indiana. He received his BA degree from DePauw University (Greencastle, Indiana) in 1958 and his MD and PhD degrees from Western Reserve University (Cleveland, Ohio) in 1965. After two years of clinical training at Massachusetts General Hospital and three years at the National Institutes of Health, he became an Associate Professor in the Departments of Medicine and Pharmacology at the University of Virginia in 1970, where he initiated his studies with cyclic GMP and nitric oxide. In 1981 he was appointed Professor of the Departments of Medicine and Pharmacology at Stanford University and Chief of Medicine of the Palo Alto Veterans Medical Center. In 1988 he became a Vice President of Abbott Laboratories. He joined the University of Texas-Houston as Professor and Chair of the Department of Integrative Biology and Pharmacology in 1997. He has received many awards including the Ciba Award in 1988, the Lasker Award in Basic Medical Research in 1996, and the Nobel Prize in Physiology or Medicine in 1998. He is a member of many societes including the National Academy of Sciences and the Institute of Medicine.

clase.[14–17] This was an exciting serendipitous observation for us, because any hormones or ligands that increased cyclic GMP accumulation in intact cells failed to increase cyclic GMP synthesis in cell-free preparations. We, therefore, thought that hormone – receptor coupling to guanylyl cyclase activation would be a complicated molecular event that was disrupted in cell-free preparations.

In order to establish the molecular events between hormone binding and guanylyl cyclase activation, it was necessary to have a hormonally responsive cell-free system and ultimately purified and reconstituted components of the signaling cascade. We thought that an understanding of the activation of guanylyl cyclase with azide, hydroxylamine, and nitrite could assist us in unraveling the complex signaling cascade, perhaps analogous to the stimulatory effects of fluoride that assisted with the characterization of the adenylyl cyclase system. This intuition was correct in ultimately leading us to the mechanism of hormonal activation of guanylyl cyclase as summarized below. Thus, we made a major commitment to understand the mechanisms of guanylyl cyclase activation by these agents.

Discovery of Proteins Required for Azide Activation and Inhibition

The activation by azide was tissue specific and had a brief time lag of several minutes before the time course became linear.[14–17] Furthermore, the activation was absent in an anaerobic environment requiring air or oxygen and could be enhanced with the addition of thiols.[14–17] These results suggested to us that azide might be converted into another compound that served as the activating species. We then did a simple and classical biochemical experiment where we mixed crude soluble cell extracts that were activated by azide with crude soluble extracts that were not activated.

In our first experiment (Table 2) we mixed crude supernatant fractions of rat liver that could be activated with azide

Table 2. Effects of sodium azide (NaN_3) on soluble guanylyl cyclase from rat liver, heart, and cerebral cortex (reprinted from references [14, 15] with permission).

Enzyme	Cyclic GMP formed[a]		$+NaN_3/-NaN_3$
	$-NaN_3$	$+NaN_3$	
liver	38.8	595.4	15.3
heart	23.0	23.2	1.0
cerebral cortex	46.0	42.0	0.9
liver + heart	27.3	23.1	0.8
liver + cerebral cortex	23.0	899.0	39.1

[a] Given in pmol per mg protein per min.

with fractions of rat brain and rat heart, tissues without azide effects on guanylyl cyclase.[14–17] Mixing extracts of liver with cerebral cortex resulted in an increased azide effect, while mixing liver extracts with heart extracts resulted in a loss of the azide effect. Our interpretation of these data was that azide activation required the presence of other factors present in liver extracts that when added to cerebral cortex permitted azide to activate the enzyme from both tissue extracts. Furthermore, heart extracts possessed a factor or several factors that blocked azide activation in the heart extracts and in liver extracts in the mixing experiments. Subsequent experiments proved these hypotheses to be true. The apparent activating factor(s) in liver and the inhibitor(s) in heart were nondialyzable and heat labile. Thus, we began to purify and characterize these activators and inhibitors.[14–21]

We thought that if we thoroughly understood the mechanism of azide activation of guanylyl cyclase, perhaps some day we could reconstitute the appropriate components and factors to observe hormonal activation of guanylyl cyclase in cell-free preparations, a major goal of the laboratory. This was a major research commitment that, while risky, turned out to be the correct approach and that led us to the discovery of the first biological effects of nitric oxide. Our University of Virginia colleagues and others thought we were crazy to waste our efforts in pursuing the effects of azide, a known metabolic poison that was often added to stored buffers and columns to inhibit bacterial growth.

Characterization of the Azide Activators and Inhibitors

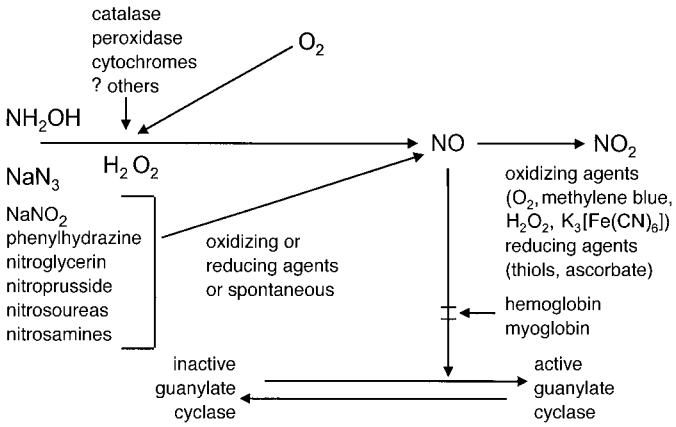
Hiroshi Kimura, Chandra Mittal, and I purified rat liver extracts and found that the macromolecular factor required for azide activation was catalase.[14–21] Other heme-containing proteins such as horseradish peroxidase and some of the cytochromes,[18–21] however, could substitute for the catalase requirement. The inhibitory factors in heart extracts were also purified and were found to be hemoglobin and myoglobin.[14–21] It rapidly became apparent that heme-containing proteins either permitted azide to activate guanylyl cyclase or blocked its activation. These stimulatory and inhibitory effects of various heme-containing proteins were important findings in that they led us to some much older literature describing the interactions of azide with catalase to generate nitric oxide.

Effects of Azide and Nitrovasodilators on Guanylyl Cyclase Activation, Cyclic GMP Accumulation in Intact Cells, and Smooth Muscle Relaxation

Azide, hydroxylamine, and nitrite not only activated guanylyl cyclase in cell-free extracts, but also increased cyclic GMP accumulation in numerous intact tissues and cell types including brain, liver, and some cell culture models.[14–21] One of the new fellows in my laboratory, Shoji Katsuki, was working with bovine tracheal smooth muscle preparations that we developed to examine the role of cyclic AMP and cyclic GMP in smooth muscle motility. We developed a rather homogenous smooth muscle preparation (about 90% smooth muscle) with a sufficient amount of tissue that permitted us to monitor motility in an organ bath after adding various agents. After quick freeze-clamping of tissue segments in liquid nitrogen, we could also measure cyclic nucleotide levels or

evaluate adenylyl or guanylyl cyclase activities.[22–25] It was obvious to us that we should also add these new guanylyl cyclase activators to bovine tracheal smooth muscle segments and evaluate motility and cyclic GMP accumulation. We found that these agents, as with other tissues, increased cyclic GMP levels as expected and simultaneously caused relaxation in precontracted muscles.[22–25] Similar results were observed when we examined clean gastrointestinal smooth muscle segments.[22–25] We intentionally avoided vascular segments due to the heterogeneity of the preparations with endothelial cells, blood cells, fibroblasts, etc. We felt that we could not determine in what cell types the cyclic GMP accumulation would occur in due to the marked cellular heterogeneity. In my previous studies with rat fat pads in Martha Vaughan's lab we had great difficulty correlating the effects of some known lipolytic agents with cyclic AMP accumulation due to the cellular heterogeneity of our preparations. The studies only become meaningful and helpful when we examined cyclic AMP accumulation in homogeneous isolated fat cells.[26–28]

After finding that these agents relaxed tracheal and gastrointestinal smooth muscle and that the increase in cyclic GMP was coincident with or preceded relaxation, we began to examine other smooth muscle relaxants such as nitroglycerin, nitroprusside, hydrazines, etc. (Scheme 2).[19–25] All of these



Scheme 2. Effects of some nitrovasodilators on cyclic GMP synthesis (reprinted with permission from references [19–21]).

agents increased cyclic GMP levels in several smooth muscle preparations as well as other tissues, caused smooth muscle relaxation, and activated guanylyl cyclase in most cell-free tissue extracts. We began to call these new guanylyl cyclase activators "nitrovasodilators", a simplified "lab jargon" that permitted us to communicate more readily. While these new nitrovasodilators did not require catalase to activate guanylyl cyclase preparations, these effects were inhibited by hemoglobin and myoglobin. The effects of altered redox conditions, thiols, and heme-containing proteins with this growing list of nitrovasodilators were to become very important for us later in determining the effects of endothelial-derived relaxant factor (EDRF) on cyclic GMP accumulation in vascular preparations subsequent to Furchgott's discovery of EDRF in 1980.[29]

Discovery of the Biological Effects of Nitric Oxide

The effects of the growing list of nitrovasodilators with nitro or nitroso functionalities and the effects of heme-containing macromolecules as factors for either activation or inhibition of these agents suggested to us that perhaps the active intermediate or proximal activator of guanylyl cyclase could be nitric oxide or one of its oxidized or reduced products. This was indeed the case.[20–25, 30] The formation of nitric oxide from various nitrovasodilator precursor molecules or "prodrugs" could be influenced by a variety of conditions as noted in Scheme 2. Furthermore, the activity of nitric oxide could also be diminished by a variety of oxidants such as methylene blue, $K_3[Fe(CN)_6]$, excess oxygen, etc. as well as sinks to trap nitric oxide such as hemoglobin and myoglobin (Scheme 2). Delightfully, our first chemical generator to produce nitric oxide and ventilate the gas into guanylyl cyclase incubations was a success (Figure 1).[22–25, 30] The experiment was conducted by

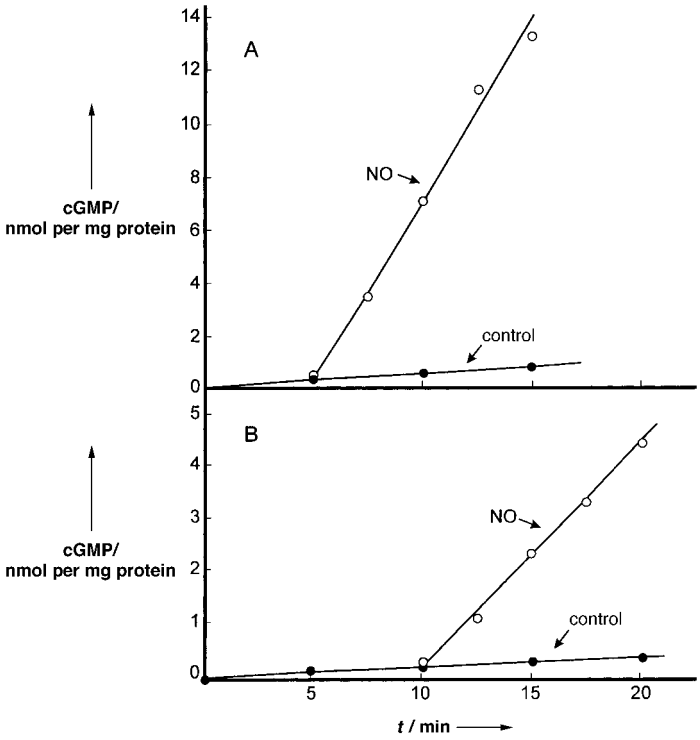


Figure 1. The first demonstration of the biological effect of nitric oxide to activate guanylyl cyclase preparations from rat lung (A) and bovine tracheal smooth muscle (B) (reprinted with permission from reference [22]).

Shuji Katsuki and William Arnold, post-doctoral fellows in my laboratory in 1976, the night before Katsuki was to complete his fellowship and return with his family to Japan. I have often thought that had Katsuki completed his fellowship earlier, our discovery of the first biological effects of nitric oxide could have been delayed considerably. Nitric oxide, unlike some other activators and nitrovasodilators, increased the activity of most guanylyl cyclase preparations and increased cyclic GMP levels in virtually all tissues tested with few exceptions (Table 3). Furthermore, the stimulatory effects of nitric oxide and various nitrovasodilators on cyclic GMP

Table 3. Effect of nitric oxide (NO) on the activity of guanylyl cyclase from various tissues.

Tissue	Fraction[a]	Cyclic GMP formed[b] −NO	+NO	+NO/−NO
rat liver	S	22.1	674.2	30.5
	P	12.4	37.3	3.9
bovine lung	S	111.7	3625.5	32.5
bovine tracheal smooth muscle	S	8.9	297.1	33.4
	P	27.0	35.3	1.3
rat heart	S	10.5	242.8	23.1
rat kidney	S	51.6	975.9	18.9
rat cerebral cortex	S	55.7	1122.6	20.2
	P	14.2	209.1	14.7
rat cerebellum	S	23.7	784.4	33.1
	P	20.6	201.2	9.8
rat skeletal muscle	S	6.1	84.0	13.8
rat spleen	S	73.5	381.7	5.2
rat small intestinal muscle	S	42.9	250.6	5.8
rat adrenal	S	37.5	394.2	10.5
rat epididymal fat	S	9.4	108.5	11.5
rat liver	D	296.2	1406.3	4.7
rat cerebral cortex	D	57.2	174.3	3.0
bovine lung	G	61.4	1672.1	27.2
rat heart	G	30.2	171.9	5.7

[a] S = soluble fraction; P = particulate fraction; D = after chromatography on DEAE-cellulose; G = after chromatography on Sephadex G-100. [b] Given in pmol per mg protein per min.

synthesis were not additive, suggesting that they all shared a similar mechanism of activation of guanylyl cyclase.[30]

Since this was the first and only example of a free radical activating an enzyme, we committed ourselves to purifying the isoforms of guanylyl cyclase to homogeneity in order to reexamine the effects of nitric oxide. We could not exclude the possibility that nitric oxide was being converted into yet some other activating species or that it was not inhibiting an inhibitor of the enzyme in our crude preparations. The inhibition of a guanylyl cyclase inhibitor could appear in our assays as a possible activation due to disinhibition.

After purification of the soluble guanylyl cyclase to homogeneity, nitric oxide activation was still observed.[21, 31] In fact, the apparent K_{act} for nitric oxide continued to decrease as we purified the enzyme. Purified soluble guanylyl cyclase has a K_{act} or EC50 for nitric oxide in the order of 1 to 10 nM, depending upon the conditions of the assay and the presence of other materials, such as thiols, proteins, sugars, lipids, etc., that can form complexes with nitric oxide and alter its effective concentration to activate guanylyl cyclase.[31] These later agents can act as traps or scavengers to form nitro or nitroso adducts and complexes to increase the K_{act} and EC50 values. In some cases these complexes can themselves also become nitrovasodilators or nitric oxide prodrugs to rerelease nitric oxide under favorable conditions.

Is Nitric Oxide a Natural Endogenous Guanylyl Cyclase Activator?

We proposed in 1978 that nitric oxide formation from some endogenous precursor could perhaps explain hormone, autacoid, and neurotransmitter effects on cyclic GMP synthesis in intact cells and tissue.[19,21] We thought that perhaps appropriate hormone treatment could influence the redox state of cells, the formation of nitric oxide from an endogenous precursor or the metabolism of nitric oxide. We also thought that nitric oxide could be an intracellular second messenger to mediate the effects of hormones. Thus, it could perhaps join the short but growing list of second messenger molecules that included cyclic AMP, cyclic GMP, Ca^{2+}, eicosanoids, lipids, proteins, etc. Again our colleagues thought we were crazy to believe that a reactive free radical could activate an enzyme and function as a second messenger. After all, nitric oxide was a known pollutant from the combustion of numerous substances that inactivated or destroyed various macromolecules and depleted the ozone layer. It surely could not be a second messenger signaling molecule, we were told by the skeptics.

In order to prove this hypothesis it would require a major technical advance. If, indeed, nitric oxide was an endogenous second messenger and regulator of guanylyl cyclase, one would expect that tissue levels would be about 1 to 10 nM (levels near the K_{act} or EC50 values) in order to be physiologically relevant and permit the fluctuations in nitric oxide concentrations to control enzyme activity. If the concentrations in tissue were much higher, one would expect the enzyme to always be activated and the physiological significance of nitric oxide as a regulator would be less convincing and meaningful. Unfortunately, the assays for nitric oxide and its oxidation products (nitrite and nitrate) were crude colormetric and spectrophotometric assays that were several orders of magnitude less sensitive than needed (about millimolar to micromolar). Thus, our hypothesis remained untestable for several years until we and others developed more specific and sensitive assays for the free radical.

Discovery of EDRF

In 1980 Furchgott presented a seminar in our Pharmacology Department while I was at the University of Virginia. He was excited about his recent discovery of endothelial-derived relaxing factor (EDRF) and the ability of endothelial cells to generate and release a labile substance that produced relaxation of the underlying smooth muscle in vascular segments.[29] The effects of EDRF shared many of the features and properties of nitrovasodilators, and I suggested to him that perhaps increased cyclic GMP levels could explain his observations with light-induced and endothelium-induced relaxation. We even planned a collaboration to test this hypothesis which never materialized because of our subsequent move to Stanford University in 1981.

At the time, Robert Rapoport was one of my new fellows working with tracheal and vascular smooth muscle preparations and impatiently waiting for the collaboration to begin. After a brief visit to our new laboratory by the late Michael Peach in 1982 and his queries about our interests in EDRF, we then proceeded to demonstrate independently that EDRF did, indeed, increase cyclic GMP synthesis in the smooth muscle compartment of rat aorta segments (Figure 2).[32–38] We observed similar effects of EDRF on cyclic GMP formation with a variety of endothelium-dependent vasodilators such as

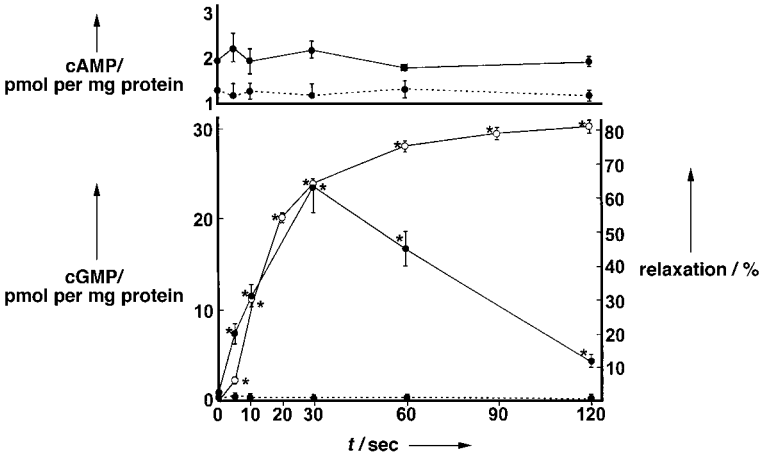


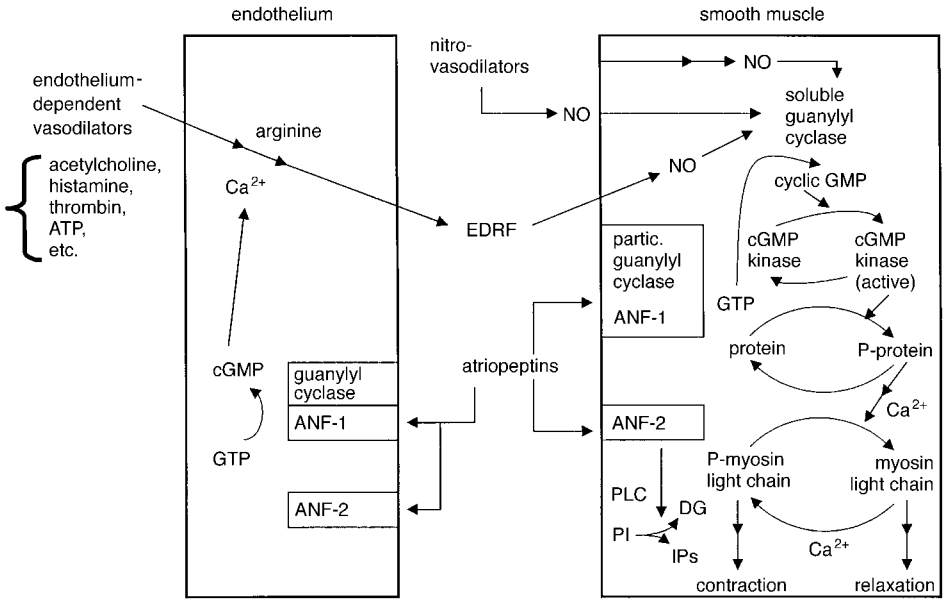
Figure 2. Effects of acetylcholine on cyclic GMP and cyclic AMP accumulation (●) and relaxation (○) of rat aorta segments with (——) and without (----) endothelium (reprinted with permission from references [32, 33]).

acetylcholine, A23187, thrombin, ATP, bradykinin, etc.[32–38] We continued the work to show that EDRF formation as well as nitrovasodilators also increased cyclic GMP dependent protein kinase activity and altered protein phophorylation of many endogenous smooth muscle proteins including the dephosphorylation of myosin light chain.[32–38]

Cyclic GMP accumulation with nitrovasodilators or endothelium-dependent vasodilators produced similar effects on cyclic GMP accumulation, cyclic GMP dependent protein kinase activation and altered $^{32}PO_4$ incorporation in the same family of proteins isolated with two-dimensional polyacrylamide gel electrophoresis.[32–38] Subsequently, we and others found that cyclic GMP decreased phosphoinoside metabolism and formation of inositol phosphates, including inositol trisphosphate, by decreasing phospholipase C activity.[39] These and other effects of cyclic GMP would be expected to decrease cytosolic free calcium and the activity of myosin light chain kinase, a calcium/calmodulin-dependent enzyme. The effect of cyclic GMP elevating agents to decrease cytosolic free calcium levels in smooth muscle and other tissues has been subsequently shown by many laboratories.

Because of the biochemical similarities of the effects of nitrovasodilators and EDRF-producing agents, we came to view EDRF as the "endogenous nitrate or endogenous nitrovasodilator".[40] About six months after this review[40] was published, Furchgott and Ignarro independently proposed that EDRF is nitric oxide at a meeting at the Mayo Clinic in the summer of 1986. I previously and continually have argued with them and others that EDRF is probably a family of nitric oxide adducts or complexes as well as nitric oxide. This controversy remains unresolved even today in that many nitro and nitroso complexes can meet the definition of EDRF as originally defined by Furchgott.[29] These complexes are readily formed between free nitric oxide in cells and the interstitial space with various cellular constituents such as thiols, lipids, proteins, etc. Presumably, many of these substances can readily dissociate their nitric oxide in the correct environment to account for the free nitric oxide found in some vascular preparations. Unfortunately, this pressing issue may not be resolved in the near future due to the low concentration of EDRF expected. As noted above, nitric oxide at concentrations of 1 to 10 mM are sufficient to activate guanylyl cyclase and permit the signaling event. Clearly endothelial cells and many other cells produce nitric oxide, and the activator of the smooth muscle guanylyl cyclase is nitric oxide. The question outstanding is: what are the intervening or intermediate molecules presumably complexed with nitric oxide that Furchgott has called EDRF?

We and a number of laboratories showed that the effects of EDRF could be blocked with methylene blue, hemoglobin, and other inhibitors of nitrovasodilator activation of guanylyl cyclase (Scheme 2). The effects of nitric oxide or EDRF can also be mimicked by atriopeptins which selectively activate particulate guanylyl cyclase (Scheme 3).[41, 42] In fact, we showed that atriopeptin receptors were heterogeneous with two receptor subclasses (ANF-R1 and ANF-R2).[43, 44] One of the atriopeptin receptors (ANF-R1) is the extracellular domain of the transmembrane particulate guanylyl cyclase.[45] This work has been elegantly confirmed by the cDNA cloning studies from Garbers' laboratory.[46]



Scheme 3. Effects of endothelium-dependent vasodilators, nitrovasodilators, and atriopeptins in rat aorta segments. PLC = phopholipase C, PI = phosphoinositides, DG = diacylglycerol, IPs = inositol phosphates (reprinted with permission from references [40, 50]).

Effects of L-Arginine on Guanylyl Cyclase Activation and Nitrite and Nitrate Formation

Degucci's laboratory was also one of the earlier few laboratories interested in guanylyl cyclase. In 1982, Degucci's laboratory reported that brain and/or neuroblastoma extracts possessed an endogenous substance that activated crude or partially purified preparations of soluble guanylyl cyclase.[47] Furthermore, this substance was identified as L-arginine. Activation by L-arginine was blocked by hemoglobin and methylhydroxylamine, which blocked the effects of nitrovasodilators. We confirmed their observations with crude or partially purified preparations of guanylyl cyclase, but found that L-arginine failed to block our highly purified guanylyl cyclase preparations.[68] These observations were the first to report arginine activation of guanylyl cyclase with effects similar to those of nitric oxide and nitrovasodilators. Unfortunately, neither they nor we pursued these arginine effects at the time, which could have led to the earlier discovery of nitric oxide synthase.

Subsequently Hibbs' laboratory in 1987 found that the cytotoxic effects of macrophages on tumor cells in culture correlated with the accumulation of nitrite and nitrate in the conditioned media.[48] The cytotoxic effects and accumulation of nitrite and nitrate were increased with L-arginine and blocked with L-arginine analogues such as *N*-methyl-L-arginine, an agent that later proved to be an effective competitive inhibitor of nitric oxide synthase.

The findings of Degucci and Hibbs were important, timely, and critical observations that subsequently permitted our laboratory and many other laboratories to move forward rapidly in understanding the role of nitric oxide in cellular signaling.[49–52] Unfortunately, their laboratories have not received sufficient recognition for these important observations.

Characterization of Nitric Oxide Synthase and Its Isoforms

Within several years many laboratories including our own began to characterize, purify, and clone this novel synthetic pathway that converted L-arginine into nitric oxide and citrulline. Initially the most active and productive laboratories included those of Marletta and Stuehr, Palmer and Moncada, Bredt and Snyder, Stuehr and Nathans, and ourselves (see references [9–11] and references therein).

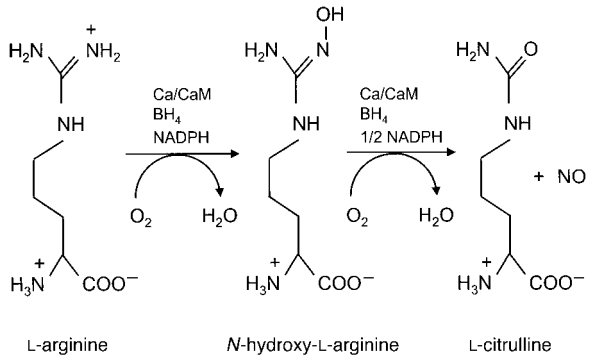
The first nitric oxide synthase (NOS) isoform to be purified was the neuronal or brain NOS or NOS-1, also called Type 1 NOS or constitutive NOS. The first investigators to purify and characterize NOS-1 were Bredt and Snyder[53] and our own laboratory.[54–57] This was followed shortly thereafter by NOS-2 or inducible NOS, also called Type II NOS, by Stuehr and Nathans,[58] and then NOS-3 or endothelial NOS, also called Type III NOS, in our lab.[59–62] Monoclonal and polyclonal antibodies to the purified isoforms and synthetic peptide fragments were produced as were the cDNA clones for these three gene products (see references [9–11] and references therein). The chromosomal location for each isoform has been identified as have the numerous cosubstrates, cofactors, and prosthetic groups (O_2, NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin, and heme; see reference [11] and references therein). All three isoforms are regulated by calmodulin, but only NOS-1 and NOS-3 have apparent and obvious regulation with altered cytosolic free calcium concentrations. NOS-2 contains calmodulin as a tightly bound component or subunit after translation and assembly such that dependency upon cytosolic free calcium levels is not demonstrable. The catalytically active isoforms exist as homodimers with a heme prosthetic group serving to facilitate dimer formation. The carboxy terminal domain has considerable homology between the isoforms, and is homologous to cytochrome P450. The amino terminal domain has less homology and may serve to bind to regulatory proteins and/or chaperones for its subcellular compartmentation. The homology of the three isoforms is only about 50 to 60%, while the homology of a given isoform between species can be as great as 85 to 92%. Some of the properties of the NOS isoforms are summarized in Table 4.

Table 4. Isoforms of nitric oxide synthase (NOS).[a]

NOS-1 (155 kDa)	neuronal, brain, Type I-NOS; central and peripheral neurons, NANC neurons, islets, endometrium, skeletal muscle, etc.
NOS-2 (125 kDa)	inducible, Type II-NOS; macrophage, liver, smooth muscle, endothelium, heart, etc.; effects of LPS, cytokines and glucocorticoids
NOS-3 (135 kDa)	endothelial, Type III-NOS; endothelium, brain, heart, etc.; acylation, phosphorylation

[a] LPS = lipopolysaccharide; NANC = nonadrenergic-noncholinergic.

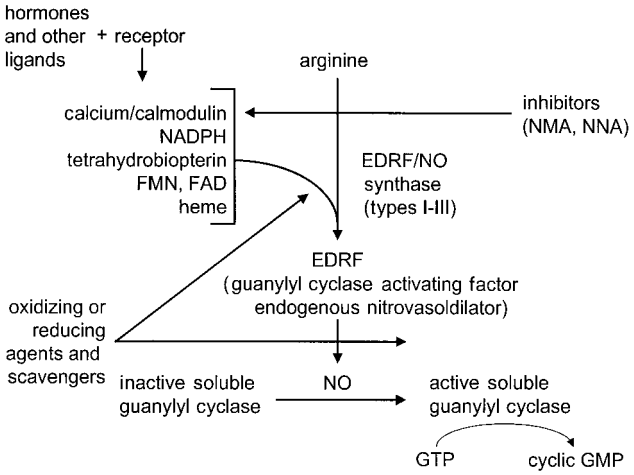
NOS converts L-arginine into L-hydroxyarginine and subsequently into nitric oxide and citruline as summarized in Scheme 4. The transfer of electrons for the oxidation of the guanidino nitrogen atom of L-arginine and the precise role of each cofactor remains unresolved. This continues as an active area of investigation in many laboratories. Recent X-ray diffraction studies with crystals of some NOS isoform fragments are currently underway in several laboratories. These studies should shed some light upon the precise roles of the cofactors and prosthetic groups. These studies may also



Scheme 4. The nitric oxide synthetic pathway. BH_4 = tetrahydrobiopterin; Ca/CaM = calcium/calmodulin.

permit the development of highly selective and effective inhibitors of each of the NOS isoforms in order to determine other important biological roles of nitric oxide and develop new and important therapeutic agents (see below).

Presumably the in situ activity of NOS can be regulated by the availability of substrate, cofactors, and prosthetic groups. However, to date most of the regulation of NOS-1 and NOS-3 has focused on the alterations of cytosolic free calcium caused by a myriad of hormones, autacoids, neurotransmitters, etc. (Scheme 5).



Scheme 5. Hormonal regulators of nitric oxide and cyclic GMP formation. NMA = *N*-methyl-L-arginine, NNA = *N*-nitro-L-arginine.

NOS-2 does not appear to be present under normal conditions in most cells. However, upon exposure to endotoxin (LPS) interferon γ, IL 1, TNF α, and other proinflammatory cytokines, the levels of mRNA, protein, and catalytic activity begin to increase within one hour and reach maximal levels in 6 to 18 hours. Various antiinflammatory cytokines and glucocorticoids can decrease the induction of NOS-2. It is thought that some of the pharmacological and biological effects of these factors may be explained by increased or decreased nitric oxide formation. For example, the hypotension observed in septic shock as well as the ensuing "multiple organ failure syndrome" (MOF) may be due to excessive nitric oxide formation from NOS-2 induction.

Translational and Posttranslational Modifications of NOS

All of the isoforms can probably be phosphorylated by a variety of protein kinases including cyclic AMP dependent protein kinase, cyclic GMP dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinase.[63] With phosphorylation, the activity of the enzyme can be increased or decreased. However, the physiological relevance of phosphorylation to regulate enzyme activity or enzyme translocation from one cellular compartment to another is unknown (see references [9–11] and references therein). In some cell-free systems with purified NOS-1, as many as 6 to 7 moles of phosphate can be incorporated into one monomer of protein.[63] Studies with intact cells and tissues with permeable and specific protein kinase inhibitors are required to determine the physiological relevance of NOS phosphorylation with regard to catalytic activity, enzyme compartmentation, enzyme regulation, and participation in various biological processes. Unfortunately, selective and permeable protein kinase inhibitors are not widely available.

NOS-3 is also myristoylated and palmitylated, and acylation probably participates in the location of this isoform in the plasma membrane and/or other organelles.[61, 64–66] Recently, Michels' laboratory has described the association of NOS-3 with the caveoloae of the plasma membrane.[65, 66] These structures also contain many other proteins participating in cell signaling events. Mechanisms to regulate and chaperon NOS-3 from the Golgi to the caveolae and the recycling of NOS-3 to the cytosol and perhaps back to the plasma membrane are areas of active investigation. These processes could perhaps serve as important sites for regulation and drug targeting.

The three gene products of NOS described to date, possible alternate mRNA splicing due to multiple promoters (i.e., NOS-1),[67] and various posttranslational modifications should permit a multitude of NOS isoforms and activities for regulation. Undoubtedly this will be a complex problem to resolve with regard to NOS function, regulation, and selective inhibition (see below). Some cells possess only one isoform while other cells have several isoforms with different subcellular locations and perhaps different functions. The precise location of pools of nitric oxide in cells and their proximity to guanylyl cyclase, thiols, fatty acids, heme-containing scavengers or sinks, other free radicals such as superoxide anions, etc. should have marked and profound effects on the biological and/or cytotoxic properties of nitric oxide. Thus, numerous question regarding nitric oxide formation, location, and function remain to be addressed.

Inhibitors of NOS

Numerous NOS inhibitors have been described. Novel arginine-based and guanidine-based antagonists are areas of active investigation in both academic and industrial laboratories (see references [9–11] and references therein). Most inhibitors to date are competitive antagonists and may be partly selective for one or another isoform. Commercially available compounds may show as much as 100- to 200-fold selectivity for one or another isoform. Unfortunately, to date, there are no highly specific inhibitors of one or another isoform. Such agents could prove invaluable to sort out the NOS isoforms in various cell types and the biological events that they regulate. Furthermore, investigations in this area could result in highly selective and perhaps specific and efficacious therapeutic agents without numerous side effects since one or more isoforms of NOS is found in virtually all cells with few exceptions. Currently, some NOS inhibitors are

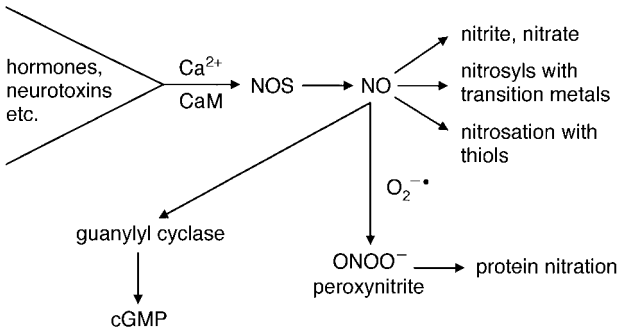
in clinical trials for patients in septic shock. Other clinical studies are under discussion and consideration.

Unfortunately, the earliest clinical studies with NOS inhibitors were not sufficiently selective as expected from previous in vitro studies with the inhibition of NOS isoforms. Thus, the initial enthusiasm for the clinical utility of NOS inhibitors is beginning to meet with some concerns and skepticism as unwanted side effects become apparent, some of which were predictable from earlier basic biological studies. Perhaps this could serve as a lesson regarding the selection of nonspecific agents for clinical development prematurely. None the less, many believe that these molecular targets will prove to be valuable approaches to some diseases with excess nitric oxide production. However, more selective agents are necessary due to the broad distribution of the many NOS isoforms and their important and diverse roles in biological regulation.

Nitric Oxide Donors or Prodrugs in Clinical Medicine

A number of NO prodrugs have been used successfully since the use of nitroglycerin and other organic nitrates for angina pectoris more than a century ago. Nitroglycerin was discovered in 1847, and it and other organic nitrates were first used clinically in the 1870s. Ironically Alfred Nobel, who discovered how to safely formulate dynamite from nitroglycerin, was prescribed nitroglycerin for his angina pectoris late in life, but he refused to take it because of the known vascular headaches of his factory workers. Other therapeutics of the "nitrovasodilator class" include nitroprusside, organic and inorganic nitrites and nitrates, nitrosamines, hydrazines, nitrosoureas, etc. Many academic and industrial laboratories are actively synthesizing additional "NO donors" that release NO at predictable rates in specific tissues or environments or can be attached to various grafts and supports for vascular surgical procedures (see reference [11] and references therein). The controlled delivery of nitric oxide with a prodrug or device at a specific target site or vascular bed without the tolerance that is developed to nitroglycerin would be a most significant contribution to therapy of several vascular disorders and other diseases. There is currently considerable promise that such agents and delivery methods can be achieved in that the work is becoming more of a medicinal chemistry and/or engineering effort with obvious targets. An important clinical indication is reperfusion injury after surgery or instrumentation for vascular patency. There are obviously many other indications that can be listed such as vascular remodeling, tissue transplantation, cancer vascularization, etc. with the known effects of nitric oxide on platelet aggregation, vascular smooth muscle proliferation, atherogenesis, angiogenesis, cytotoxicity, etc.

One of the most rapidly advancing areas of clinical application is the inhalation of low concentrations of the NO gas (see reference [11] and references therein). Interestingly, nitric oxide at low concentrations is quite stable and minimally reactive. Its reactivity and toxicity is a second-order reaction dependent upon the concentration of nitric oxide and its interaction with other free radicals and reactive oxygen species such as superoxide. At higher concentrations NO can interact with many transition metals, heme-containing proteins, and thiol groups, oxidize functionalities on polynucleotides (RNA and DNA) and proteins, and form strand breaks in polynucleotides (Scheme 6). There is a rapid and almost diffusion-limited interaction of nitric oxide with superoxide to form the very reactive peroxynitrite. This is becoming a very interesting area of investigation that undoubtedly relates to the toxic properties of these free radicals.



Scheme 6. Formation and fate of nitric oxide.

When low concentrations of nitric oxide are administered to premature infants with low birth weights by nasal catheters, nitric oxide has marked beneficial effects on infant mortality. The pulmonary hypertension is improved as is the pulmonary and systemic oxygenation and systemic hypertension resulting from the right-to-left shunting through the patent ductus arteriosus. There may also be beneficial effects in infants and children with congenital heart defects with right-to-left shunting and hypoxia. The beneficial effects in adult patients with various pulmonary disorders including pulmonary hypertension and/or acute respiratory distress syndrome (ARDS) are less convincing and more controversial. The controversy and variability of the response to inhaled nitric oxide in adults undoubtedly relates to the etiology of their disease and the presence or absence of some "reversible components" of their pathology. Some of the molecular targets and processes regulated by NO and/ or cyclic GMP are summarized in Tables 5 and 6. These targets can obviously suggest other therapeutic uses for NOS inhibitors and NO prodrugs.

Table 5. Targets of nitric oxide and/or cyclic GMP.

guanylyl cyclase
cyclic nucleotide protein kinases
cyclic nucleotide phosphodiesterses
cyclooxygenase (COX II)
heme proteins, iron centers, and thiol groups
DNA modifications
regulation of *N*-methyl D-aspartate(NMDA)-activated glutamate receptors
phospholipase C

Table 6. Some processes regulated by nitric oxide and/or cyclic GMP.

relaxation of smooth, cardiac, and skeletal muscle
retinal phototransduction
intestinal secretion and ion transport
renal tububar-glomerular feedback
endothelial permeability
smooth muscle proliferation
platelet adhesin and aggregation
insulin secretion
hormone production and secretion
neurotransmission
long-term potentiation and memory
regulation of transcription
tissue injury and inflammation
pathogen cytotoxicity
tumor cytotoxicity
calcium transport and redistribution

Summary

Since our discovery of the first biological effects of nitric oxide more than two decades ago, more than 20000 publications have appeared on this topic. There have been more than 4500 to 5000 publications per year in the recent several years, and the number of publications continues to grow exponentially. It has become almost impossible for investigators to read all the abstracts of these publications, let alone the full papers. At times it can be difficult to keep up with just the titles, authors, and laboratories and remain current. This will undoubtedly test the mentorship of many of us as we train students and fellows who need exciting and novel publications and the priorities required to find positions in our competitive research society and remain funded. However, there seems to be no obvious saturation of the talent required in this exciting and growing field. Numerous important questions remain to be addressed and answered. I have attempted to raise some of these areas and questions here. What excitement it has been for those of us who joined the field early and were able to see such major advances and progress in recent years. There still remains many nuggets available in a vast gold mine of nitric oxide biology. By no means has the field been over-mined with a paucity of gems to be found.

In recent years a number of laboratories have made significant advances in nitric oxide research. Some current areas of active research are listed in Table 7. Undoubtedly, other interesting areas will also emerge and require increasing research and attention.

Table 7. Some areas of active nitric oxide research.

transcriptional regulation
overexpression and gene therapy
gene knockouts
histochemistry and hybridization
acylation, phosphorylation, etc.
cell types with NOS isoforms and functions
cyclic GMP mediated effects
noncyclic GMP mediated effects
selective/specific NOS inhibitors
novel NO donors

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[1] "The formation and metabolism of cyclic GMP": J. G. Hardman, J. A. Beavo, J. P. Gray, T. D. Chrisman, W. D. Patterson, E. W. Sutherland, *Ann. N.Y. Acad. Sci.* **1971**, *185*, 27 – 35.
[2] See "Guanylate cyclase activity in heart and lung": A. A. White, *Adv. Cyclic Nucleotide Res.* **1975**, *5*, 353 – 373.
[3] "Regulation of cyclic nucleotide phosphodiesterase": M. Appleman, W. Terasaki, *Adv. Cyclic Nucleotide Res.* **1975**, *5*, 153 – 162.
[4] "Cyclic nucleotides, protein phosphorylation and neuronal function": P. Greengard, *Adv. Cyclic Nucleotide Res.* **1975**, *5*, 585 – 602.
[5] "Evidence for two different forms of guanylate cyclase in rat heart": H. Kimura, F. Murad, *J. Biol. Chem.* **1974**, *249*, 6910 – 6919.
[6] "Two forms of guanylate cyclase in mammalian tissues and possible mechanisms for their regulation": H. Kimura, F. Murad, *Metabolism* **1975**, *24*, 439 – 445.
[7] "Increased particulate and decreased soluble guanylate cyclase activity in regenerating liver, fetal liver, and hepatoma": H. Kimura, F. Murad, *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 1965 – 1969.
[8] "Cyclic GMP synthesis and function": S. A. Waldman, F. Murad, *Pharm. Rev.* **1987**, *39*, 163 – 96.
[9] "Cyclic GMP Synthesis, Metabolism and Function": *Advances in Pharmacology*, *Vol. 26* (Ed.: F. Murad), Academic Press, New York, **1994**, pp. 1 – 335.
[10] "The role of nitric oxide in modulating guanylyl cyclase": F. Murad, *Proc. Int. Congr. Pharmacol*. **1994**, *10*, 1 – 4 (Neurotransmission).
[11] "Nitric Oxide: Biochemistry, Molecular Biology, and Therapeutic Implications": *Advances in Pharmacology*, *Vol. 34* (Eds.: L. Ignarro, F. Murad), Academic Press, New York, **1995**, pp. 1 – 516.
[12] "Signal Transduction by guanylyl cyclase": M. Chinkers, D. Garbers, *Annu. Rev. Biochem.* **1991**, *60*, 553 – 575.
[13] "The membrane form of guanylate cyclase": D. L. Garbers, D. G. Lowe, L. J. Dangott, M. Chinkers, D. S. Thorpe, J. K. Bentley, C. S. Ramarao, D. V. Goeddel, S. Singh, *Cold Spring Harbor Symp. Quant. Biol.* **1988**, *53*, 993 – 1003.
[14] "Activation of guanylate cyclase from rat liver and other tissues with sodium azide": H. Kimura, C. K. Mittal, F. Murad, *J. Biol. Chem.* **1975**, *250*, 8016 – 8022.
[15] "Requirement for a macromolecular factor for sodium azide activation of guanylate cyclase": C. K. Mittal, H. Kimura, F. Murad, *J. Cyclic Nucleotide Res.* **1975**, *1*, 261 – 269.
[16] "Increases in cyclic GMP levels in brain and liver with sodium azide, an activator of guanylate cyclase": H. Kimura, C. K. Mittal, F. Murad, *Nature* **1975**, *257*, 700 – 702.
[17] "Appearance of magnesium guanylate cyclase activity in rat liver with sodium-azide activation": H. Kimura, C. K. Mittal, F. Murad, *J. Biol. Chem.* **1976**, *251*, 7769 – 7773.
[18] "Purification and properties of a protein required for sodium azide activation of guanylate cyclase": C. K. Mittal, H. Kimura, F. Murad, *J. Biol. Chem.* **1977**, *252*, 4348 – 4390.

[19] "Guanylate cyclase: Activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin": F. Murad, C. K. Mittal, W. P. Arnold, S. Katsuki, H. Kimura, *Adv. Cyclic Nucleotide Res.* **1978**, *9*, 145 – 158.

[20] "Properties and regulation of guanylate cyclase: Activation by azide, nitro compounds and hydroxyl radical and effects of heme containing proteins": F. Murad, C. K. Mittal, W. P. Arnold, K. Ichihara, E. Braughler, M. El-Zayat in *Molecular Biology and Pharmacology of Cyclic Nucleotides* (Eds.: G. Folca, R. Paoletti), Elsevier, Amsterdam, **1978**, pp. 33 – 42 (Proc. NATO Adv. Study Inst. Cyclic Nucleotides, Milan, Italy, **1977**).

[21] "Effect of nitro-compound smooth muscle relaxants and other materials on cyclic GMP metabolism": F. Murad, C. K. Mittal, W. P. Arnold, J. M. Braughler in *Advances in Pharmacology and Therapeutics*, *Vol. 3 Ions*, *Cyclic Nucleotides*, *Cholinergy* (Ed.: J. C. Stocklet), Pergamon, New York, **1978**, pp. 123 – 132 (Proc. 7th Int. Congr. Pharmacol., Paris, France, July, **1978**).

[22] "Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine": S. Katsuki, W. P. Arnold, C. K. Mittal, F. Murad, *J. Cyclic Nucleotide Res.* **1977**, *3*, 23 – 35.

[23] "Stimulation of formation and accumulation of cyclic GMP by smooth muscle relaxing agents": S. Katsuki, W. P. Arnold, C. K. Mittal, F. Murad, *Proc. of the 2nd Japanese Cyclic Nucleotide Conference* (Osaka) **1977**, pp. 44 – 50.

[24] "Regulation of cyclic 3′,5′-adnosine monophosphate and cyclic 3′,5′-guanosine monophosphate levels and contractility in bovine tracheal smooth muscle": S. Katsuki, F. Murad, *Mol. Pharmacol.* **1977**, *13*, 330 – 41.

[25] "Effect of sodium nitroprusside, nitroglycerin and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues": S. Katsuki, W. P. Arnold, F. Murad, *J. Cyclic Nucleotide Res.* **1977**, *3*, 239 – 247.

[26] "Effects of guanosine 3′,5′-monophosphate on glycerol production and accumulation of adenosine 3′,5′-monophosphate during incubation of fat cells": F. Murad, V. Manganiello, M. Vaughan, *J. Biol. Chem*. **1970**, *245*, 3352 – 3360.

[27] "Effects of lipolytic and antilipolytic agents on cyclic 3′, 5′-adenosine monophosphate in fat cells": V. Manganiello, F. Murad, M. Vaughan, *J. Biol. Chem*. **1971**, *246*, 2195 – 2102.

[28] "Adenyl cyclase activity in particles from fat cells": M. Vaughan, F. Murad, *Biochemistry* **1969**, *8*, 3092 – 3099.

[29] "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle to acetylcholine": R. Furchgott, J. Zarwodski, *Nature* **1980**, *288*, 373 – 376.

[30] "Nitric oxide activates guanylate cyclase and increases guanosine 3′,5′-monophosphate levels in various tissue preparations": W. P. Arnold, C. K. Mittal, S. Katsuki, F. Murad, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3203 – 3207.

[31] "Purification of soluble guanylate cyclase from rat liver": J. M. Braughler, C. K. Mittal, F. Murad, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 219 – 222.

[32] "Agonist-induced endothelial-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP": R. M. Rapoport, F. Murad, *Circ. Res.* **1983**, *52*, 352 – 57.

[33] "Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: Role for cyclic GMP": R. M. Rapoport, F. Murad, *J. Cyclic Nucleotide Protein Phosphorylation Res.* **1983**, *9*, 281 – 296.

[34] "Endothelium-dependent vasodilator- and nitrovasodilator-induced relaxation may be mediated through cyclic GMP formation and cyclic GMP-dependent protein phosphorylation": R. M. Rapoport, M. B. Draznin, F. Murad, *Trans. Assoc. Am. Physicians* **1983**, *96*, 19 – 30.

[35] "Endothelium dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation": R. M. Rapoport, M. B. Draznin, F. Murad, *Nature* **1983**, *306*, 274 – 276.

[36] "Endothelium-dependent and nitrovasodilator-induced activation of cyclic GMP-dependent protein kinase in rat aorta": R. R. Fiscus, R. M. Rapoport, F. Murad, *J. Cyclic Nucleotide Protein Phosphorylation Res.* **1983**, *9*, 415 – 425.

[37] "Effect of cyanide on nitrovasodilator-induced relaxation, cyclic GMP acumulation and guanylate cyclase activation in rat aorta": R. M. Rapoport, F. Murad, *Eur. J. Pharmacol*. **1984**, *104*, 61 – 70.

[38] "Sodium nitroprusside-induced protein phosphorylation in intact rat aorta is mimicked by 8-bromo-cyclic GMP": R. M. Rapoport, M. Draznin, F. Murad, *Proc. Natl. Acad. Sci. USA* **1982**, *79*, 6470 – 6474.

[39] "Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells": M. Hirata, K. Kohse, C. H. Chang, T. Ikebe, F. Murad, *J. Biol. Chem*. **1990**, *265*, 1268 – 1273.

[40] "Cyclic guanosine monophosphate as a mediator of vasodilation": F. Murad, *J. Clin. Invest*. **1986**, *78*, 1 – 5.

[41] "Atrial natriuretic factor elicits an endothelium independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle": R. M. Winquist, E. P. Faison, S. A. Waldman, K. Schwartz, F. Murad, R. M. Rapoport, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 7661 – 7664.

[42] "Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues": S. A. Waldman, R. M. Rapoport, F. Murad, *J. Biol. Chem*. **1984**, *259*, 14332 – 14334.

[43] "Atrial natriuretic peptide receptors and the guanylate cyclase-cyclic GMP system": D. C. Leitman, C. R. Molina, S. A. Waldman, F. Murad, *UCLA Symp. Mol. Cell. Biol*. **1988**, *81*, 39 – 56 (Proc. of the UCLA Symposium on Atrial Natriuretic Factor in Biological and Molecular Aspects of Atrial Factors).

[44] "Cyclic GMP synthesis and function": S. A. Waldman, F. Murad, *Pharm. Rev*. **1987**, *39*, 163 – 96.

[45] "Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung": T. Kuno, J. W. Andresen, Y. Kamisaki, S. A. Waldman, L. Y. Chang, S. Saheki, D. C. Leitman, M. Nakane, F. Murad, *J. Biol. Chem*. **1986**, *261*, 5817 – 5823.

[46] "Guanyl cyclase linked receptors": D. L. Garbers, *Pharmacol. Ther*. **1991**, *50*, 337 – 345.

[47] "L-arginine identified as an endogenous activator for soluble guanylate cyclase from neuroblastoma cells": T. Degucci, M. Yoshiako, *J. Biol. Chem*. **1982**, *257*, 10147.

[48] "Macrophage cytotoxicity. Role for L-arginine deaminase and imino nitrogen activation to nitrate": J. Hibbs, R. Traintor, Z. Vanin, *Science* **1987**, *235*, 473.

[49] "The role of cyclic GMP in the mechanism of action of nitrovasodilators, endothelium-dependent agents and atrial natriuretic peptide": F. Murad, *Biochem. Soc. Trans.* **1988**, *16*, 490 – 492.

[50] "Effects of nitrovasodilators, endothelium-dependent vasodilators and atrial peptides on cGMP": F. Murad, D. Leitman, S. Waldman, C. H. Chang, M. Hirata, K. Kohse, *Cold Spring Harbor Symp. Quant. Biol.* **1988**, *53*, 1005 – 1009.

[51] "Modulation of the guanylate cyclase-cGMP system by vasodilators and the role of free radicals as second messengers": F. Murad in *Vascular Endothelium* (Eds.: J. D. Catravas, C. N. Gillis, U. S. Ryan), Plenum, New York, **1989**, pp. 157 – 164 (Proc. of the NATO Advanced Studies Institute on Vascular Endothelium: Receptors and Transduction Mechanisms. Porto Carros, Greece, June, **1988**).

[52] "Mechanisms for hormonal regulation of the different isoforms of guanylate cyclase": F. Murad in *Proceedings of the 40th Mosbach Colloquium on Molecular Mechanisms of Hormone Action* (Eds.: Y. Gehring, E. Helmreich, G. Schultz), Springer, Heidelberg, **1989**, pp. 186 – 194.

[53] "Isolation of nitric oxide synthase, a calmodulin-requiring enzyme": D. Bredt, S. Snyder, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 682 – 685.

[54] "Production of an EDRF-like activity in the cytosol of N1E-115 neuroblastoma cells": L. Gorsky, U. Förstermann, K. Ishii, F. Murad, *FASEB J.* **1990**, *4*, 1494 – 1500.

[55] "The cytosol of N1E-115 neuroblastoma cells synthesizes and EDRF-like substance that relaxes rabbit aorta": U. Förstermann, K. Ishii, L. D. Gorsky, F. Murad, *Naunyn-Schmiedberg's Arch. Pharmacol*. **1989**, *340*, 771 – 774.

[56] "Hormone induced biosynthesis of endothelium-derived relaxing factor/nitric oxide-like material in N1E-115 neuroblastoma cells requires calcium and calmodulin": U. Förstermann, L. Gorsky, J. Pollock, K. Ishii, H. H. H. W. Schmidt, M. Heller, F. Murad, *Mol. Pharmacol.* **1990**, *38*, 7 – 13.

[57] "Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase": H. H. H. W. Schmidt, J. Pollock, M. Nakane, L. Gorsky, U. Förstermann, M. Heller, F. Murad, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 365 – 369.
[58] "Purification and characterization of the cytokine-induced macrophage nitric oxide synthase": D. Stuehr, H. Cho, N. Kwon, M. Weise, C. Nathans, *Proc. Natl. Acad. Sci. USA*. **1991**, *88*, 7773 – 7777.
[59] "Enzymes synthesizing guanylyl cyclase activating factor (GAF) in endothelial cells, neuroblastoma cells and rat brain": U. Förstermann, H. H. H. W. Schmidt, J. S. Pollock, M. Heller, F. Murad, *J. Cardiovasc. Pharmacol*. **1991**, *17* (Supplement 3), 557 – 564.
[60] "Subcellular localization and regulation of the enzymes responsible for EDRF synthesis in endothelial cells and N1E-155 neuroblastoma cells": U. Förstermann, L. Gorsky, J. S. Pollock, H. H. H. W. Schmidt, K. Ishii, M. Heller, F. Murad, *Eur. J. Pharmacol*. **1990**, *183*, 1625 – 1626.
[61] "Purification and characterization of particulate EDRF synthase from cultured and native bovine aortic endothelial cells": J. S. Pollock, U. Förstermann, J. A. Mitchell, T. D. Warner, H. H. H. W. Schmidt, M. Nakane, F. Murad, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 10480 – 10484.
[62] "Isoforms of EDRF/NO synthase: Characterization and purification from different cell types": U. Förstermann, H. H. H. W. Schmidt, J. S. Pollock, H. Sheng, J. A. Mitchell, T. D. Warner, M. Nakane, F. Murad, *Biochem. Pharmacol*. **1991**, *42*, 1849 – 1857.
[63] "Phosphorylation by calcium/calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase": M. Nakane, J. A. Mitchell, U. Förstermann, F. Murad, *Biochem. Biophys. Res. Commun*. **1991**, *180*, 1396 – 1402.
[64] "Endothelial nitric oxide synthase is myristylated": J. Pollock, V. Klinghofer,U. Förstermann, F. Murad, *FEBS Lett*. **1992**, *309*, 402 – 404.
[65] "Agonist-modulated palmitoylation of endothelial nitric oxide synthase": L. J. Robinson, L. Busconi, T. Michel, *J. Biol. Chem*. **1995**, *270*, 995 – 998.
[66] "Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae": P. W. Shaul, E. J. Smart, L. J. Robinson, Z. German, I. S. Yuhanna, Y. Ying, R. Anderson, T. Michel, *J. Biol. Chem*. **1996**, *271*, 6518 – 6522.
[67] "Two closely linked but separate promoters for human neuronal nitric oxide synthase gene transcription": J. Xie, P. Roddy, T. Rife, F. Murad, A. Young, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 1242 – 1246.
[68] F. Murad, J. Lewicki, unpublished results.