Mini Review

Molecular Mechanisms of Nitric Oxide Regulation

Potential Relevance to Cardiovascular Disease

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Nitric oxide (NO) is a recently appreciated regulator molecule for leukocytes, endothelial cells of blood vessels, and neurons in the brain and peripheral nervous system. NO is handled in the body quite differently from other autacoids and neurotransmitters such as the biogenic amines. Whereas the latter are generally stored in vesicles, released by exocytosis, and act at specific receptor proteins on adjacent cells, NO is formed on demand by the action of NO synthase (NOS) on arginine and diffuses to adjacent target cells, where its best characterized "receptor" is the iron at the active site of guanylyl cyclase. This type of intercellular signaling is similar to that used by autacoid messengers, such as prostaglandins.

Biologic NO formation was discovered as a physiological regulator molecule independently in blood vessels, macrophages, and, subsequently, neurons. Classic studies of Furchgott and Zawadzki[1] addressed the obligatory role of endothelium in the vascular relaxation produced by acetylcholine. In vessel segments precontracted with norepinephrine, acetylcholine elicited relaxation only if the endothelium was present, suggesting endothelial release of a substance that diffuses to the smooth muscle. This "endothelium-derived relaxing factor" (EDRF), which in some physiological systems has a half-life of only 3 to 5 seconds, was eventually determined to be NO[2,3] and has subsequently been shown to be the active vasodilating metabolite of nitroglycerin and other organic nitrates.[4] More recently, NO has been shown to circulate as an *S*-nitrosoadduct of serum albumin, which may serve to increase its half-life in blood.[5]

In macrophages, NO may account for tumoricidal and bactericidal actions. These effects are blocked by deletion of the NO precursor arginine from the medium or by adding derivatives of arginine, such as N^G-monomethyl-L-arginine (L-NMMA), which compete with arginine for NOS and thus inhibit NO formation. In addition to macrophages, neutrophils possess active NOS. Whether NO formed from inducible NOS fully accounts for the tumoricidal and bactericidal actions of neutrophils and macrophages is not clear. Activated neutrophils and macrophages produce oxygen-free radicals, which can combine with NO to form peroxynitrite, which decomposes to hydroxide free radical and NO^2 free radical, substances much more toxic than NO itself.[6,7] These decomposition products may be the cytotoxic effectors, but since NO generation may be rate limiting, NOS inhibitors prevent their formation.

In the brain the demonstration that NO can be formed was followed by studies showing that it accounts for many of the actions of the major excitatory neurotransmitter glutamate.[8,9] Moreover, immunohistochemical localization of NOS shows it to be exclusively localized to discrete neuronal populations throughout the brain.[10] In the peripheral nervous system NOS occurs in many groups of autonomic fibers. In the gastrointestinal system, NO formed by NOS fulfills all of the requirements of the neurotransmitter responsible for the regulation of nonadrenergic, noncholinergic (NANC) responses such as relaxation of the stomach,[11] ileocolonic junction,[12] and internal anal sphincter.[13] In the penis NOS is localized to the parasympathetic neurons that mediate erection. Treatment of rats with NOS inhibitors, such as nitroarginine or L-NMMA, completely blocks erection induced by stimulation of the pelvic nerves, establishing that NO is the physiological neurotransmitter that mediates erection.[14]

In some large blood vessels of the brain, eye, and penis, NOS occurs in autonomic nerve fibers of the adventitial layer.[10,14] For cerebral blood vessels and those of the eye, these fibers, which derive from cells in the sphenopalatine ganglia, likely regulate blood vessel reactivity.[15]

Nitric Oxide Synthase

Quantitative studies of NO levels have been difficult because of its lability, although the recent development of a sensitive NO microsensor may markedly facilitate investigations.[16] Intracellular recordings from a microsensor inserted into a single vascular myocyte and one on the surface of adjacent endothelial cells demonstrate NO production by endothelial cells in response to bradykinin, with subsequent elevation of NO levels in underlying smooth muscle.

Many molecular advances in the understanding of the NO system have come from studies of NOS. There appear to be at least three distinct forms of NOS. Macrophages have negligible NOS activity under basal

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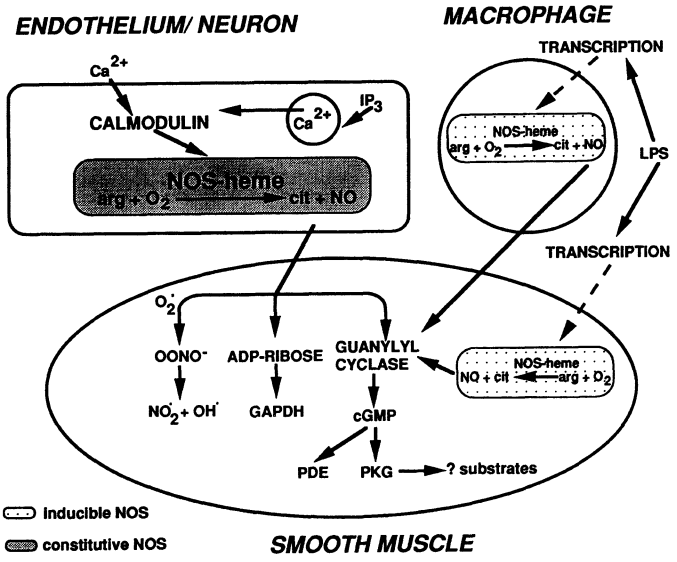

FIG 1. *Molecular mechanisms of nitric oxide (NO) synthesis and actions. IP_3, inositol 1,4,5-trisphosphate; NOS, NO synthase; arg, L-arginine; cit, L-citrulline; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase.*

conditions, but after stimulation with lipopolysaccharide and/or interferon gamma, massive increases in NOS activity occur within 2 to 4 hours.[17-20] The macrophage enzyme has thus been referred to as "inducible NOS" (iNOS) in contradistinction to the enzyme in neuronal tissues (nNOS) or endothelium (eNOS), which appear to be expressed constitutively. Many cell types throughout the body, including hepatocytes, neurons, neutrophils, endothelial cells, vascular smooth muscle cells, and cardiac myocytes, appear capable of iNOS expression.[21-23] In understanding the role of NOS in vascular function and pathology, discrimination between the involvements of the various isoforms of NOS may be important.

How is NO formation regulated in blood vessels and neurons if NOS is not inducible? In these systems NOS is activated by Ca^{2+}, which binds to calmodulin, forming a complex that is a crucial cofactor for enzyme activity.[24,25] In vascular endothelium this Ca^{2+} may be made available through stimulation by agents, such as acetylcholine and bradykinin, that generate inositol 1,4,5-trisphosphate (IP_3) production via the phosphoinositide second-messenger system. IP_3 elicits Ca^{2+} release from intracellular stores by binding to IP_3 receptors on the endoplasmic reticulum. Additionally, a portion of mobilized Ca^{2+} is thought to arise extracellularly. Alternatively, agonist-independent NO release may also contribute to vascular tone. Both shear stress[26] and deformation of vascular endothelium,[27] which accompany pulsatile flow through blood vessels, stimulate NO release through poorly defined mechanisms. After its production, NO relaxes blood vessels by binding to iron in the heme at the active site of guanylate cyclase, thereby activating the enzyme to generate cGMP. cGMP may elicit muscle relaxation through influences on a Na^+-Ca^{2+} exchange, by stimulating the phosphorylation of poorly defined substrates by cGMP-dependent protein kinase, via direct action at a cGMP-gated channel, or as a consequence of cGMP-mediated activation or inhibition of phosphodiesterases[28] (Fig 1). In the brain, the excitatory neurotransmitter glutamate acts at the *N*-methyl-D-aspartate subtype of receptor to open Ca^{2+} channels and cause subsequent influx of Ca^{2+}-stimulating NOS activity and elevating cGMP levels.[29,30]

NO may also act independent of cGMP. NO enhances ADP ribosylation of platelet proteins.[31] The target of the NO-stimulated ADP ribosylation is glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).[32,33] GAPDH is auto–ADP-ribosylated, a process that is stimulated by NO. Since ADP ribosylation involves the specific cysteine associated with NAD-dependent catalysis, ADP ribosylation inhibits GAPDH activity. The decreased glycolysis associated with NO enhanced GAPDH-ADP ribosylation could well mediate a number of actions of NO, such as myocardial stunning and hibernation, reperfusion injury, and neurotoxicity. The physiological relevance of NO-mediated ADP ribosylation awaits further investigation. Effects of NO on mitochondrial iron-sulfur enzymes may also mediate NO actions.[19]

Many insights into NOS function have come from studies of nNOS, which was the first form of the enzyme to be purified[24] and molecularly cloned.[34] More recently both endothelial[35-37] and inducible macrophage forms have also been cloned.[38-40] Whereas many oxidative enzymes use a single electron donor, the oxidation of arginine to NO by NOS involves multiple oxidative cofactors with associated binding sites. The cloned enzyme displays binding sites for NADPH, flavin adenine dinucleotide, and flavin mononucleotide. Quite recently we[41] and others[42] have demonstrated that nNOS and iNOS possess tightly bound heme (Fig. 2). After treatment with carbon monoxide, NOS absorbs light at 450 nm, indicating properties of a cytochrome P-450 enzyme. NOS activity is also enhanced by tetrahydrobiopterin.[43,44] The sequential transfer of electrons between the various cofactors facilitates catalytic function, culminating in heme directly donating the electrons that oxidize the guanidine moiety of arginine.

All isoforms of NOS cloned thus far possess a recognition site for calmodulin (Fig 2). How does this accord with the apparent absence of Ca^{2+} dependence for iNOS? Calmodulin is very tightly bound to iNOS even in the absence of added Ca^{2+} (C. Nathan, personal communication). Thus, exogenous Ca^{2+} is not essential

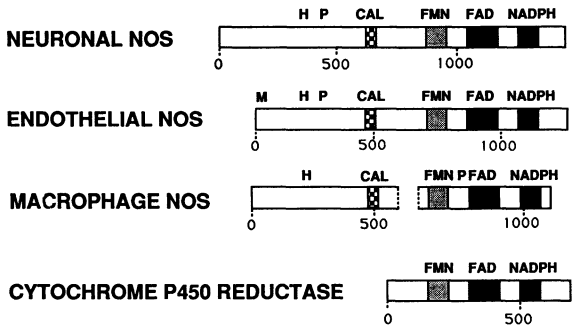

FIG 2. *Schematic alignment of neuronal, endothelial, macrophage nitric oxide synthase (NOS) and cytochrome P-450 reductase. Consensus binding sites include the following: CAL, calmodulin; H, heme; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate. Potential sites for N-terminal myristoylation (M) and phosphorylation by cAMP-dependent protein kinase (P) are noted. Please note that an additional consensus cite for cAMP-dependent protein kinase exists within the NADPH binding domain of the macrophage isoform.*

for calmodulin to associate with iNOS, and extremely rigorous conditions are required to dissociate calmodulin from iNOS.

The three forms of NOS all have consensus sites for cAMP-dependent phosphorylation. We have directly demonstrated phosphorylation of nNOS by cAMP-dependent protein kinase, protein kinase C, and Ca^{2+}/calmodulin protein kinase.[45] Protein kinase C phosphorylation substantially diminishes NOS catalytic activity, whereas the effects of other types of phosphorylation are less apparent.[45] Unique among NOS isoforms, eNOS possesses a site for myristoylation that conceivably could account for the association of eNOS with membranes, whereas nNOS and iNOS are largely cytoplasmic. Although unproven, association of eNOS with the cell membrane may direct delivery of NO both to underlying smooth muscle for efficient vasoregulation and to the blood-endothelial interface, where NO inhibits platelet aggregation.

A striking feature of NOS is its ability to produce superoxide as well as NO.[46] Superoxide is generated in the absence of arginine, whereas arginine addition reduces superoxide formation concomitant with enhanced production of NO. Because endothelial superoxide has been implicated in myocardial and vascular damage following reperfusion,[47] NOS may participate in such effects. However, it is unclear whether the low arginine levels associated with in vitro superoxide generation by NOS occur in intact cells in the microenvironment of NOS protein. It is notable that endothelium-dependent coronary dilation is impaired after ischemia-reperfusion[48-50] and that this impairment may be ameliorated by administration of L-arginine[51] or pharmacological donors of NO at doses not associated with vasodilation.[52] Thus, inactivation of superoxide radical by NO may be considered cardioprotective.[51] Alternatively, because combinations of NO and superoxide generate toxic free radicals, it is also conceivable that these molecules may be cytotoxic effectors associated with reperfusion injury.

The three forms of NOS display about 50% amino acid sequence identity. iNOS and eNOS, each about 130 kD, are shorter than nNOS, which is 150 kD. The three NOS isozymes possess substantial sequence homology with only a single other mammalian enzyme, cytochrome P-450 reductase (CPR) (Fig 2). CPR donates electrons for the cytochrome P-450 drug-metabolizing enzymes. NOS can be thought of as resembling a fusion of CPR and a cytochrome P-450–like enzyme, which may reflect links in evolution that account for the cytochrome P-450–like properties of NOS. Interestingly, CPR also donates electrons for heme oxygenase, which converts heme to biliverdin and carbon monoxide. With the exception of the liver, the relative concentrations of CPR in various organs parallel those of heme oxygenase, suggesting that regulation of heme oxygenase is a major physiological function for CPR. In situ hybridization reveals that mRNA for the noninducible form of heme oxygenase is highly concentrated in discrete populations of neurons in the brain as well as in vascular smooth muscle throughout the body.[53] Like NO, carbon monoxide stimulates cGMP formation and may thereby modulate vascular tone by relaxing smooth muscle of blood vessels.[54] In the brain, mRNA for the noninducible form of heme oxygenase displays localizations closely resembling guanylyl cyclase and implying a role for carbon monoxide in cGMP regulation.

NO in Cardiovascular Disease

Numerous studies indicate that NO is the major physiological regulator of basal blood vessel tone. These include the vasoconstrictive action of NOS inhibitors, which is accompanied by a substantial increase in blood pressure.[55] In comparison, inhibitors of angiotensin converting enzyme and other endogenous mediators of vasorelaxation have minimal effects on basal blood pressure. The vasoconstrictive action of hemoglobin, which binds NO and thus prevents its access to target tissues, appears to predominate in resistance vessels in situ, where basal production of NO has been suggested to coordinate the aggregate hemodynamic properties of vascular networks through the maintenance of a fourth-power relation between diameter and flow.[56] As a result, basal NO release may act to maintain similar flow distributions at variable flow rates as well as limit the work of perfusion.[56] Administration of NOS inhibitors in vivo has been shown to result in constriction and decreased conductance of various vascular beds in the rat.[57]

The participation of NO in the maintenance of vascular tone in various tissues has been shown to result from its release from NANC nerves,[58-60] via cholinergic stimulation[61] and in response to shear stress as discussed earlier. Furthermore, NO decreases central sympathetic outflow[62] and mediates L-glutamate–elicited decreases in blood pressure and heart rate through baroreceptor-like reflexes in the nucleus tractus solitarius.[63] NOS inhibitors administered at doses that do not affect blood pressure diminish renal excretion of sodium and water, suggesting additional mechanisms through which NO affects blood pressure.[64]

Clinical studies suggesting abnormalities in the NO system in cardiovascular disease preceded the discovery that NO is responsible for EDRF activity. Atherosclerotic coronary arteries display diminished vasodilation in

response to acetylcholine compared with normal coronary arteries, although the response to nitroglycerine is the same for both.[65] A similar hyporesponsiveness to acetylcholine occurs in blood vessels of hypertensive patients.[66] It is not clear whether these abnormal responses are primary or secondary to the disease. The fact that diminished responsiveness to acetylcholine occurs in coronary arteries with only minimal atherosclerosis suggests a primary role. On the other hand, studies in hypertensive animals with impaired endothelium-related vasodilator responses indicate that normalization can occur after antihypertensive pharmacotherapy.[67,68]

Atherosclerosis and hypertension are well-recognized pathophysiological contributors to the progression of coronary artery disease. Diminished NO production may accelerate coronary artery disease by promoting interactions between platelets and the vessel wall through loss of NO-mediated platelet inhibition.[69] In diseased vessels, products of activated platelets such as thromboxane, serotonin, adenine nucleotides, and platelet-derived growth factor may lead to vasoconstriction and the proliferation of vascular smooth muscle, processes that tend to accelerate the progression of coronary artery disease.[70] In addition to limiting platelet-endothelial interactions, NO may play an important role in maintaining the normal mitogenic state of vascular smooth muscle. By stimulating the production of cGMP, NO may inhibit the proliferation of vascular smooth muscle.[71]

NOS activity may also play a role in cardiovascular consequences of hypoxia. Since basal NO production contributes to vascular tone, the acute arterial pressor response stimulated by hypoxia is augmented by inhibitors of NOS in the pulmonary,[72] coronary,[73] and systemic vasculature.[74] In pulmonary vessels evidence suggests that NO activity is increased to modulate the pulmonary pressor response. Chronic hypoxia is associated with diminished endothelium-dependent relaxation suggestive of impaired NO synthesis or release.[75] Evidence both in vitro demonstrating decreased NOS activity[76] and in vivo demonstrating reduced exhaled NO production during hypoxia[77] are suggestive of an important relation between available oxygen and NOS activity. Precise quantification of the amount of hypoxia required to affect NO synthesis in intact cells and tissues awaits further investigation.

To determine whether abnormal NOS genes play a role in the etiology of atherosclerosis, hypertension, and other diseases, the structure of NOS genes and their expression in blood vessels and other tissues of patients is being studied. Advances in molecular cloning may also permit therapeutic approaches such as the direct insertion of cDNA for eNOS into the walls of coronary and peripheral resistance vessels.

Whereas diminished NO may be relevant to abnormalities in hypertension, coronary artery disease, and hypoxia, excessive NO levels may mediate other cardiovascular pathologies. Septic shock is associated with release of numerous cytokines, which can activate iNOS in cells such as macrophages and vascular smooth myocytes. Once induced, NO production may mediate the hypotension associated with septic shock. Direct evidence of this comes from animal studies in which the NOS inhibitor L-NMMA reversed the hypotension caused by bacterial lipopolysaccharide[78] and tumor necrosis factor[79] and from the limited the clinical experience in patients with septic shock administered a NOS inhibitor[80] or methylene blue.[81]

Cytokines exert negative inotropic effects on the heart that appear to involve the synthesis of NO, since they can be blocked by NOS inhibitors.[82] These effects may account for the depression of myocardial contractility noted in cardiomyopathies associated with inflammation (viral, AIDS-related, drug-induced, or idiopathic) as well as stunned myocardium after reperfusion of ischemic cardiac tissue. Hypotensive effects of cytokines have necessitated a limit on the dosages used for oncological therapy.[83] The use of NOS inhibitors to prevent such hypotension may facilitate therapeutic applications of cytokines.

Finally, it is conceivable that endogenous NOS inhibitors play a role in cardiovascular regulation, at least in certain disease states. N^G,N^G-dimethyl-arginine (ADMA) is formed by the methylation of arginine in nonhistone nuclear proteins. Circulating levels in humans of ADMA are about 1 μM, whereas at 10 μM ADMA substantially reduces NOS activity. In patients with chronic renal failure, plasma ADMA accumulates to levels of about 10 μM and thus may be a factor in the hypertension associated with chronic renal disease.[84]

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