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THE REGULATION AND PHARMACOLOGY OF ENDOTHELIAL NITRIC OXIDE SYNTHASE

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■ **Abstract** Nitric oxide (NO) is a small, diffusible, lipophilic free radical gas that mediates significant and diverse signaling functions in nearly every organ system in the body. The endothelial isoform of nitric oxide synthase (eNOS) is a key source of NO found in the cardiovascular system. This review summarizes the pharmacology of NO and the cellular regulation of endothelial NOS (eNOS). The molecular intricacies of the chemistry of NO and the enzymology of NOSs are discussed, followed by a review of the biological activities of NO. This information is then used to develop a more global picture of the pharmacological control of NO synthesis by NOSs in both physiologic conditions and pathophysiologic states.

NITRIC OXIDE

Nitric oxide (NO) is structurally one of the simplest biological molecules, yet NO participates in some of the most intricate and versatile signaling behaviors known. NO consists merely of a single oxygen atom bonded to a nitrogen atom through a chemical bond that exhibits partial double bond and partial triple bond character, resulting from the unpaired electron occupying the $2p\pi^*$ antibonding molecular orbital. The free radical character of NO confers specific chemical reactivity properties and determines the interaction of NO with numerous in vivo targets. As a highly lipophilic and diffusible gas, NO can readily disperse from its source and traverse multiple cell membranes en route to its final target. Unlike many other signaling molecules, the targets of NO often are at some distance from the sites of NO synthesis. The molecular receptors for NO include a variety of intracellular and extracellular proteins, and NO itself may serve as a second

messenger as a direct result of its diffusibility and lipophilicity. NO plays key roles in mammalian biology that reflect these special chemical properties.

NITRIC OXIDE SYNTHASES

A family of nitric oxide synthase (NOS) enzymes catalyzes the stoichiometric five-electron oxidation of the terminal guanidino group of L-arginine to produce NO and L-citrulline. Three mammalian NOS archetypal isoforms are distinguished: nNOS (NOS I), or neuronal NOS; iNOS (NOS II), an inducible NOS isoform expressed in a variety of activated tissues; and eNOS (NOS III), or endothelial NOS. The three mammalian NOS isoforms are the products of distinct genes but share approximately 50%–60% sequence identity. Although the isoforms share similar overall enzymatic and chemical properties, distinctive catalytic and regulatory properties are characteristic of the different isoforms.

The NOSs appear to be bifunctional enzymes: The N terminus exhibits homology principally to the other NOS isoforms and has limited similarity to cytochrome P450 monooxygenase enzymes; the C-terminal domain has significant sequence homology to various cytochrome P450 reductases. The NOS N terminus binds tetrahydrobiopterin and heme; L-arginine binds in the enzyme active site near heme, while molecular oxygen is coordinated directly to the ferrous heme iron. The binding sites for tetrahydrobiopterin and heme are localized along the interface of the monooxygenase domains of the dimeric, active form of NOS. Binding of heme and tetrahydrobiopterin, especially for iNOS, may be crucial in promoting dimerization and NOS catalytic activity. The C-terminal domain binds NADPH, FAD, and FMN cofactors (1). The N- and C-terminal domains are linked by a short sequence that binds calmodulin, an allosteric effector that is essential for full NOS activity.

After limited proteolysis, the isolated monooxygenase or reductase domains of NOS can function independently. The intact NOS functions as a mixed function monooxygenase/reductase enzyme and has the capability to synthesize a number of molecules besides NO, most notably superoxide (2). In all NOS catalysis reactions, electrons are shuttled within the reductase domain sequentially from NADPH to FAD and finally to FMN. Calmodulin is believed to function in facilitating the flow of electrons from the reductase domain to the monooxygenase domain as well as from FAD to FMN (3). Because electrons appear to flow from the reductase domain of one NOS monomer to the oxygenase domain of another NOS monomer, enzyme dimerization is required for full enzymatic activity (4).

The actual synthesis of NO utilizes this basic schema but is believed to proceed via two distinct monooxygenase reaction steps. In the first step, one molecule of NADPH delivers electrons to molecular oxygen, which then reacts with bound L-arginine to release one molecule of water and form L-omega-N-hydroxyarginine (LHA) (5). LHA is the substrate for the second step of NOS catalysis, although it is unclear whether LHA remains bound in the active site or interchanges with

solvent between the two steps. In the second step, one electron (or 0.5 equivalents of NADPH) is required to form a second equivalent of reduced molecular oxygen (6), which attacks the guanidino carbon of LHA to form a tetrahedral intermediate. This tetrahedral intermediate collapses to form the carbonyl group of citrulline with concomitant expulsion of NO. The overall stoichiometry of NO catalysis thus consumes 1 mole of L-arginine, 1.5 moles of NADPH, and 2 moles of molecular oxygen to generate 1 mole of NO, 1 mole of L-citrulline, and 2 moles of water.

The efficiency of this reaction requires that NOS effectively coordinate the binding of multiple substrates and cofactors. For example, if the enzymatic reduction of oxygen becomes uncoupled from other catalytic function in the monooxygenase domain, the product of NOS would then generally be superoxide. nNOS, eNOS, and iNOS can produce superoxide when levels of tetrahydrobiopterin or arginine are insufficient (2, 7, 8). Release of superoxide by NOS may result in the rapid reaction of superoxide with NO to yield peroxynitrite ($ONOO^-$). Nitroxyl ion, or NO^-, is another postulated NOS product based on other possible NOS reaction stoichiometries. Like NO, NO^- could lead to formation of peroxynitrite, but through its combination with molecular oxygen instead of superoxide. Finally, it is entirely plausible that in vivo NOS synthesizes a mixture of these reactive nitrogen and oxygen species (RNS, ROS) as a consequence of cofactor binding and cellular conditions, including oxidative stress, which alter the redox state of tetrahydrobiopterin (9). Together, such factors likely determine NOS's relative output of RNS/ROS and thus the physiologic and pathophysiologic consequences of NOS activation.

INTRACELLULAR ASPECTS OF THE REGULATION OF NOS AND NO

Because NO is a relatively labile free radical with an in vivo half-life of less than 5 s, NO cannot be stored in free form and must generally be synthesized on demand or from more stable adducts that have specific biological effects. Consistent with temporally and spatially precise NO-dependent signaling, the activity of NOS enzymes is subject to carefully controlled activation and inactivation developed through multiple interconnected mechanisms of regulation.

Expression and Transcription of NOS

The most fundamental level of NOS regulation is reflected in the tissue-specific expression of the different isoforms. nNOS is widely expressed in neurons of the central and peripheral nervous systems, but is also found in skeletal muscle, the adventitial layer of some blood vessels, pulmonary epithelium, the gastrointestinal system, and the genitourinary system. iNOS was first recognized in activated macrophages, but has since been identified in numerous activated cell types, including monocytes, neutrophils, eosinophils, hepatocytes, vascular smooth muscle,

myocytes, osteoblasts, fibroblasts, epithelium, and endothelium. eNOS is expressed in vascular endothelium as well as blood platelets and cardiomyocytes.

All three NOS isoforms are subject to transcriptional controls. As originally identified, eNOS and nNOS were believed to be only constitutively expressed in their characteristic tissues, whereas expression of iNOS was inducible upon immunoactivation, for example, in response to LPS, interferon γ, TNF-α, and IL-1. However, nNOS is now known to be upregulated in diverse tissue models, including cutaneous wound repair and ischemic preconditioning (10, 11). Similarly, eNOS transcription can be actively modulated in endothelial cells, for example, in response to laminar shear stress, regulated in part by the transcription factor KLF2 (12, 13). The rho/rho kinase pathway is another mediator of eNOS transcription, and it may function in a variety of cardiovascular physiologic and pathophysiologic states (14). Rho represents a family of small GTPases anchored to membranes by geranylgeranyl anchors. Rho regulates rho kinase, which helps to modulate and reorganize the actin cytoskeleton by inhibiting myosin light-chain phosphatase (15). The rho-mediated effects on the actin cytoskeleton have been connected to modulating gene expression of a number of proteins, including eNOS. Thus, all three NOS isoforms exhibit inducible and constitutive patterns of expression in different tissue environments.

Especially with respect to eNOS, regulation at the posttranscriptional level of mRNA stability is being increasingly viewed as an important locus of control. Although eNOS mRNA exhibits notable stability under normal physiologic conditions, numerous disparate influences that destabilize eNOS mRNA are now known (16). For example, the stability of eNOS mRNA is diminished in the presence of oxidized LDL, thrombin, inflammation, and hypoxia (16–18). nNOS may be differentially spliced to result in at least three possibly tissue-specific variants (19, 20).

Subcellular Localization: Protein-Protein Interactions and Acylation of eNOS

nNOS and eNOS are localized to specific subcellular domains. nNOS, unlike iNOS and eNOS, harbors a 250–amino acid PDZ/GLGF motif at its N terminus. The GLGF motif binds dystrophin and, at least in skeletal muscle, targets nNOS to the sarcolemma. In neuronal tissue, nNOS localizes to both the rough endoplasmic reticulum and electron-dense postsynaptic areas (21). eNOS is also associated with membranes; in resting endothelial cells, most eNOS protein is localized to specialized invaginated domains of the cell membrane called caveolae. Caveolae are characterized by the presence of a transmembrane scaffolding protein known as caveolin (caveolin-1 isoform in endothelial cells, caveolin-3 isoform in cardiomyocytes) (22). Relative to the surrounding plasma membrane, caveolae are enriched with cholesterol and sphingolipids, which together decrease the fluidity of these discrete membrane regions (23). The distinct fluid phase properties of caveolae seem to help draw together proteins involved in a variety of signaling pathways,

and thus may function to facilitate protein-protein and protein-membrane interactions necessary for cellular signal transduction (24). eNOS is specifically targeted to caveolae, and this localization appears to be key to modulating its activity by situating eNOS in close physical proximity to other upstream signaling proteins colocalized to caveolae (25). The caveolar targeting of eNOS is entirely dependent on irreversible myristoylation at its N-terminal glycine (26). Myristoylation also appears to initially target eNOS to the cell membrane, where the enzyme is doubly palmitoylated at N-terminal cysteine residues 15 and 26, a modification that further helps anchor eNOS in caveolae (27). However, because these thiopalmitoyl bonds are labile, palmitoylation and the resultant caveolar targeting are reversible and controllable, thus creating an additional level of dynamic control of eNOS activity (28). In endothelial cell caveolae, eNOS strongly and directly interacts with caveolin-1; this protein-protein interaction tonically inhibits eNOS activity in part by occupying the calmodulin binding site (as well as by interfering with other caveolin-associated receptors that stimulate eNOS activity). It was previously believed that the eNOS-caveolin protein-protein interaction was an essential factor in eNOS caveolar localization (29). However, it has been demonstrated that membrane targeting of eNOS, whether by N-terminal myristoylation or CD8 tagging at the eNOS N terminus without myristyolation, and not specficially an eNOS-caveolin protein-protein interaction, is necessary and sufficient for eNOS palmitoylation and caveolar localization (30).

Regulation of NOS Enzymatic Activity by Effects on the NOS Protein: Calcium/Calmodulin and Reversible Palmitoylation

eNOS, nNOS, and iNOS share some fundamental similarities in the archetypal molecular pathways that underlie NOS catalysis. Each of the three isoforms requires all redox cofactors, cosubstrates, and calmodulin for maximal NO synthesis. Of these, calmodulin is a key NOS activator essential for activity that specifically connects control of NOS activity to cellular calcium levels. Even at calcium levels far below ambient concentrations, iNOS fully binds calmodulin and is thus not particularly subject to regulation by intracellular calcium levels (31). eNOS and nNOS, however, contain a 40–50 amino acid loop located within the FMN binding domain that destabilizes calmodulin binding at subphysiologic calcium levels (8). In the absence of bound calmodulin, the transfer of electrons from the reductase domain to the oxygenase domain is impeded and the catalytic activity is blunted. eNOS is further inhibited by the binding of caveolin in a reversible protein-protein interaction that serves to attenuate basal enzyme activity. Thus, eNOS achieves maximal activity only when intracellular calcium reaches a sufficient level to promote calmodulin binding and facilitate the electron flux necessary for catalytic activity. In addition, calmodulin itself is under control of the kinase CK2; because phosphorylation of calmodulin attenuates its stimulatory effect on eNOS, the response to intracellular calcium is inhibited (32). CK2-mediated phosphorylation of calmodulin may thus serve to selectively displace calmodulin from eNOS and

reduce eNOS activity without decreasing free cellular calmodulin levels or affecting other calmodulin-dependent signaling pathways (32, 33).

Regulation of intracellular calcium levels provides the most rapid induction of eNOS activity, and a multitude of diverse pathways mobilize calcium to activate eNOS. In general, calcium is released from cellular storage pools as the downstream effect of phospholipase C (PLC)-mediated cascades. Once activated, PLC cleaves phosphatidylinositol 4,5-triphosphate into diacylglycerol and inositol 1,4,5-triphosphate (IP3), the former leads to the activation of protein kinase C and the latter to the activation of IP3-receptors, which themselves are enriched in caveolae (34). Initial activation of IP3-receptors leads to the increase of intracellular calcium concentration in a complex way, involving the activation of numerous ion channels.

Prolonged agonist stimulation may induce eNOS depalmitoylation, whereupon it may be translocated into the cytosol (35). Additional evidence suggests that calmodulin may promote the cytosolic translocation of eNOS by increasing the suitability of eNOS as a substrate for the enzyme APT-1, which depalmitoylates eNOS (28). Cytosolic translocation may be a mechanism for downregulating the activity of eNOS following prolonged agonist stimulation.

Phosphorylation

In addition to regulatory pathways involving acylation and calcium/calmodulin, the dynamic regulation of eNOS involves numerous pathways of phosphorylation and dephosphorylation. All NOS isoforms are subject to regulation by phosphorylation. eNOS is known to be phosphorylated at multiple sites, including Ser 1177 and Ser 635, which are stimulatory, and Thr 495 and Ser 116, which are inhibitory. (Note that this numbering system makes reference to phosphorylation sites in human eNOS isoform to Ser 1177 and Thr 495, which correspond to Ser 1179 and Thr 497 in bovine eNOS.) The protein kinase Akt (also known as protein kinase B) appears to be a key determinant of eNOS phosphorylation at Ser 1177 and consequent eNOS activation at basal levels and in response to agonists (36). Kinase Akt is under the direct control of phosphoinositide-3-kinase (PI3K) (37). PI3K is itself controlled by a number of eNOS agonists, and these PI3K/Akt pathways are significant in the coordinate regulation of eNOS phosphorylation state and enzymatic activity.

In contrast to phosphorylation at Ser 1177, phosphorylation at Thr 495 appears to attenuate eNOS enzyme activity by preventing calmodulin binding (38). Ser 635 may represent a second stimulatory phosphorylation responsive to basal stimuli, such as shear stress, and agonists downstream of protein kinase A (39). Ser 617 may be another Akt phosphorylation site and may serve to sensitize eNOS to calmodulin binding and modulate phosphorylation at other eNOS sites (36, 40).

S-Nitrosylation

S-Nitrosylation of the eNOS protein has been recently recognized as another level of dynamic receptor-mediated posttranslational control of eNOS activity (40a).

The source of NO for nitrosylation is eNOS, in a process that occurs when eNOS is membrane localized. Quiescent eNOS in endothelial cells is inhibited as a result of tonic S-nitrosylation at two of the cysteine residues, Cys 94 and Cys 99 (Cys 96 and Cys 101 in bovine eNOS), that form the zinc tetrathiolate cluster (40a,b). In response to eNOS agonists, eNOS is rapidly but transiently denitrosated on a time course that parallels the increase in eNOS catalytic activity.

The mechanism by which S-nitrosylation inhibits the activity of eNOS remains unknown. Given S-nitrosylation of zinc cluster cysteine and chemical assay evidence of zinc release upon eNOS S-nitrosylation, a mechanism involving destruction of the zinc cluster with subsequent dissociation of the eNOS homodimer was proposed to account for the decline in activity (40b). More recent studies have suggested that S-nitrosylation does not necessarily cause eNOS monomerization in intact cells (40a); it is possible that S-nitrosylation modifies the interface between the eNOS homodimers and blunts the efficiency of electron transfer between the two subunits. Interestingly, there is evidence that S-nitrosylation of iNOS inhibits the enzyme by destruction of the zinc cluster with formation of inactive monomers (40c).

Further work is needed in order to elucidate the mechanisms of S-nitrosylation-mediated eNOS inhibition; the effects of cellular redox state; the mechanism of denitrosylation and the significance of transnitrosylation reactions in the cytosol; and the consequences of cross-talk between eNOS nitrosylation, acylation, and phosphorylation pathways.

PHARMACOLOGIC PARADIGMS OF NO ACTION

The unique properties of NO define its versatile signaling roles. As a lipophilic gas, it easily traverses cell and organelle membranes to directly affect downstream signaling functions. NO interacts readily with various types of protein bound metal centers and also has a propensity to interact with cysteine thiol groups and other nucleophilic centers, such as tyrosine residues. The known repertoire of ultimate targets and functions of NO has consistently expanded along with increased understanding of its diverse biochemical interactions.

Reactivity with Metal Centers

The interaction of NO with metal centers represents the paradigmatic mechanism of NO. NO is known to react with numerous hemoproteins, the most well characterized of which is guanylate cyclase. Further study demonstrates additional reactivity of NO toward iron-sulfur clusters and zinc and copper centers.

SOLUBLE GUANYLATE CYCLASE The most well-recognized function of NO is the activation of heme containing enzyme soluble guanylate cyclase (sGC), a protein ubiquitously expressed in mammalian cells. sGC is a heterodimeric protein

consisting of 73 kDa α_1 and 70 kDa β_1 subunits, both of which are required for catalytic activity. In general, the N-terminal domain of sGC monomers (especially the β monomer) binds heme, whereas catalytic functions reside at the sGC C-terminal domain. sGC is distinguished from various transmembrane isoforms of guanylate cyclase, which possess a variable N-terminal extracellular receptor domain/transmembrane domain linked to a conserved C-terminal catalytic domain. The C-terminal catalytic domain of membrane-bound guanylate cyclases is similar to that of sGC.

sGC catalyzes the conversion of GTP to the second messenger cGMP at a low basal rate. Exposure of sGC to 10–100 nM NO results in binding to the heme group by displacing the axial histidine ligand, leaving a pentacoordinate iron, and shifting the iron atom out of the plane of its porphyrin ring. This conformational change leads to allosteric activation of dimeric sGC and a 500-fold enhancement of its activity. Only NO is able to bind the sGC heme group, as neither NO^- nor NO^+ activate sGC. In addition, carbon monoxide, while able to bind the sGC heme group, does not break the axial histidine linkage and thus leaves a hexacoordinate iron; upon carbon monoxide binding, sGC activity only rises by a factor of 6, underscoring the requirement for the particular NO-mediated conformational change.

cGMP directly and indirectly modulates numerous targets, including protein kinases such as protein kinase G (PKG), GPCRs, ion channels, PLC, phosphodiesterases (which can modulate either cellular cGMP or cAMP levels), tyrosine kinases, and tyrosine phosphatases (41).

In the vessel wall, eNOS-derived NO diffuses from the endothelium across cell membranes into underlying vascular smooth muscle as well as into the vascular lumen where it affects platelets. The primary downstream target of vascular smooth muscle cGMP is PKG, which itself activates the myosin light-chain phosphatase (MLCP) (42). The cGMP-mediated dephosphorylation of smooth muscle myosin abrogates tonic contraction of the contractile apparatus and results in relaxation and vasodilatation (43). The relaxant properties of cGMP are enhanced by the ability of MLCP to desensitize the smooth muscle contractile elements to calcium (44). Not surprisingly, eNOS knockout animals are hypertensive in both the systemic and pulmonary circulations and lose characteristic responses to eNOS agonists (45). In addition to its effects in vascular smooth muscle, NO-induced cGMP synthesis in platelets reduces intraplatelet calcium and negatively regulates molecules like glycoprotein IIb/IIIa that are essential for platelet aggregation and activation.

INTERACTION OF NO WITH OTHER METAL CENTERS Although its reaction with the heme group of sGC was the first signaling pathway investigated in detail, the interaction of NO with other hemoproteins, nonheme proteins, copper proteins, and zinc proteins are now well known. Besides sGC, NO regulates catalase and cytochrome c oxidase via interaction with heme. In contrast to the common heme ligands molecular oxygen and carbon monoxide, which bind only

ferrous heme, NO appears to bind ferrous and ferric forms of heme iron. NO also possesses the ability to bind and alter the activity of iron-sulfur cluster enzymes, including ferrochelatase, aconitase, complex I, and complex II. Generally, 4Fe-4S clusters are ligated to the protein via cysteinyl residues; one of the solvent accessible iron atoms binds hydroxide. Binding of NO displaces that hydroxide and may stimulate dissociation of the apical iron from the cluster, thereby inactivating the enzyme. The inhibitory interaction of NO with ferrochelatase may be a mechanism of negative feedback on NO levels, as ferrochelatase inhibition limits production of heme, which is necessary for NOS dimerization and catalysis. On the other hand, the regulation of aconitase by NO exhibits not only inhibitory effects on the citric acid cycle but also iron metabolism. NO binding to the aconitase iron-sulfur cluster exposes a RNA binding site that recognizes mRNA of both ferritin and transferrin; this form of aconitase involved in iron homeostasis is known as the iron response element. Binding of the iron response element stabilizes transferrin mRNA and facilitates transcription, whereas binding to ferritin mRNA inhibits its transcription. NO is also known to recognize copper centers, as for example in cytochrome c oxidase (46).

INTERACTIONS OF NO WITH METAL CENTERS IN THE MITOCHONDRIA: EFFECTS ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION The reactivity of NO toward various metallic centers and its capability to inhibit NADH:quinone oxidoreductase (complex I), succinate:quinone oxidoreductase (complex II), and cytochrome c oxidase (complex IV) may be critical physiologic determinants of respiration kinetics. Complex I is characterized by numerous 4Fe-4S and 2Fe-2S clusters; complex II has 2Fe-2S, 3Fe-4S, and 4Fe-4S clusters; and complex IV hosts two heme groups, cyt_a and cyt_{a3}, and two copper clusters, Cu_A and Cu_B. Exposure to submicromolar NO causes potent but reversible inhibition of electron transport, oxygen consumption, and cellular respiration. Thus, under physiologic conditions, the precise balance between NO and oxygen gas levels may coordinately determine flux through cellular respiratory pathways (47). One context in which transient reversible inhibition of mitochondria by NO may regulate physiologic processes is in vascular smooth muscle, where depletion of ATP may result in passive vasodilatation (48). In contrast, iNOS-derived NO, released from macrophages at micromolar quantities, may exert much of its cytotoxic effect through preventing respiration of the target pathogen. This macrophage-induced block on energy production in the pathogen is compounded by the inhibitory effects of NO on aconitase and the citric acid cycle, as well as inhibition of catalase.

MITOCHONDRIAL NOS Evidence for production of NO in the mitochondria via a NOS-like protein (dubbed mtNOS, for mitochondrial NOS) began to emerge in the late 1990s (49–51). mtNOS has been reported to be widely distributed in various mammalian tissues, and appears to be constitutively active and have similar cofactor requirements as eNOS and nNOS. Although some reports identify mtNOS as an nNOS splice variant, others posit a novel NOS isoform distinct from

the three paradigmatic forms (52, 53). Other reports suggest that mtNOS may be myristoylated like eNOS, a property that could serve to anchor mtNOS to the inner mitochondrial membrane (53). mtNOS has been suggested to function as a mitochondrial source of NO that reversibly interacts with metalloproteins involved in mitochondrial respiration, including cytochrome c, to modulate oxygen consumption and cellular energetics (53).

Covalent Interaction with Proteins

NO forms covalent bonds with protein thiol and tyrosine groups. The interaction of NO with thiols is generally readily reversible, an unusual characteristic among covalent bonds and postulated to be important in cell signaling. In contrast, the formation of nitrotyrosine would not be expected to be reversible. As a free radical, NO also is reactive with both low-molecular-weight and protein-bound free radicals.

NITROSOTHIOLS Cysteine thiol groups in proteins serve as important determinants of tertiary structure and native conformation, may function as sites for attachment of other covalently bound factors, and can set the structural geometry and chemical environment of the active site. NO and its oxidized derivatives can covalently modify protein thiols in a specific and reversible manner; this reaction, called S-nitrosylation (termed S-nitrosation by some investigators), yields nitrosothiol species (RSNO), which can induce changes in protein structure and function. Although NO does not readily react with thiols, the actual S-nitrosylation reaction is believed to proceed with oxidized NO derivatives, such as N_2O_3 produced under physiologic aerobic conditions. N_2O_3 is effectively NO^+, which does have affinity for and reactivity toward RSH groups, and is formed from the oxidation of NO to NO_2, followed by the addition of another molecule of NO to the NO_2. Other plausible mechanisms for generating a thiol-reactive NO^+ species include formation of peroxynitrite or metal-NO complexes, such as dinitrosyl iron (53a). Additionally, nitrosothiols can donate their NO functionality to other thiols in a process termed transnitrosylation (54).

Specificity of the S-nitrosylation reaction is recognized because not every cysteinyl protein is nitrosated. Furthermore, not every individual cysteine in a protein becomes nitrosated. For example, in the ryanodine receptor (RyR), only 12 specific cysteines out of 84 are subject to reversible S-nitrosylation. The structural determinants of S-nitrosylation for individual cysteine residues have not yet been definitively elucidated. A nitrosylation consensus sequence has been proposed in which the cysteine substrate for nitrosylation resides within a sequential four-amino-acid motif containing a polar amino acid, an acidic or basic amino acid, cysteine, and an acidic amino acid; presumably, this microenvironment would contribute to particular acid-base chemistry necessary to facilitate transnitrosylation and denitrosylation (55). Others, however, have proposed instead that S-nitrosylation may be more dependent on tertiary structure broadly and how it affects the pK of the

particular thiol group, local three-dimensional hydrogen bonding networks, and local electrostatic properties (54, 56).

Despite an expanding literature describing the roles of nitrosylation, relatively few nitrosoproteins, such as nitrosoalbumin and nitrosohemoglobin, have been isolated from otherwise untreated in vivo sources. Many other nitrosothiols are studied in the context of exogenous NO donors like S-N-acetylpenicillamine. Given such experimental procedures, it is critical to carefully interpret results when extrapolating and applying data to understanding nitrosylation that may actually occur under physiologic conditions (53a). Formation of nitrosothiols is quite facile when proteins are exposed to high concentrations of exogenous NO donors. Experimental conditions that employ high concentrations of either reactant, namely NO "donor" or thiol "acceptor," may lead to spurious findings that do not reflect the intracellular levels of these reactants. Furthermore, the in vivo milieu contains copious quantities of metals and other thiols like glutathione that may prevent protein nitrosylation. Thus, although nitrosylation demonstrated in vitro might suggest the possibility of dynamic regulation of nitrosoproteins in cells and/or biological fluids, additional proof is required before concluding that the protein is nitrosated in vivo and that such nitrosylation is relevant. It is therefore critical to distinguish between the effects of exogenous donors and endogenous NOS as sources of NO. Unfortunately, the lability of nitrosoproteins renders their analysis difficult, so chemically rigorous methods must be devised for their analysis. One such method is the biotin switch assay, which specifically converts nitrosated cysteines, but not cysteines or cystine disulfide bonds, to biotinylated cysteines that are then detectable with immunoblotting (57). Another variant of this assay involves reacting nitrosated cysteines with a radiolabeled pyridyl disulfide, detectable via autoradiography (58).

POSSIBLE EXAMPLES OF S-NITROSYLATION AS A MODULATOR OF PROTEIN ACTIVITY

S-nitrosylation may constitute a key regulatory post-translational modification that modulates enzymatic and protein function. Although nitrosothiols can enhance guanylate cyclase activity, they may also modulate the principal cGMP-independent modes of action of NO. However, the mechanism(s) by which nitrosothiols exert NO-like effects remains under investigation; plausible pathways include nonenzymatic scission of the nitrosothiol bond to release NO (59), transnitrosylation of other proteins, or direct signaling by the nitrosothiol species (60).

Although the roles of S-nitrosylation and nitrosothiols are only beginning to be understood, diverse examples of the possible effects of nitrosylation on the control of protein activity are appreciated, as shown in Table 1. Protein S-nitrosylation may directly affect enzyme activity, perhaps by altering the active sites of substrate and cofactor binding, protein-protein interactions, and/or subcellular localization (53a, 53b).

Proteins that seem to be activated by physiologic S-nitrosylation include ras, the RyR, tissue plasminogen activator, and calcium-dependent potassium channels. In the presence of reactive nitrogen species, ras is directly modified and activated to

TABLE 1 Nitrosylated proteins (40a,b,c; 53a; 57)

Class	Examples
Ion channel proteins	Calcium-dependent potassium channel Ryanodine receptor L-type calcium channels NMDA receptor Na^+/K^+ ATPase
Kinases/phosphatases	Protein kinase C Numerous tyrosine phosphatases
Signal transducers	Ras eNOS
Transcription factors	fos jun c-Myb
Proteins mediating oxidative stress	Glutathione γ-Glutamylcysteinyl synthetase Catalase iNOS
Energy transduction	Creatine kinase Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Glycogen phosphorylase Dihydrolipoamide dehydrogenase Malate dehydrogenase Hydroxymethylglutaryl-CoA synthase
Plasma proteins	Albumin Hemoglobin Factor XIII
Proteolytic enzymes	Papain Cathepsin B Caspsases
Structural	β Tubulin Actin
Enzyme involved in nitrovasodilator tolerance	Aldehyde dehydrogenase
Miscellaneous	Rb Hsp72 Glutamate oxaloacetate transaminase 2Sarcosine dehydrogenase Methione adenosyltransferase

bind GTP even without its normal upstream signals. Activated ras may serve as a sensor and response element for oxidative stress, stimulating further signaling through MAPK and NFκB pathways.

Proteins inactivated by physiologic S-nitrosylation include eNOS, iNOS, GAPDH, caspases, L-type calcium channels, delayed rectifier potassium channels,

and factor XIII. eNOS is tonically inhibited in endothelial cells by S-nitrosylation at the cysteine residues that comprise the zinc tetrathiolate cluster at the homodimer interface (40a,b). Although eNOS is not necessarily dissociated following S-nitrosylation, iNOS appears to be inhibited by its dissociation into monomers following S-nitrosylation (40a,c). Inactivation of the glycolytic enzyme GAPDH at Cys199 reversibly inhibits its catalytic activity. Note then that a macrophage's respiratory burst showering a target pathogen with iNOS-derived NO can jointly inhibit glycolysis, the citric acid cycle, and electron transport and oxidative phosphorylation. The role of GADPH S-nitrosylation is not, however, limited to the microbicidal context, as nitrosylation may protect critical cysteines from irreversible adenylylation and inactivation in normal cells. Caspases are a family of cysteine-aspartate proteases that function as important pro-apoptotic signals by transducing TNFα and interleukin-1 cascades. At relatively low NO levels ($<$50 μM), NO functions in an antiapoptotic manner most likely by inhibiting the active site cysteines in caspases 1, 2, 3, 4, 6, 7, 8, and 9. Thus in the endothelium, shear stress may be an important antiapoptotic factor by maintaining basal eNOS tone and NO levels (63). It has been recently shown using the biotin switch methodology that procaspase 9 is nitrosylated in vivo and this nitrosylation is responsive to NOS inhibitors; S-nitrosylation of procaspase 9 inhibits signaling through downstream proteins and apoptosis (64). However, at levels exceeding 300 μM, NO appears to be proapoptotic, most likely owing to caspase-independent oxidative stress mechanisms such as peroxynitrite damage, DNA damage, and mitochondrial permeability.

NITROSYLATION AND CATION CHANNELS IN ENDOTHELIUM AND MYOCARDIUM

Numerous examples of the control of the tone of vascular smooth muscle and myocardial excitation-contraction coupling through the effect of NO on cation channels have emerged. S-nitrosylation of key thiols in specific cation channels can interfere with the channel's electrostatic and hydrogen bonding network, thus altering ion transductance. Such S-nitrosylation may affect the stability of the channel protein, and those effects may be partially mediated by affecting the status of disulfide bonds in the channel protein or creating disulfide bonds by oxidizing free cysteines.

In the vessel wall, calcium-dependent potassium channels (CDKC) allow potassium efflux and serve to hyperpolarize vascular smooth muscle, resulting in vasodilatation. NO can augment the activity of CDKCs directly via nitrosylation or indirectly via PKG. Evidence for the direct interaction of NO and CDKC was provided by patch clamp experiments of the channels in reconstituted lipid bilayer systems free of cGMP, ATP, or PKG and subjected to pharmacologic sources of NO (65, 66).

The RyR is a sarcoplasmic reticulum channel that allows calcium to reach the sarcoplasm as a means to facilitate muscular contraction. By specific S-nitrosylation of 12 of its 84 cysteine sites, NO directly stimulates the RyR to amplify calcium flow into sarcoplasm. It is possible that this nitrosylation of

this protein occurs in vivo, as nitrosated RyR was extracted from purified canine hearts. The mechanism of the effect of NO on the RyR may be a means for controlling excitation-contraction coupling and modulating the force of muscular contraction. Interestingly, the effects seem specifically connected to nitrosylation, as mere oxidation of the same RyR thiol sites does not augment channel activity (67).

NO affects several other ion channels in endothelium and myocytes, but for many of them the role for direct NO interaction with the channel protein has not yet been firmly established. For example, NO affects L-type calcium channels, but these effects appear to be completely mediated through the cGMP-dependent actions on both PKG-induced inhibition of L-type channels as well as inhibition of cAMP phosphodiesterases.

NITROSOTHIOLS AS STORAGE FORMS OF NO Some nitrosothiols, particularly nitrosoalbumin, may constitute a physiologic storage pool of nitric oxide equivalents, as nitrosothiols generally have a significantly longer half-life than free NO (68). S-nitrosylation of tissue plasminogen activator (S-NO-t-PA) may affect its interaction with fibrin and subsequent catalytic activation of plasmin. As a nitrosothiol, S-NO-t-PA exhibits vasodilatory, antiplatelet, and antiinflammatory effects (antiinflammatory effects result from interference with leukocyte-endothelial interaction by downregulating expression of cell surface glycoproteins such as P selectin). S-NO-t-PA may act as a storage form to deliver NO to areas where the local endothelium may be dysfunctional and incapable of producing sufficient quantities of NO to maintain normal vascular homeostasis (69). In a similar fashion, nitrosoglutathione may exert antiplatelet effects (70), and nitrosohemoglobin, carrying NO as a covalent adduct at β Cys 93 or at a heme moiety, may also serve as a storage and transport form of NO (71).

COVALENT INTERACTION OF NO WITH PROTEIN RADICALS NO and related RNS, such as N_2O_3, are cross-reactive with other nucleophilic foci in proteins, such as protein sheltered radicals or certain aromatic systems. Two examples of NO acting in particular with protein radicals involve cyclooxygenase 2 (prostaglandin H synthase 2) and ribonucleotide reductase. Tyrosyl radicals characterize the active sites and mechanisms of both of these enzymes. Interaction of NO with these tyrosyl radicals results in formation of a 3-nitrotyrosine species. Micromolar concentrations of NO quench the tyrosyl radical of ribonucleotide reductase and thereby impede DNA synthesis. As such, NO-induced inhibition of this enzyme may represent yet another mechanism of NOS-mediated microbicidal activity. The effect of nitrosylation of the cyclooxygenase 2 tyrosyl radical remains unclear, as enhanced and reduced enzymatic activity have both been observed; conflicting results may represent artifacts of the specific cell models and experimental conditions employed, reactivity of NO with the cyclooxygenase heme or thiol groups, or effects of NO on cyclooxygenase gene expression (72, 73).

NITROTYROSINE IN MODELS OF INFLAMMATION 3-Nitrotyrosine results from the attack on the nucleophilic tyrosine phenolic systems in proteins by NO^+-like species, particularly peroxynitrite. Free NO does not have the capability to attack the phenolic rings and form nitrotyrosine. Another possible mechanism of tyrosine nitration involves neutrophil myeloperoxidase-mediated oxidation of nitrite to nitrogen dioxide (74–76).

Unlike nitrosylation of specific cysteinyl residues, it is not known whether nitration of protein tyrosines occurs at specific tyrosine residues. There is some evidence of temporal specificity in nitration that correlates with the time course of inflammatory processes (77). Because 3-nitrotyrosine is a stable covalent adduct, measurement of nitrotyrosine levels has been widely used to assess peroxynitrite levels, especially as a general marker of inflammation and RNS/ROS associated oxidative damage (78). For example, elevated nitrotyrosine levels have been observed in disparate disease states and involving inflammatory processes, including atherosclerosic plaques; viral myocarditis and myocardial inflammation (79, 80); ischemia-reperfusion injury (81, 82); preeclampsia; septic shock; rheumatoid arthritis and even osteoarthritis (83); vasculitides (84); various inflammatory bowel and liver diseases (85); and numerous states of pulmonary inflammation, including cystic fibrosis, asthma, and obliterative bronchiolitis (86). In general, in these inflammatory conditions, induction of iNOS may be the source of NO and the ultimately high concentrations of peroxynitrite required for extensive nitrosylation of tyrosine residues.

Whether nitrotyrosine is merely a result of inflammatory processes or actually facilitates the pathophysiology of inflammation remains to be established, although proteins involved in oxidative stress and apoptosis are in fact nitrated (87). Nitration of tyrosine residues obviously changes the structures of target proteins and could theoretically affect their function. For example, glutathione S-transferases, which contain an active site tyrosine, are subject to inactivation by tyrosine nitration (88). The major endothelial protein targeted by tyrosine nitration is prostacyclin synthase; inhibition of this enzyme can lead to adhesion of leukocytes and platelets to the vascular endothelium, integral parts of some inflammatory and vascular processes (89). Nitrotyrosine itself has also been demonstrated to result in peroxide formation and to induce DNA oxidative damage (90).

EXTRACELLULAR REGULATION OF eNOS AND ACTIVITY OF NO

The focus of this section is to elucidate those extracellular signals that control the processes of eNOS palmitoylation and acylation as well as calcium flux in the target cell and unravel the mechanisms by which these processes connect an extracellular signal to eNOS activation and NO function.

The finer aspects of the molecular regulation of NOS isoforms are in fact best characterized for eNOS regulation in endothelial cells, where a picture of

truly dynamic stimulation and inhibition responsive to a network of physiologic stimuli has emerged. In resting endothelial cells, eNOS is targeted to caveolae, bound to caveolin-1 in favor of calmodulin, and thus tonically inhibited. Basal NO production is maintained via extracellular signals, including shear stress and receptor-modulated calcium transients. Dynamic activation of eNOS occurs generally through agonist-modulated increases in intracellular calcium that lead to eNOS-calmodulin binding, with these receptor-dependent changes in enzyme activity importantly influenced by eNOS subcellular targeting; protein associations; and posttranslational modifications, including phosphorylation, palmitoylation, and S-nitrosylation.

Activation of eNOS typically results in a relatively rapid phase of increased enzymatic catalysis coupled with a relatively slower mechanism of inactivation, ostensibly to provide the cell with a rapid response to stimuli followed by an eventual return to baseline. Persistent activation of eNOS is accompanied by dissociation from caveolin and translocation to the intracellular sites through the effects of calmodulin and reversible depalmitoylation, respectively. Subsequently, eNOS is translocated back to caveolae as calmodulin dissociates and eNOS is repalmitoylated.

Classical Ligands of G Protein–Coupled Receptors that Activate eNOS

The widest array of mammalian cell surface receptors, encompassing approximately 1000 receptor subtypes, are members of the superfamily of seven transmembrane guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs). These receptors are intimately linked on their intracellular side to heterotrimeric G-proteins that consist of α and $\beta\gamma$ subunits. Each subunit is further subgrouped into classes comprising at least 20 Gα, 6 Gβ and 12 Gγ, thereby allowing a wide variety of combinations between receptor subtypes to downstream G-protein subunits. Indeed, a given receptor subtype is often able to transmit its signals through different G-protein subunits, adding additional levels of complexity and possible signaling diversity to the system of eNOS control. It can be difficult to extrapolate findings in a particular experimental system to other biological contexts; such extrapolation is complicated by the lack of understanding of the significance of the possible quantitative ratios of receptors to signaling proteins in distinct cell systems.

One of the best-characterized eNOS agonists is bradykinin. Bradykinin is a nonapeptide hormone that represents the archetypal endogenous ligand for the bradykinin B_2 receptors, which are widely expressed in mammalian vascular endothelium (91). Local concentrations of bradykinin are regulated by kininogenases that cleave kininogens (the precursor proteins for bradykinin) as well as by kininases that degrade bradykinin to biologically inactive peptide fragments. Among various kininases, angiotensin converting enzymes (ACEs) represent an important cardiovascular regulatory locus that controls the concentration of bradykinin

in the vicinity of the vessel wall (92). Bradykinin and other endogenous ligands for GPCR that mobilize calcium and activate eNOS include acetylcholine (which signals via m_2 muscarinic receptors), histamine, adenosine, ATP/ADP, and thrombin, among others (93). These ligands activate GPCRs coupled to G-proteins that activate PLCβ, thereby mobilizing intracellular calcium and promoting rapid and robust eNOS activation (94). High-resolution microscopy has been used to visualize calcium waves following GPCR stimulation that occur initially and specifically at the caveolin-rich cell edges where significant quantities of eNOS protein, and some GPCRs, are anchored (95).

Sphingosine 1-Phosphate and Lysophosphatidic Acid as Novel Ligands for G Protein–Coupled Receptors

Platelet-derived lipid mediators, including sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), also stimulate eNOS through GPCR-dependent pathways (96). Unlike other GPCR ligands, data suggests that S1P and LPA mediate their effects on eNOS activity not necessarily through PLC/calcium pathways, but through kinase Akt phosphorylation cascades.

S1P, an endogenous sphingolipid species found in biological fluids of numerous organisms from yeasts to humans, was first implicated in signal transduction machinery of mammals as an intracellular second messenger that mediated fibroblast mitogenic responses in response to platelet-derived growth factor stimulation (97). S1P is released by stimulated platelets and facilitates endothelial cell functions like vasorelaxation and angiogenesis through its interaction with G protein–coupled S1P receptors (formerly called EDG receptors) (98, 99). Studies in endothelial cell culture conclusively demonstrate the ability of S1P to activate eNOS in a GPCR-dependent manner (100–102). Activation of the S1P receptor associated G$\beta\gamma$ subunit activates PI3Kβ, which leads to activation of kinase Akt resulting in eNOS phosphorylation at Ser 1177; S1P does not activate Akt through PI3Kα, unlike other eNOS agonists (such as VEGF, see below) (103, 104). Although the physiologic consequences of eNOS activation by S1P remain to be studied in vivo, experiments suggest that the S1P/Akt pathway may mediate endothelial cell survival and migration (105). Experiments further suggest that GPCRs for S1P may have key roles in normal development and cardiovascular morphogenesis (105, 106). The physical proximity of sphingolipids, their receptors, and eNOS may be significant: Plasmalemmal caveolae are specifically enriched in sphingolipids, whereas S1P receptors and eNOS are targeted to caveolae. In resting endothelial cells, approximately half of the cellular complement of S1P receptors are localized to caveolae. Exposure to S1P induces their caveolar translocation such that within 60 min, more than 90% of S1P receptors become localized in caveolae (107). This translocation may bring the S1P receptors closer to downstream effectors and eNOS, although it may also plausibly have a role in attenuating cellular responses to S1P.

In addition to its effects on eNOS phosphorylation, S1P can activate nonselective cation channels, resulting in an influx of calcium into the endothelium and

providing a supplementary route to eNOS activation (108). Interestingly, the calcium chelator BAPTA abrogates S1P-mediated activation of PI3Kβ, suggesting that the two paradigmatic pathways of eNOS regulation—PLC/calcium/calmodulin and PI3K/Akt phosphorylation—may actually exhibit cross-talk and/or synergy downstream of S1P, and possibly other eNOS agonists (100).

Lysophospholipids, including LPA, also represent GPCR ligands connected to eNOS activation. LPA signal transduction occurs through different but structurally similar GPCRs as compared to S1P (109–111).

Polypeptide Growth Factors: VEGF

Vascular endothelial growth factor (VEGF) is an angiogenic polypeptide growth factor and potent eNOS stimulus that binds to at least four independent receptor subtypes, most notably the KDR receptor (112). KDR and the other VEGF receptors are members of a superfamily of receptor tyrosine kinases. VEGF was initially implicated in eNOS regulation when it was observed to induce endothelium-derived vasorelaxation in freshly isolated canine coronary arteries in a manner sensitive to both NOS inhibitors and tyrosine kinase inhibitors (113). Subsequent studies have revealed that VEGF potently promotes eNOS activity in endothelial cells by stimulating kinase Akt and eNOS Ser 1177 phosphorylation (114). The effect of VEGF on Akt is likely mediated via both PI3Kα and PI3Kβ pathways owing to its tyrosine kinase mechanism, although it may also involve protein kinase C. eNOS activation by VEGF appears to participate in regulation of angiogenesis (115); however, the relative importance of acute eNOS activation by VEGF/phosphorylation versus chronic gene regulation of eNOS by VEGF in vivo remains to be established (116).

VEGF sensitizes endothelium to the effects of sphingolipid mediators, for example, by inducing S1P receptor mRNA, thus augmenting the capacity for endothelial response to S1P (117). It is attractive to hypothesize that such synergy between S1P and VEGF may function in vivo in angiogenesis. However, in vitro experiments demonstrate that endothelium must be pretreated for 60–90 min with VEGF to realize synergistic effects with S1P, suggesting possible complex kinetic interactions.

VEGF also stimulates eNOS by activating the phosphatase calcineurin, leading to dephosphorylation of Ser 116 in eNOS. The calcineurin inhibitor cyclosporin prevents VEGF-induced dephosphorylation at Ser 116 and may explain this drug's side effect of arterial hypertension. Although membrane-associated phosphorylation at Ser 1177 and cytosolic dephosphorylation at Ser 116 appear to be stimulatory to eNOS, at present no evidence for synergy or cross-talk links these pathways (114). Nevertheless, there is an important temporal distinction between these two processes as Ser 1177 phosphorylation achieves maximal response by 5 min, whereas Ser 116 is maximally dephosphorylated at 30 min; thus, these two pathways may plausibly have different roles in the kinetic control of eNOS activity (114).

Like S1P, VEGF can affect eNOS through mobilization of calcium in addition to the more prominent influences on phosphorylation cascades. The tyrosine kinase c-Src, downstream of the VEGF receptor KDR activates PLCγ, an alternative PLC isoform that nevertheless raises intracellular calcium in endothelial cells (118).

Finally, reversible denitrosylation of eNOS has been identified as a mechanism by which agonists such as VEGF regulate eNOS activity (40a). eNOS in resting endothelial cells is robustly S-nitrosylated. However, in response to agonists, eNOS is rapidly denitrosylated concomitant with enzyme activation; in over 60 min eNOS is renitrosylated, as the enzyme returns to the quiescent state. The time course of VEGF-induced denitrosylation/renitrosylation parallels that of phosphorylation at Ser 1177, and the relative amounts of nitrosylated eNOS versus Ser 1177 phosphorylated eNOS are inversely proportional over this time course (40a). Importantly, membrane targeting of eNOS is necessary for its S-nitrosylation. It is possible that the translocation of eNOS from plasmalemmal caveolae to intracellular membranes is required for enzyme denitrosylation, and that the return of eNOS to caveolae is a key determinant of the enzyme's renitrosyation. This suggests that the cycle of eNOS S-nitrosylation and denitrosylation could be impacted by processes that alter cellular redox state in specific subcellular compartments.

Hormones

Insulin is a key hormone that principally regulates glucose metabolism in the liver and skeletal muscles. Insulin has also been shown to exert direct actions in the vasculature, such as vasodilatation in humans that is sensitive to NOS inhibitors, as well as activation of eNOS in cultured endothelial cells via an insulin receptor tyrosine kinase, upstream of PI3K (119–121). Insulin can activate endothelial cell eNOS by Akt-mediated Ser 1177 phosphorylation and eNOS denitrosylation (40a, 122). Insulin also activates platelet eNOS by activating PI3K and AMP-activated protein kinase (123). The role of insulin-dependent eNOS activation in diabetic vasculopathy remains to be elucidated. However, eNOS, like many other endothelial proteins, is known to become irreversibly and nonspecifically glycosylated in response to elevated serum glucose levels to form O-linked N-acetylglucosamine conjugates (124). Formation of these O-linked N-acetylglucosamine derivatives of eNOS has been reported to abrogate eNOS Ser 1177 phosphorylation and contribute to endothelial dysfunction and atherogenesis in diabetes, although this pathway is controversial (125).

Estrogen is a key sex steroid hormone that may have cardioprotective effects through genomic actions in the cardiovascular system by maintaining favorable lipid profiles, regulating vascular tone, and inhibiting coagulation (126). Estrogen is also believed to exert acute, nongenomic actions in the vasculature by increasing eNOS activity (127–129). Estrogen activates PI3K/Akt and MAP kinase via nonnuclear signaling pathways that may involve its nuclear ERα receptor (130, 131). In addition, estrogen, like classical GPCR eNOS agonists, may stimulate eNOS

by inducing calcium transients (129, 132). Experiments with ERα-deficient mice demonstrate that estrogen and ERα also regulate longer term vascular processes, especially matrix deposition and vascular smooth muscle proliferation in the response to vascular injury; specifically, estrogen acting via ERα attenuates the robustness of the vascular response to injury (133). The estrogen receptor ERβ, also expressed in myocardium, endothelium, and vascular smooth muscle cells, exerts effects on the cardiovascular system and blood pressure regulation by regulating eNOS as well as activating iNOS. ERβ mediates vasodilatory responses to estrogen even in endothelium denuded vessels; in contrast, ERβ-deficient mice are hypertensive compared to wild-type controls, and endothelium denuded vessels from ERβ-deficient mice were more potently constricted by alpha agonists versus wild type mice despite treatment with estrogen (134).

Thrombin affects eNOS via GPCRs and also inhibits eNOS phosphorylation at Ser 1177 via rho/rho kinase–mediated inhibition of Akt (135). Thrombin also downregulates eNOS mRNA in a rho/rho kinase–dependent manner (18). The inhibitory effects of thrombin on eNOS may contribute to mechanisms of endothelial dysfunction in cardiovascular pathophysiology.

Receptor-Independent Pathways of eNOS Activation

Besides biochemical ligands that bind to and activate endothelial receptors, mechanical stimulation, especially resulting from fluid shear stress along vascular endothelial cells, is known to activate eNOS. The firm identification of putative mechanoreceptors that sense such mechanical forces and convert them to intracellular chemical phenomenon remains elusive. Some GPCRs, like heterologously expressed P2X4 purinoreceptors in HEK293 cells, may sense fluid shear stress (136). On the other hand, some G-protein subunits seem to be directly activated in response to fluid shear stress, independently of GPCR expression, thereby suggesting their function as mechanoreceptors (137). It is noteworthy that shear stress also upregulates transcription of eNOS as well as S1P receptors (138, 139), indicating the possibility of cross-talk of shear stress transduction with more well-characterized biochemical pathways in the endothelium. Similarly, part of the response to shear stress is transduced through calcium-independent PI3K/Akt kinase pathways that may serve to maintain basal vascular tone (140, 141).

Integrated Phosphorylation/Dephosphorylation Mechanisms of eNOS Control

Kinase Akt, a serine/threonine kinase that activates eNOS by phosphorylation at Ser 1177, integrates signals from a number of factors, including platelet-derived lipid mediators, VEGF, insulin, estrogen, and shear stress (120, 140, 141). All of these factors generally stimulate Akt by acting on one or more isoforms of its upstream regulator, PI3K. PI3K activation leads to the recruitment of several molecules, including kinase Akt, to membranous fractions within cells; this is a crucial step because cytosolic Akt is normally inactive and must be translocated to

the membrane to become activated and subsequently phosphorylate its diverse substrates, including eNOS (26). The activation of different isoform of PI3K by eNOS agonists demonstrates that eNOS signaling pathways seem to diverge proximally, although ultimately converging at eNOS enzyme upregulation. For example, although VEGF and S1P stimulate different isoforms of PI3K, both agonists induce calcium mobilization, leading to complementary effects on phosphorylation and calmodulin binding (Figure 1) (103).

Interfering with PI3K isoforms, kinase Akt, or eNOS Ser 1177 phosphorylation abrogates the ability of eNOS agonists to promote eNOS activity (140, 141). In contrast, an eNOS Ser1177Asp mutant, which structurally mimics Ser1177-phosphorylated eNOS, exhibits increased catalytic activity presumably owing to enhanced electron flux in the reductase domain and increased resistance to calmodulin dissociation (142).

PI3K/Akt pathways are but one of a number of phosphorylation cascades that regulate eNOS. VEGF, S1P, and bradykinin can all stimulate various mediators in the MAP kinase pathway, such as ERK1 and ERK2, which may inhibit eNOS via phosphorylation at sites other than Ser 1177 (114). Such a function could be consistent with agonists inducing a rapid activation of eNOS followed by a longer term inactivation so as to return NO output to the original level (143).

In addition to phosphorylation, several lines of experimentation have elucidated roles for phosphatases in eNOS regulation. Calcineurin-dependent pathways lead to dephosphorylation of Ser 116 to increase eNOS activity. Part of the eNOS-activating effect of bradykinin may derive from calcineurin-mediated dephosphorylation of Thr 495 (144). Protein phosphatase 2A (PP2A) likely functions as an overall negative effector of eNOS activity owing primarily to dephosphorylation of Ser 1177, despite its ability to also dephosphorylate Thr 495. Reciprocal control of phosphorylation at Thr 495 may contribute to the balance of production of NO and superoxide by eNOS.

The dynamic receptor-regulated S-nitrosylation and denitrosylation of eNOS may also represent key determinants of eNOS activity, yet the relationships between eNOS S-nitrosylation and phosphorylation are incompletely understood (40a). Although the time course of agonist-induced eNOS denitrosylation parallels that of the enzyme's Ser 1177 phosphorylation, it appears that phosphorylation of eNOS is not required for enzyme denitrosylation, and conversely, that denitrosylation of eNOS is not essential for the enzyme phosphorylation (40a). By contrast, eNOS acylation does appear to be a key determinant of S-nitrosylation: eNOS targeting to cellular membranes facilitates the enzyme's S-nitrosylation. The fact that eNOS renitrosylation occurs at the membrane may indicate that renitrosylation is one of the final steps in the return of eNOS to its basal state, after the enzyme has been relocated back to plasmalemmal caveolae following agonist-mediated eNOS activation and translocation. Because eNOS undergoes dynamic cycles of reversible subcellular translocation, thiopalmitoylation, and S-nitrosylation, these processes may importantly interact in normal cells and in disease states.

PATHOPHYSIOLOGIC STATES AFFECTING eNOS AND NO

Dyslipidemia and Atherosclerosis

Prior to the development of overt atherosclerotic lesions, hypercholesterolemia impairs vessel relaxation and other homeostatic vasculoprotective functions (145, 146). Indeed, coronary arteries harvested from pigs fed a high-cholesterol diet for 9 weeks lack atherosclerotic changes, as evaluated by light and electron microscopy, but have impaired relaxation in response to endothelium-dependent agonists (147). Yet, these vessels respond normally to nitroprusside and to other direct vascular smooth muscle vasodilators. Furthermore, the coronary arteries of humans with risk factors for atherosclerosis, but lacking gross disease as evaluated by angiography and intracoronary ultrasound, vasoconstrict in response to acetylcholine, in contradistinction to the normal vasodilatory response of coronary arteries from humans free of atherosclerosis risk factors (146, 148).

The observed vascular abnormalities in dyslipidemias are at least partly due to impaired NO synthesis. Native LDL (n-LDL), at a concentration of 2 mg/ml, attenuates acetylcholine-induced relaxation of rabbit aortic rings, and at 1 mg/ml n-LDL inhibits ATP-induced endothelial-dependent relaxation (149). In contrast, lower concentrations of LDL (0.1 mg/ml) appear to have no effect on acetylcholine-mediated vascular relaxation (150). Upon incubation with cultured cells, such as endothelium, smooth muscle cells, or macrophages, LDL undergoes structural and metabolic changes that dramatically alter its effects and atherogenicity (151). These changes are initiated by peroxidation of polyunsaturated fatty acids and involve hydrolysis of phosphatidylcholine to lysophosphatidylcholine (LPC) (151, 152). Oxidized LDL (ox-LDL) and LPC are found in atherosclerotic arteries and have been shown to severely attenuate acetylcholine-mediated rabbit aortic strip relaxation (150, 153).

There is growing evidence indicating that ox-LDL and LPC modulate endothelial NOS at the transcriptional, posttranscriptional, and posttranslational levels. Exposure of endothelium to 50–100 μg/ml ox-LDL for 24 h decreases eNOS mRNA transcript levels, although the effects of lower concentrations of ox-LDL are less clear (154, 155). The decreased expression of eNOS mRNA at 24 h of exposure to ox-LDL appears to result from a marked increase in the rate of transcript degradation, as the transcriptional activity of the eNOS gene is more than two times higher than basal activity (17, 154). In advanced atherosclerotic lesions, the overlying endothelial cells are also characterized by reduced expression of eNOS (156). As a result, atherosclerotic human coronary arteries respond to acetylcholine by vasoconstricting (157, 158).

Unlike treatment with LDL, treatment of endothelial cells with LPC actually seems to upregulate eNOS gene transcription, protein expression, and enzymatic activity (155, 159). LPC may activate eNOS via protein phosphatase 2A–mediated dephosphorylation of the transcription enhancing factor Sp1, which augments the

binding efficiency and transcriptional control of the eNOS gene by Sp1 (160, 161). LPC may mediate the stimulatory effects of ox-LDL on eNOS gene transcription, whereas the destabilization of the eNOS transcript may be mediated by other factors. In addition, it may seem contradictory that LPC treatment increases eNOS activity of cultured endothelial cells but inhibits acetylcholine-mediated relaxation of rabbit aorta. However, it should be emphasized that LPC could affect other processes that impinge on NO-mediated vascular dilation, such as production of superoxide, which affects NO stability or smooth muscle cell relaxation (162, 163).

Ox-LDL also appears to exert inhibitory effects on eNOS by altering the caveolar microenvironment in which eNOS resides. Incubation of bovine aortic endothelial cells with hypercholesterolemic human serum for 5 min strengthens the association of eNOS with caveolin and inhibits NO synthesis (164). Treatment of porcine pulmonary artery endothelial (PPAE) cells with in vitro ox-LDL for 1 h attenuates acetylcholine-induced eNOS activity and promotes translocation of eNOS and caveolin from caveolae to intracellular membranes (165). Despite the translocation to intracellular membranes, caveolin and eNOS appear to remain colocalized. Furthermore, within 30 min of treatment with ox-LDL, cholesterol is transferred from endothelial caveolae to ox-LDL particles, a process mediated through the CD36 scavenger receptor (166). The depletion of caveolar cholesterol is thought to be specifically responsible for the eNOS and caveolin translocations because treatment of PPAE cells with native or oxidized LDL has no effect on eNOS palmitoylation, myristoylation, or phosphorylation (165).

In contrast to the inhibitory effects of LDL on eNOS, HDL enhances eNOS activity. The effects of ox-LDL on eNOS localization and activity are inhibited in the presence of HDL (166). When endothelial cells are exposed to both ox-LDL and HDL, the cholesterol content of caveolae is maintained because HDL apparently donates cholesterol to caveolae and thereby compensates for the LDL-induced cholesterol removal. Class B scavenger receptors (SR-BI receptor), which bind HDL, mediate the activation of eNOS in response to HDL treatment in cultured endothelial cells and intact vessels (167). HDL appears to activate eNOS through Src/PI3K/Akt and MAP kinase pathways downstream of SR-BI docking (168). Moreover, responses to HDL are abrogated by antibodies that block the SR-BI scavenger receptor (166).

Interestingly, it may not be HDL per se that mediates eNOS activation and vasodilatation, but instead lipophilic factors, such as S1P, sphingosylphosphorylcholine, lysosulfatide, and estrogen, which are carried in HDL and delivered to endothelial cells upon HDL docking with SR-BI receptors. Because S1P and related lysophospholipids are enriched in HDL fractions, it has been proposed that S1P may be at least partly responsible for the observed protective actions of HDL on the vascular endothelium (169, 170). These lysophospholipids stimulate eNOS in Akt and calcium-dependent pathways downstream of the S1P3 receptor, and the vascular response to HDL is severely attenuated in receptor-deficient mice (171). In addition, HDL particles enriched with estradiol, such as from serum of female mice, premenopausal human women, and postmenopausal human women

receiving estrogen replacement therapy, stimulate eNOS; this stands in contrast to serum from male mice and humans as well as postmenopausal women, which all were relatively inactive toward eNOS (172). HDL may also upregulate eNOS activity in calcium- and Akt-independent pathways by reversibly increasing intracellular ceramide levels after docking with SR-BI (173).

Further studies are needed to explore the interrelationships of HDL/SR-BI, S1P, and estrogen pathways in the context of eNOS regulation and vascular physiology. Nevertheless, these actions may represent a novel locus of cardioprotective effects of HDL independent of LDL.

Hypoxia

The effect of hypoxia on eNOS expression remains controversial. In their landmark study describing the obligatory role of the endothelium in acetylcholine-mediated relaxation of arterial smooth muscle cells, Furchgott & Zawadzki demonstrated that anoxia abrogates acetylcholine-induced vasodilatation (174). Analogous effects are seen in the pulmonary vessels of rats exposed to hypoxia (10%–11% O_2) for three weeks (175). Furthermore, exposure of cultured endothelial cells to anoxia or hypoxia (3% O_2) for 48 to 72 h impairs both eNOS mRNA transcription rates and transcript stability, resulting in diminished eNOS protein levels and NO-producing activity (176, 177). The presence of inflammatory mediators and cytokines in hypoxic conditions further decreases expression of eNOS and production of NO (178). There is growing evidence that rho/rho kinase pathways can mediate hypoxia-induced inhibition of eNOS transcription (15). As discussed below, statins, including simvastatin, inhibit this rho-mediated mechanism of inhibition of eNOS transcription and thus increase eNOS levels under hypoxic conditions (178, 180). Additionally, hypoxic conditions may attenuate Akt-mediated phosphorylation at Ser 1177, alter calcium metabolism, and alter the balance of eNOS protein-protein interactions with caveolin and calmodulin (181).

In contrast, several other experiments have concluded that hypoxia can upregulate eNOS. Two independent studies showed that exposure of rats to 10% O_2 for 1–3 weeks stimulates eNOS mRNA expression, protein levels, and catalytic activity in the adult rat lung (182, 183). These experiments are possibly explainable as an organ-specific eNOS response to hypoxia in lung. However, exposure of bovine aortic endothelial cells to 1% O_2 for 6–24 h also stimulates eNOS transcription, without changing total eNOS activity (184). Notably, this experiment differs from the studies documenting a hypoxia-induced decrease in endothelial eNOS expression by the shorter duration of hypoxia and the source of endothelial cells. Discrepancies observed in the effect of hypoxia on eNOS may result from differences between in vitro and in vivo systems, different cellular and disease models, and evaluation of the models at different points along the time course of hypoxia.

Other studies have reported that eNOS may be induced through the involvement of two hypoxia-inducible proteins, erythropoietin and VEGF. Erythropoietin has the capacity to upregulate both erythropoietin receptors and eNOS in hypoxic

endothelial cells (185). Hypoxia is one of the more potent stimuli of VEGF expression, which ultimately induces VEGF-eNOS-NO-mediated angiogenesis. In chronic hypoxia models in hearts, eNOS becomes more tightly associated with heat shock protein 90, a mechanism that may serve to increase NO generation and limit superoxide production by eNOS (186). The role of hypoxia in eNOS regulation clearly awaits further analysis of the important physiologic and molecular mechanisms.

Oxidative Stress

ROS can affect and modulate vascular tone through diverse molecular mechanisms. Recall that superoxide inactivates NO by forming peroxynitrite, which itself is a marker of oxidative stress and inflammation occurring in diverse pathological conditions. For example, enhanced superoxide production may contribute to atherogenesis as evidenced by increased superoxide anion content in vessels from animals fed high-cholesterol diets (187). Superoxide may reduce expression of eNOS protein, as well as decrease the number of caveolae in endothelial cells (188). The most significant source of superoxide in the vascular wall is NADPH oxidase (189). Production of superoxide by this enzyme system is elevated in response to angiotensin II, which may constitute a specific feature of endothelial dysfunction. In dysfunctional endothelium, superoxide production can result in formation of peroxynitrite or in uncoupling of eNOS catalytic function via tetrahydrobiopterin oxidation, thus decreasing NO output in favor of increasing ROS output (9).

The enzyme superoxide dismutase acts to detoxify superoxide and releases hydrogen peroxide (H_2O_2). H_2O_2 has been shown to directly relax vascular smooth muscle cells, as evidenced by its ability to vasodilate endothelial-denuded, as well as endothelial-intact, rabbit aortic rings (190). H_2O_2 also may induce phosphorylation of eNOS Ser 1177 and dephosphorylation of eNOS Thr 495, apparently involving distinct tyrosine kinase–dependent PI3K/Akt mechanisms (191). H_2O_2 may therefore serve as a compensatory mechanism in endothelial dysfunction associated with high levels of angiotensin II and NADPH oxidase–derived superoxide (192).

ROS may directly affect eNOS through either transcriptional mechanisms or by altering the redox state of the tetrahydrobiopterin cofactor. Hydrogen peroxide has been reported to upregulate eNOS mRNA transcription and enhance eNOS mRNA stability in aortic endothelial cells; by contrast, superoxide does not exhibit these effects (193). It is possible that one function of H_2O_2 is to promote eNOS expression and activity in an attempt to maintain normal NO and vascular homeostasis despite oxidative stress likely associated with inflammatory and vascular disease states. Another mechanism whereby ROS may inhibit eNOS activity may reflect the dependence of eNOS denitrosylation on cellular redox state, such that decreases in cellular reduced thiol content might lead to increased levels of S-nitrosylated eNOS and a suppression of enzyme activity.

Given the central role of oxidative stress in modulating NO synthesis and degradation, it is not surprising that the effects of antioxidants on eNOS and NO signaling have been extensively explored. In human plasma, ascorbic acid (vitamin C) is the major water-soluble antioxidant that scavenges superoxide and other ROS, and it plays a key role in regulating the intracellular oxidation-reduction state (194). Oral vitamin C treatment improves endothelial-dependent vasomotor function of brachial arteries of patients with coronary artery disease (195, 196). Exogenously administered ascorbic acid accumulates intracellularly in cultured porcine aortic endothelial cells and enhances eNOS V_{max} by 50% without changing the enzyme's K_m for L-arginine (197). The activation of eNOS activity by ascorbic acid may reflect its direct affect on eNOS S-nitrosylation (40a) and may be a consequence of increased bioavailability of tetrahydrobiopterin (2, 198).

PHARMACOLOGIC INTERVENTION IN NO PHYSIOLOGY

Nitrovasodilator Drugs

Numerous drugs in clinical practice are utilized for their NO-donating properties, including glyceryl trinitrate (nitroglycerin), isosorbide dinitrate, and sodium nitroprusside. These drugs have been dubbed "endothelial cell replacement therapy" for their ability to recreate at least part of the biological effects of endogenous NO (199). Nitrovasodilators have been successfully employed for many years in the management of coronary artery disease, angina pectoris, and congestive heart failure.

The many NO-donating drugs differ in the chemical functionality that serves as the source of NO, as some drugs release NO from a nitrate group (nitroglycerin), a nitrite group (amyl nitrite), or an organometallic NO ligand (sodium nitroprusside). Although the generation of NO from sodium nitroprusside is apparently a spontaneous nonenzymatic process, the putative enzymatic processes by which most other nitrovasodilators like nitroglycerin and isosorbide dinitrate release NO remain under study. Within the family of nitrovasodilators, clinicians can choose among a selection of drugs with different bioavailability, dosing, kinetics, and side effects (200).

Although quite efficacious, the most significant problem with extended nitrovasodilator therapy is tolerance, by which the endothelial response and the clinical benefit to nitrovasodilators is markedly attenuated. The exact biochemical events that cause tolerance are still poorly understood. However, several theories have sought to explain the observation of tolerance, including physiologic vascular tolerance via feedback inhibition, depletion of cellular stores of sulfhydryl equivalents, neurohumoral reflexes that result in sodium and water retention, increased endothelial production of superoxide and other free radicals, and alteration in the

local vascular production of vasoactive compounds (200). Recently, nitroglycerin tolerance has been linked to downregulation of an enzyme, mitochondrial aldehyde dehydrogenase, which may be involved in the generation of NO from nitroglycerin (201).

Drugs Affecting NOS mRNA and/or Protein Levels

Cardioprotective drugs, including statins, angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), and some calcium channel blockers (CCB), are being increasingly recognized for their pleiotropic effects, which may include cardioprotective effects through the modulation of eNOS. It is becoming increasingly appreciated that the effects on NO may importantly complement the agent's primary mode of action and shift the vascular system more toward normal homeostatic equilibrium.

In addition to their well-characterized effects on in vivo cholesterol biosynthesis, serum lipid profiles, and atherogenesis, the statin drugs influence the transcriptional control of eNOS. Statins may help to maintain eNOS mRNA levels in potentially compromised atherosclerotic endothelium by preventing the ox-LDL-mediated degradation of eNOS mRNA (17, 202). Statins may additionally increase eNOS mRNA levels through the inhibition of rho-GTPases, which appear to destabilize eNOS mRNA (15). The mechanism appears to involve inhibition of the posttranslational prenylation of rho-GTPases, as statins inhibit synthesis of the isoprenoid geranylgeranyl anchor compounds (17, 202–204). Statins also may induce posttranslational modifications of eNOS protein by activating Akt and phosphorylating eNOS Ser 1177 (205). Finally, statins have been reported to reduce nitrotyrosine levels in atheromas in patients with coronary artery disease, and the comprehensive effects of statins on inflammatory processes continue to be investigated (206, 207).

The therapeutic benefit of statins in protecting against fatal and nonfatal cardiovascular events in patients with asymptomatic hypercholesterolemia is independent of baseline lipoprotein levels and is not correlated with extent of LDL decrease beyond a 25% reduction (208, 209). In addition, it has been shown that patients treated daily with a statin for one or six months exhibit enhanced acetylcholine-mediated dilation of the forearm and coronary arteries (210, 211). Taken together, these studies indicate that the cardioprotective effects of statins may be mediated through not only LDL-dependent mechanisms but also through modulation of eNOS. Protective effects of statin therapy in preventing cerebrovascular accidents are also apparently mediated by eNOS, as they are not observed in eNOS knockout mice (212).

ACEI powerfully inhibit the renin-angiotensin system and limit formation of the potent endogenous vasoconstrictor angiotensin II. ACEI also inhibit the proteolytic activity of a family of kininases that are responsible for the catabolism of bradykinin. By prolonging the half-life of bradykinin, ACEI presumably help

to stimulate endothelial NO generation by preserving an important stimulus of eNOS activity (213). However, ACEI have also been demonstrated to augment eNOS expression in vivo (214). ARBs likewise inhibit the effects of angiotensin II and increase eNOS mRNA levels in vivo, although they are incapable of preventing bradykinin catabolism. The possible mechanism for the effects of these two classes of drugs on eNOS upregulation may involve remediation of endothelial damage caused by elevated angiotensin II levels in certain cardiovascular disease states.

Two of the three major chemical classes of CCB, the dihydropyridines and the benzothiazepines, which include nifedipine and diltiazem, respectively, also upregulate eNOS expression in isolated endothelial cells as well as in vivo (215, 216). The mechanism of action of these drugs on expression remains unknown. However, the dihydropyridine amlodipine may upregulate eNOS function by stimulating phosphorylation at Ser 1177 while limiting phosphorylation of Thr 495 (217).

Drugs Affecting NOS Activity: NOS Inhibitors

As might be anticipated for an enzyme involving so many cofactors, cosubstrates, and allosteric modifiers, NOS catalysis can be inhibited by a broad range of compounds, including antagonists of any of the five essential redox cofactors, calmodulin antagonists, calcium chelators, appropriate phosphatases and kinases, and inhibitory substrate analogs. A group of L-arginine substrate analogs that inhibit NOS activity have proven useful in not only biochemical assays but also clinical studies. These arginine analogs generally feature substitutions at the guanidino nitrogen, and include, for example, N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine acetate (L-NMMA), and N^G-amino-L-arginine. In general, these inhibitors do not exhibit profound selectivity for a specific NOS isozyme, although L-NNA and L-NAME are moderately selective for eNOS and nNOS. In addition to arginine analogs, citrulline analogs, including L-thiocitrulline and S-methyl-L-thiocitrulline, inhibit various NOS isozymes. These thiocitrullines may exert part of their inhibitory effect by compromising the reducing potential of the NOS heme iron. L-thiocitrulline is partially selective for eNOS and nNOS, whereas S-methyl-L-thiocitrulline is selective for nNOS.

Infusion of the arginine-based NOS inhibitor L-NMMA into brachial arteries of human subjects results locally in vasoconstriction and decreased blood flow, underscoring the function of NO in the regulation of arterial hemodynamics (94, 199, 218).

NOS inhibitors have also been proposed as beneficial therapy in certain shock syndromes. In septic shock, massive elaboration of cytokines results in overstimulation of iNOS and voluminous release of NO. NO synthesis is believed to contribute to the systemic hypotension and refractoriness to pressor agents in septic shock.

NOVEL ROLES FOR NO: A LESSON FROM FIREFLIES

NO is a superb example of the beautiful irony in biology in that such a structurally simple molecule can exhibit robust and pleiotropic effects. The unique and flexible chemistry of NO can be exploited for diverse functions across organisms and organ systems, for regulating both intracellular and extracellular processes. As the list of biological functions attributable to NO continues to grow, we can literally expect to uncover unexpected and completely novel roles for NO owing to its unique chemistry and biological capabilities. This expectation held true as a multidisciplinary team consisting of cardiologists, engineers, chemists, mitochondrial biochemists, microscopists, evolutionary biologists, entomologists, and insect neurophysiologists applied the principle of NO-mediated mitochondrial inhibition to explain the control of the bioluminescent flash of fireflies—a mystery that had long piqued the curiosity of scientists and philosophers alike.

In fireflies, bioluminescent flashing is a complex behavior essential for sexual selection and reproduction. The flash itself results from a peroxisomal reaction catalyzed by luciferase in which the polycyclic substrate luciferin is adenylylated. In the presence of oxygen, luciferyl-AMP transiently achieves an unstable high-energy state before decaying with release of light. The control of the firefly flash is fundamentally control of the stoichiometry of the luciferin reaction by gating the availability of oxygen to peroxisomes, thus trapping the luciferyl-AMP intermediate. The distinctive anatomy of the firefly lantern facilitates this gating of oxygen to peroxisomes by the active respiration of mitochondria. In the firefly lantern organ, the photocyte cells are characterized by an inner zone of peroxisomes that are literally surrounded by an outer perimeter of mitochondria. Oxygen enters the lantern organ via spicules and courses between photocytes through a vast network of tracheoles that line the photocytes and thus deliver the gas directly to mitochondria. Typically, mitochondria consume all oxygen delivered from the tracheoles such that none reaches peroxisomes in the center. Oxygen can, however, reach the peroxisomes in the center of the photocyte only when mitochondrial respiration is temporarily inhibited by reversible binding of NO to electron transport chain metalloproteins (Figure 2).

The proximal signal for local NO generation is octopamine discharge from the firefly central nerve, which synapses at numerous tracheolar end organs, or the regions encompassing the convergence of several tracheoles. Several lines of evidence suggested the existence of NOS expression localized in the tracheolar end organ area in or near the mitochondria (219). The neuronal octopamine signal stimulates this NOS isoform to release a transient burst of NO, thus inhibiting local mitochondrial respiration, transiently allowing oxygen to reach peroxisomes, and leading to light emission via oxygen-mediated oxidation and decay of luciferyl-AMP. The flash is terminated by the rapid decomposition of the various inhibitory metalloprotein-NO complexes, thus limiting oxygen availability to peroxisomes.

NO and NOS are intensely studied and are of paramount importance in diverse biological processes. Future work in this expansive field is certain to shed light on other fundamental biological mysteries.

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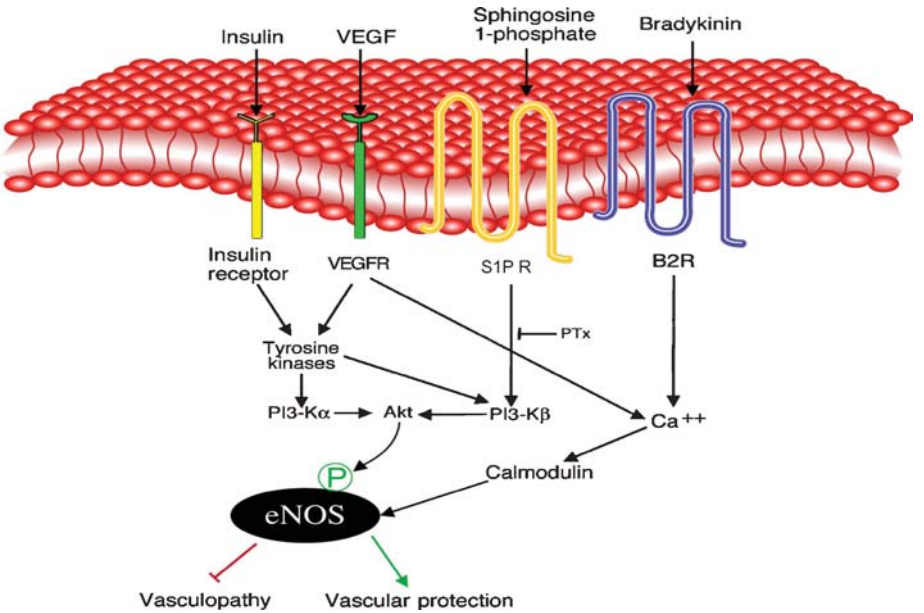

Figure 1 Diverse agonists affect various endothelial cell receptors and signaling pathways that promote eNOS activity as a result of increased intracellular calcium and/or eNOS phosphorylation. Agonists that activate eNOS by increasing intracellular calcium include bradykinin and other GPCR ligands as well as S1P and VEGF. VEGF, S1P, and insulin activate PI3K isoforms, which then activate kinase Akt and ultimately phosphorylate eNOS at Ser 1177. Cross-talk between the various agonist-mediated signaling pathways represents a possibly significant aspect of eNOS regulation, as for example, VEGF induces both calcium- and phosphorylation-dependent pathways of eNOS activity. Figure adapted from Reference 220.

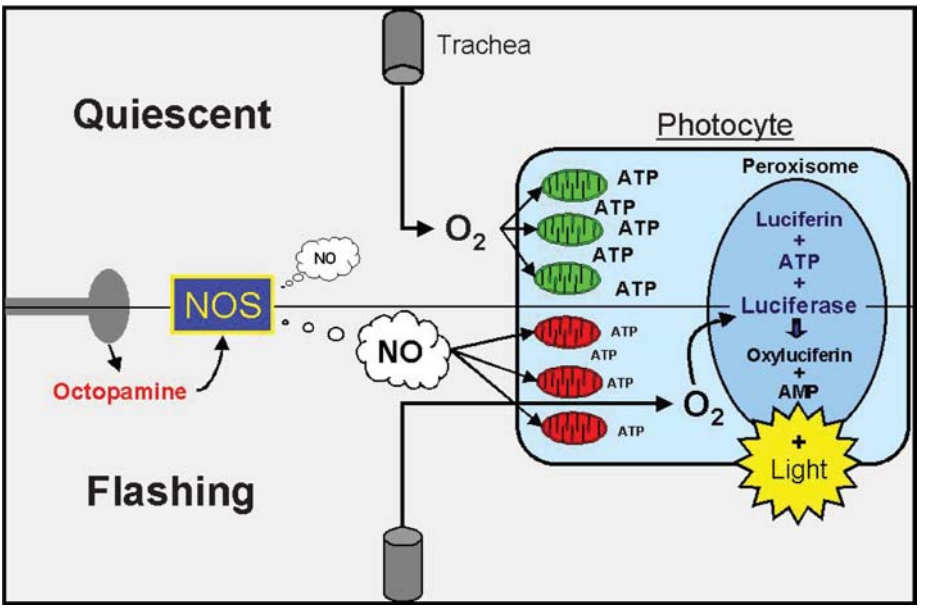

Figure 2 The figure depicts a highly schematized version of the physiology of the firefly lantern organ and control of the firefly flash by NO. In its quiescent state, oxygen enters the organ via an extensive tracheolar network. Mitochondria contained in photocyte cells abutting the tracheoles consume sufficient oxygen to prevent its dissociation into the interior of the photocytes where peroxisomes reside. Within the peroxisomes, luciferin is adenylated to luciferyl-AMP but is trapped in that state without oxygen. Because bioluminescence in this system requires the oxygen-mediated decay of luciferyl-AMP, no light is produced. Upon stimulation, however, an octopamine neural impulse terminates at numerous junctions of tracheoles, called tracheolar end organs. Octopamine discharge in these areas is believed to stimulate a mitochondrial NOS variant, which generates a short, local burst of NO that diffuses into the photocytes. The NO inhibits mitochondrial metalloproteins and blunts oxygen consumption, leaving oxygen to reach the peroxisomes in the photocyte interior. Luciferyl-AMP is oxidized to peroxyluciferin and then decays with release of light. Generation of light may be switched off by NO dissociation from mitochondrial metalloproteins or photolysis. Adapted from Reference 219 (unpublished figure).

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