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REVIEW

Subcellular Localization of Oxidants and Redox Modulation of Endothelial Nitric Oxide Synthase

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Reactive oxygen species (ROS) have long been viewed as deleterious chemicals that lead to oxidative stress. More recently, ROS, especially the stable ROS hydrogen peroxide (H_2O_2), have been shown to have roles in normal physiological responses in vascular cells. Endothelial nitric oxide synthase (eNOS) is dynamically targeted to plasmalemmal caveolae, and represents the principal enzymatic source of nitric oxide ($NO^{\bullet}$) in the vascular wall. eNOS maintains normal vascular tone and inhibits the clinical expression of many cardiovascular diseases. Increases in oxidative stress are associated with eNOS dysfunction. In a paradigm shift in the conceptual framework linking redox biochemistry and vascular function, H_2O_2 has been established as a physiological mediator in signaling pathways, yet the intracellular sources of H_2O_2 and their regulation remain incompletely understood. The subcellular distributions of ROS and of ROS-modified proteins critically influence the redox-sensitive regulation of eNOS-dependent pathways. ROS localization in specific subcellular compartments can lead to selective oxidative modifications of eNOS and eNOS-associated proteins. Likewise, the dynamic targeting of eNOS and other signaling proteins influences their interactions with reactive nitrogen species and ROS that are also differentially distributed within the cell. Thus, the subcellular distribution both of eNOS and redox-active biomolecules serves as a critical basis for the control of the "redox switch" that influences $NO^{\bullet}$- and oxidant-regulated signaling pathways. Here we discuss the biochemical factors, cellular determinants, and molecular mechanisms that modulate redox-sensitive regulation of eNOS and $NO^{\bullet}$ signaling under normal and pathological conditions. (*Circ J* 2012; **76:** 2497–2512)

Key Words: Nitric oxide; Nitric oxide synthase; Reactive oxygen species

Pathological perturbations of the intracellular redox potential affecting nitric oxide ($NO^{\bullet}$)-dependent cell signaling pathways in vascular endothelial and smooth muscle cells can lead to the development of vascular dysfunction across a broad spectrum of cardiopulmonary and cardiovascular diseases.[1] The association of oxidative stress with chronic disease states ranging from atherosclerosis to neurodegeneration has led to numerous studies exploring the effects of antioxidant vitamins and dietary supplements. Although some clinical trials have found beneficial effects of these, meta-analyses of numerous clinical trials have found no net benefit, and there may possibly be detrimental effects of nonspecific antioxidant therapies.[2] These unsuccessful clinical efforts to use antioxidant vitamins or dietary supplements to prevent or ameliorate the detrimental effects of increased vascular oxidant stress in humans have led to a resurgence of investigations that aim to define with greater specificity the precise mechanism(s) by which oxygen free radicals promote vascular diseases.[3–7] The recognition of physiological roles for reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) may indicate that the broad use of nonspecific antioxidant supplements may interfere with normal cellular responses and could undermine any possible health benefit that might theoretically derive from a decrease in oxidative stress.

At the center of this larger scientific effort is an evolving paradigm shift in the conceptual framework by which oxidative, nitrosative, and reductive stresses and signals affect subcellular proteins to influence vascular function. It has long been known that mitochondria represent a critical subcellular source of ROS, and of course this organelle represents a discrete subcellular compartment that is a distinct locale for many key signaling molecules. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) represent another important source for cellular ROS production in cells, and most if not all NOX isoforms are targeted to cellular membranes, including the plasma membrane, plasmalemmal caveolae, and endoplasmic reticulum. In turn, NOX-derived oxidants can target spatially-restricted lamellipodia in endothelial cells to modulate cell migration[8] among many other cellular responses. Importantly, endothelial nitric oxide synthase (eNOS) and proteins associated with eNOS-dependent cell signaling have been identified as key redox-sensitive targets. The subcellular distribu-

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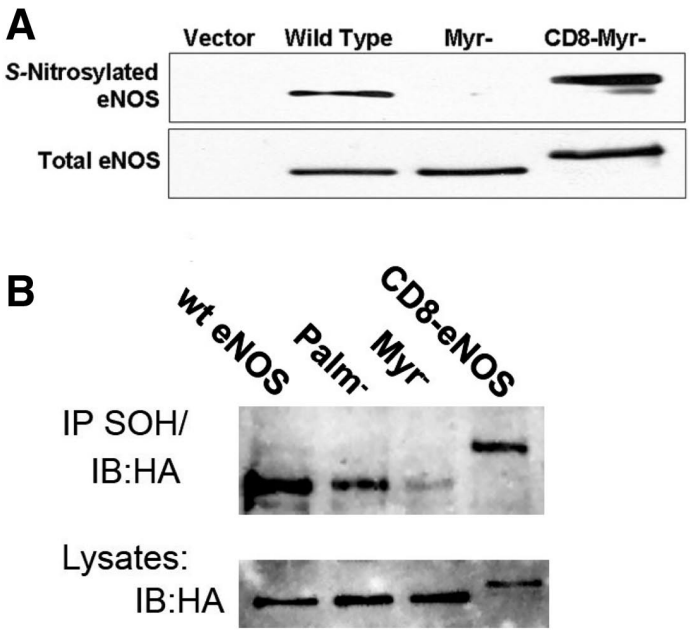

Figure 1. Endothelial nitric oxide synthase (eNOS) subcellular targeting and oxidative/nitrosative modifications. (**A**) COS-7 cells transfected with cDNA encoding recombinant wild-type eNOS, Myr-eNOS (localized to the cytosol), or CD8-Myr-eNOS (localized to the cell membrane), as indicated. Recombinant proteins were immunoprecipitated with anti-eNOS antibody from lysates of transfected cells and processed by the biotin switch method. *S*-nitrosylation levels of the various recombinant eNOS constructs and total starting eNOS levels in cell lysates (lower blot) were detected by probing immunoblots with anti-eNOS antibody. (Modified from Erwin et al.[19]) (**B**) Endothelial cells transfected with HA epitope-tagged cDNA constructs encoding wild-type eNOS, palmitoylation-deficient mutant eNOS (Palm-), myristoylation-deficient eNOS (Myr-) or CD-eNOS fusion proteins, as indicated. Cells were lysed in the presence of dimedone and immunoprecipitated with a dimedone antibody (IP SOH) and analyzed in immunoblots probed with antibody against the HA epitope tag. Shown below is a loading control of transfected lysates immunoblotted with HA antibody to document similar levels of expression of the various eNOS constructs. Collectively, these findings demonstrate that only membrane-associated eNOS – but not cytosolic eNOS – undergoes sulfenation and *S*-nitrosylation. IP, immunoprecipitation; IB, immunoblot.

tion of eNOS is dynamically regulated[9] and the enzyme is thus exposed to different concentrations of ROS depending upon where in the cell the protein is localized. Alterations in eNOS function due to changes in ROS levels may reflect either physiological or pathological responses in both normal cells and in vascular disease states. The relationship between impaired eNOS activity and the development of vascular dysfunction has been extensively studied: $NO^{\bullet}$ is a potent vasodilator molecule that also attenuates platelet aggregation, vascular smooth muscle cell (VSMC) proliferation, and negative vascular remodeling.[10] Collectively, these properties contribute to the prevention of intermediate pathophenotypes of vascular disease, including cardiac and/or vascular hypertrophy, fibrosis, and atherosclerosis. In counterpoise to these deleterious effects of ROS, there also appears to be a key physiological role for H_2O_2 in eNOS regulation.[11] Elucidating the redox-dependent mechanisms involved in the physiological and pathophysiological regulation of eNOS by ROS will provide critical new insights into vascular disease states and may lead to the identification of novel treatment targets related to eNOS and eNOS-modulated vascular responses.

The Intracellular Redox Potential: Subcellular Localization of Oxidants to Modulate Vascular Function

Vascular endothelial and smooth muscle cells normally maintain a net reductive electrochemical potential in the intracellular space, despite numerous environmental, dietary, and other acquired factors that challenge this homeostasis through increased ROS levels. Importantly, distribution of the intracellular redox potential is inhomogeneous: specific subcellular compartments have different reductive environments leading to distinct patters of protein oxidative modifications in disparate regions of the cell. One proposed hierarchy of redox potential in eukaryotic cell structures is as follows: [most oxidizing] mitochondrion>nucleus>cytoplasm>endoplasmic reticulum>extracellular [most reductive].[12] The biological importance of these differences in redox potential has direct implications for cellular signaling; for example, the local redox milieu and hydrophobicity of the cell membrane favors the formation of *S*-nitrosothiols. Indeed, many key oxidants and nitrosylating agents are non-polar, and may interact freely within the hydrophobic membrane lipid bilayer and be concentrated in biological membranes.[13] This fact has direct implications for eNOS, which is dynamically targeted to plasmalemmal caveolae. Caveolae are membrane invaginations that have a distinct lipid composition consisting of sphingolipids, glycosphingolipids, and cholesterol, while being relative depleted of phospholipids. It is not yet fully understood if and how the distinct lipid composition of caveolae affects the distribution and chemical properties of reactive nitrogen and ROS. Moreover, caveolae contain the scaffolding/regulatory protein, caveolin, a transmembrane protein that interacts with and modulates the activity of diverse signaling proteins, including eNOS. It is plausible that the unique protein and lipid environment in caveolae might play a role in modulating the oxidative and nitrosative modifications of signaling molecules that are targeted to this distinct subcellular organelle.

The development of highly specific and sensitive chemical sensors and biosensors have enhanced the specificity and sen-

Table. Reactivity of Selected Biologically Important Oxidants

Radical	Name	1- or 2-electron oxidant	Reduction potential (1 electron) (V) at pH 7	Rate constant (M/s) with GSH
$\bullet O_2^-$	Superoxide anion	1		6.7×10^{9},*
$OH^\bullet$	Hydroxyl radical	1	2.31	
$Cys^\bullet/CysS^-$	Cysteine/cystine	1	0.92	
$RO^\bullet$	Alkoyl	1	1.6	
$NO_2^\bullet$	Nitrogen dioxide	1	1.04	
HOCL	Hypochlorous acid	2		3×10^{7}
$ONOO^-$	Peroxynitrite anion	2		700**
O_2NOO^-	Peroxynitrate anion	2		3×10^{4}
H_2O_2	Hydrogen peroxide	2		0.9
$ROO^\bullet$	Peroxyl radical	1	0.77–1.44	
$NO^\bullet$	Nitric oxide	1		

Reduction potentials ($E^{o\prime}$) and reaction rates with reduced GSH are presented for various radicals and oxidants implicated directly or indirectly in the redox regulation of endothelial nitric oxide synthase. These data (adapted from Winterbourn[168]) outline the wide variation in oxidant potential; for example, superoxide anion ($\bullet O_2^-$) is 10^8-fold more reactive than hydrogen peroxide (H_2O_2). *Derived from Huie and Padmaja.[169] **Derived from Gupta et al with permission.[170]
GSH, glutathione.

sitivity whereby ROS flux can be measured in individual subcellular compartments.[14] These advances have identified intramitochondrial compartments that are responsible for ROS generation under specific (patho)biological conditions, and have also documented NOX translocation as a determinant of spatially-restricted ROS generation.[15,16] In addition to the spatially heterogeneity of ROS synthesis, it is important to note that pathways involved in ROS catabolism may also be differentially targeted within cells: it is not sufficient to consider only ROS synthesis without also analyzing ROS degradation.[17] Taken together, these novel discoveries have translated into the identification of new mechanisms that link acquired/environmental stimulators of oxidant stress with changes in vascular function. For example, Waypa et al[18] used ratiometric fluorescent protein biosensor methods to demonstrate intramitochondrial compartment-specific changes in ROS levels within pulmonary artery smooth muscle cells under hypoxic conditions. Cell exposure to hypoxia leads to an increase in ROS levels within the mitochondrial intramembrane space, but not within the mitochondrial matrix, possibly representing a redox-sensitive mechanism controlling hypoxic pulmonary vasoconstriction.

A growing body of evidence supports the hypothesis that the subcellular compartmentalization of redox-active molecules may critically regulate eNOS itself. For example, eNOS targeting plasmalemmal caveolae is a key determinant of its *S*-nitrosylation, which serves to deactivate the enzyme (see later section on *S*-nitrosylation of eNOS). By contrast, the reductive potential of the cytosol favors eNOS denitrosylation and enhances enzyme activation.[19] Whereas wild-type caveolae-targeted eNOS is robustly *S*-nitrosylated, the acylation-deficient eNOS mutant (Myr^-), which is expressed exclusively in the cytosol, fails entirely to undergo this modification (**Figure 1A**). Similarly, wild-type eNOS undergoes sulfenation, whereas the Myr^- mutant cytosolic eNOS shows no sulfenation whatsoever (**Figure 1B**). Thus, two critical redox-modulated post-translational modifications of eNOS are entirely dependent on the enzyme's subcellular localization. Because eNOS undergoes dynamic agonist modulated translocation (see review by Dudzinski et al[9]), the marked differences in the subcellular distribution of redox potential may exert a significant effect on eNOS-dependent signaling responses. In addition to differences in the local redox potential between the cell membrane and the cytosol, changes to the 3-dimensional structure of target proteins in various intracellular compartments and relative abundances of enzymes involved in *S*-nitrosylation are postulated as contributing factors in the differential activation of eNOS.[20,21]

ROS and Factors Influencing Their Subcellular Localization

The term "reactive oxygen species" describes a group of molecules derived from $\bullet O_2$ (formed from molecular oxygen) and synthesized through a series of 1-electron reduction reactions.[22] For example, superoxide anion may be reduced spontaneously[23] or dismutated by superoxide dismutase (SOD) to form the weaker oxidant H_2O_2. In turn, proton abstraction by xanthine oxidase-generated $\bullet O_2^-$ results in the formation of the strong oxidant perhydroxyl radical ($HOO^\bullet$), which may initiate fatty acid (LOOH) peroxidation in a process implicated in the pathobiology of atherogenesis and atherothrombosis.[24] Importantly, each specific ROS is associated with a unique biochemical profile, oxidizing potential, capacity to induce 1- or 2-electron oxidation reactions, and propensity for reduction in the presence of antioxidants (**Table**). Key enzymatic sources of $\bullet O_2^-$ generation include the electron transport chain in mitochondria, xanthine/xanthine oxidase, NOX (the NOX type-4 enzyme isoform may catalyze the 2-electron reduction of oxygen to directly synthesize H_2O_2), and uncoupled eNOS (reviewed by Sugamura and Keaney[25]) (**Figure 2**).

The net charge of nitrosylating agents and some oxidants (eg, H_2O_2) is a key factor in determining the subcellular compartmentalization of *S*-nitrosylation and post-translational protein oxidative modifications. For example, H_2O_2 is non-polar and lipophilic; these biochemical properties may facilitate the concentration of H_2O_2 in cell membrane-bound structures and/or permit H_2O_2 to traverse the cell membrane to transduce intercellular signaling functions.[23] Another key biochemical factor that contributes to subcellular localization is the proclivity of H_2O_2 to rapidly interact with heme-containing proteins and thiolates ($R\text{-}SH^-$).[26–28] These rapid reaction rates, which are determined in vitro in solution, support observations of the interaction of H_2O_2 with various heme-containing proteins and cysteinyl thiol(s) important in modulating eNOS-dependent cell signaling, including eNOS,[1,29,30] soluble guanylyl cyclase

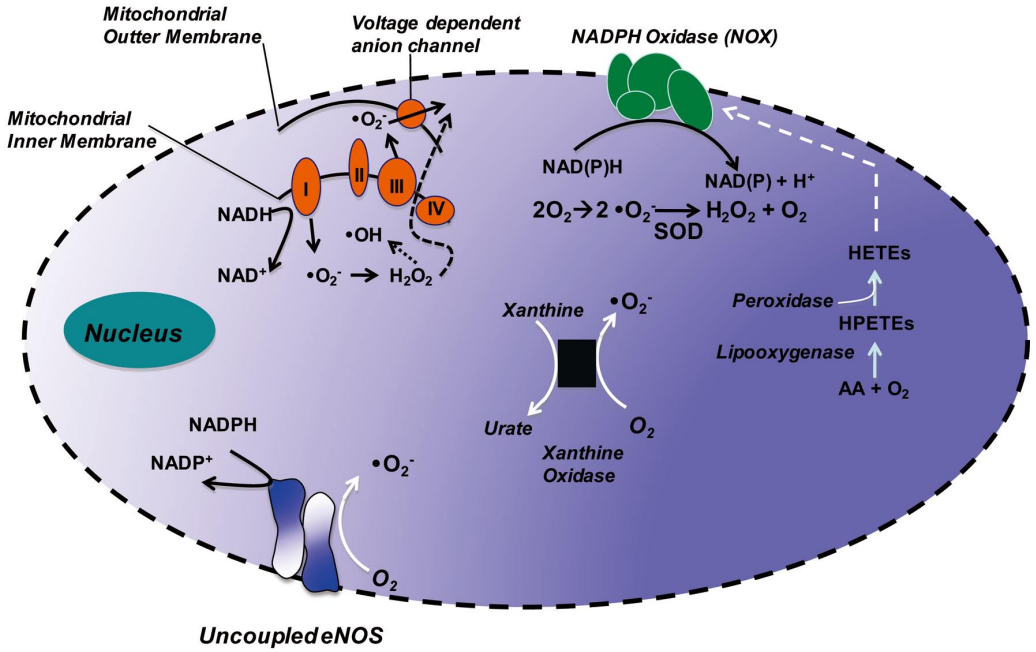

Figure 2. Key enzymatic sources of reactive oxygen species (ROS). Mitochondrial complexes I and III are important components of the electron transport chain in mammalian mitochondria that are involved in the generation of ROS. Under physiological conditions, the vast majority of all electrons that pass through this system reach the final step to react with molecular oxygen and participate in the synthesis of water. However, under pathological conditions, incomplete flow of electrons results in the preferential formation of superoxide anion ($\bullet O_2^-$) to water. Superoxide may be dismutated to hydrogen peroxide (H_2O_2) and, subsequently, to the hydroxyl radical (•OH). Importantly, H_2O_2 is able to diffuse freely across the inner/outer mitochondrial membrane (dotted line), whereas $\bullet O_2^-$ flux into the intracellular compartment is receptor-dependent. Additional important enzymatic sources of intracellular ROS generation include lipooxygenases, uncoupled eNOS, xanthine oxidase and the multimeric protein complex NADPH oxidase (NOX). I, II, II, IV are the different mitochondrial complexes. NADPH, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, 5-hydroperoxyeicosatetraenoic acid; SOD, superoxide dismutase.

(sGC),[31] glutathione,[32] thioredoxin,[33] vascular endothelial growth factor receptor-2 (VEGFR-2),[34] endothelin type-B receptor (ET_B),[35] and peridoredoxin,[36] among others. Importantly, several of these potential H_2O_2 targets are localized in cellular membranes, where the hydrophobic environment may facilitate the interaction of these proteins with H_2O_2.

Other subcellular locales have been studied in the context of site-specific targeting by oxidant stress. Mitochondrial ROS flux is complexly determined, reflecting the intricate network of enzymes regulating the metabolism of oxidants, including the electron transport chain, NOX-4, glutathione peroxidase-1, peroxiredoxins (see later), thioredoxin, and SOD. Although eNOS does not appear to localize to mitochondria, cross-talk between pathways that regulate both mitochondrial bioenergetics and eNOS have been described through the Silent mating type information regulation homolog (SIRT1) protein.[37,38] (see later section on the structure and function of eNOS).[38] SIRT1 upregulation in mitochondria occurs at the expense of increased levels of the oxidizing agent NAD^+,[39] which contributes to enhanced mitochondrial respiration. The downstream consequences of increased mitochondrial ROS flux with respect to SIRT-1-eNOS signaling are not fully resolved.

Subcellular Localization of Oxidants in a Reductive Environment

A long-standing puzzle in redox biochemistry and human disease has been the observation that oxidants, particularly H_2O_2, may regulate protein function despite the intrinsically reductive redox state of the cell and an abundance of H_2O_2-detoxifying enzymes such as catalase and peroxiredoxin.[40] Woo et al demonstrated that NOX-derived H_2O_2 generation induces phosphorylation of (membrane-bound) peroxiredoxin I (PrxI) through a mechanism involving Src, which phosphorylates and deactivates PrxI, thereby promoting the local accumulation of bioactive H_2O_2.[17] These elegant observations provide support to linking H_2O_2 and vascular disease despite normal antioxidant defenses: redox-dependent signal transduction may be governed by local regulatory mechanisms that offset the tendency for vascular cells to detoxify peroxides, thus enabling oxidant-mediated signaling.

eNOS and NO• Under (Patho)physiological Conditions

Structure and Function of eNOS

NO is synthesized in endothelial cells by eNOS, a homodimeric enzyme containing an N-terminal heme-binding (oxygenase) domain and a C-terminal reductase domain (Figure 3).

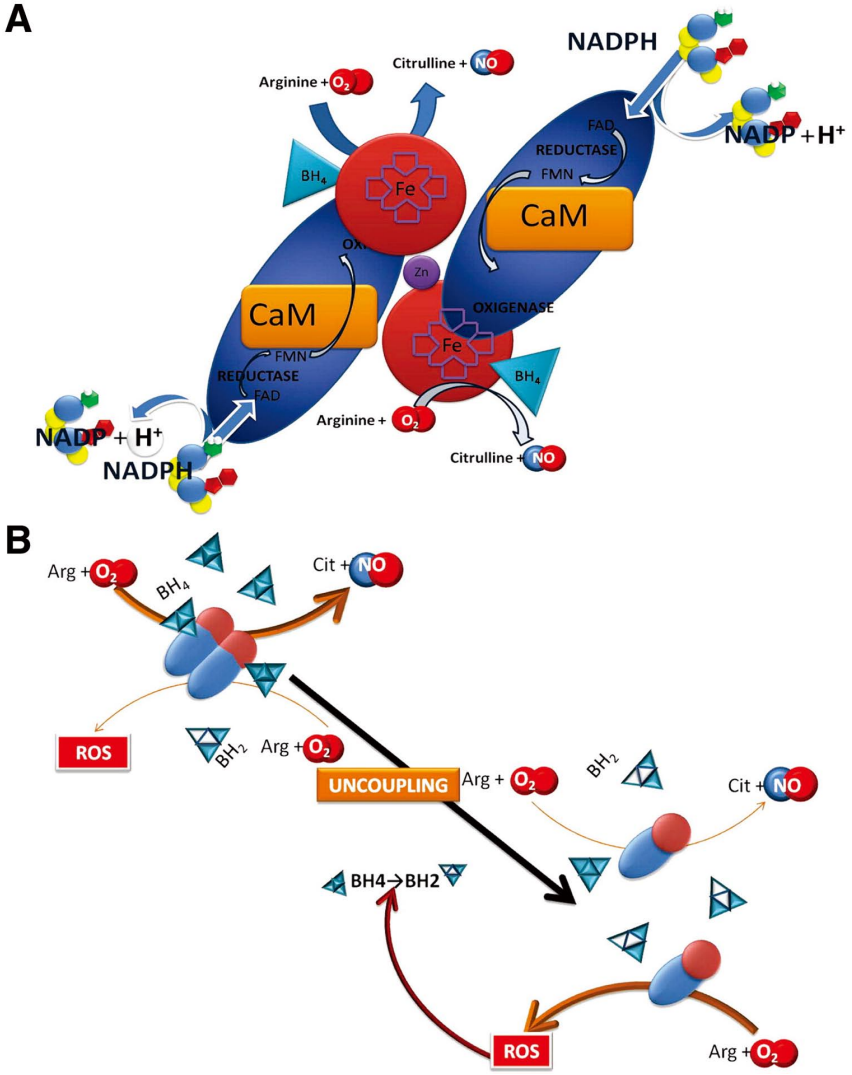

Figure 3. Molecular structure of endothelial nitric oxide synthase (eNOS). (**A**) The secondary structure of eNOS consists of a C-terminal reductase domain and an N-terminal oxidase domain, which bind heme (Fe^{2+}) and 5,6,7,8-tetrahydrobiopterin (BH_4) that is synthesized from 7,8-dihydrobiopterin (BH_2). Molecular oxygen and L-arginine bind the reductase domain, whereas the NADPH, FAD, and FMN cofactors bind the C-terminal domain. Importantly, the reductase and oxygenase domains are linked by a calmodulin-binding sequence, which is essential for maximal NOS activity. The synthesis of nitric oxide ($NO^{\bullet}$) proceeds in 2 monooxygenation steps in which 1 mole of NADPH supplies electrons to O_2, which then interacts with L-arginine to release 1 mole of water and form L-omega-N-hydroxyarginine (LHA). In the second monooxygenation step, 0.5 mole of NADPH forms reduced O_2, which utilizes LHA to form a tetrahedral intermediate, and, ultimately citrulline and $NO^{\bullet}$. (**B**) Reactive oxygen species (ROS) oxidize eNOS cofactors, such as BH_4, resulting in a shift from the dimeric to monomeric form of the enzyme. In the monomeric conformation, eNOS is uncoupled and superoxide anion ($\bullet O_2^-$) is synthesized in preference to $NO^{\bullet}$. Arg, L-arginine; CaM, calmodulin; Cit, L-citrulline; FAD, flavin adenine dinucleotide; Fe, heme; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Zn, zinc. (Reproduced with permission from Gielis et al.[171])

The reductase domain is structurally similar to cytochrome P450 reductase; however, NO synthases have the distinctive property of the oxygenase and reductase domains being separated by a calmodulin-binding region. The oxygenase domain of eNOS contains binding sites for heme, tetrahydrobiopterin (BH_4), and the substrate L-arginine.[9] The C-terminal flavoprotein oxidoreductase domain binds flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH. Under basal conditions, electron transfer between NOS domains is suppressed; however, in the setting of increased intracellular $[Ca^{2+}]_i$, the formation of a Ca^{2+}/calmodulin complex disrupts eNOS suppression from the eNOS-caveolin interaction, thereby permitting the transfer of electrons from the reductase to the oxygenase domains of eNOS.[41] The oxidation of the guanidine nitrogen of L-arginine is catalyzed by eNOS, which proceeds in 2 distinct monooxygenation steps, each involving electron transfer to molecular oxygen, ultimately resulting in the release of 1 mole each of NO and L-citrulline.[41]

Nitric Oxide Biology

Nitric oxide is a small (30 kDa) gaseous and lipophilic molecule, key properties that enable its function as a juxtacrine mediator of cell signaling processes that modulate blood vessel relaxation.[42–44] These characteristics contribute to the diffusion-limited movement of $NO^{\bullet}$ between vascular endothelial and smooth muscle cells,[45] which favors rapid intercellular signaling that is not regulated by the kinetic constraints of receptor-mediated signal transduction. The free radical structure of $NO^{\bullet}$ affords its interaction with biomolecules, including thiols (R-SH), metals (ie, Fe^{2+}), and other free radicals such as superoxide anion ($\bullet O_2^-$).[45,46] The range of $NO^{\bullet}$ molecular targets reflect the versatile biochemical framework that governs $NO^{\bullet}$ biology. For example, protein cysteine-$NO^{\bullet}$ interactions form nitrosothiols adducts, which offset the rapid degradation of $NO^{\bullet}$ and allow $NO^{\bullet}$ to target remote sites; the interaction of $NO^{\bullet}$ with the heme ligand of sGC functions as the principle mechanism underlying vascular relaxation; and the formation of peroxynitrite anion ($ONOO^-$) through the rapid interaction of $NO^{\bullet}$ with $\bullet O_2^-$,[47] functions as a key redox-sensitive counter-regulatory mechanism that may serve to limit the bioavailability of $NO^{\bullet}$.

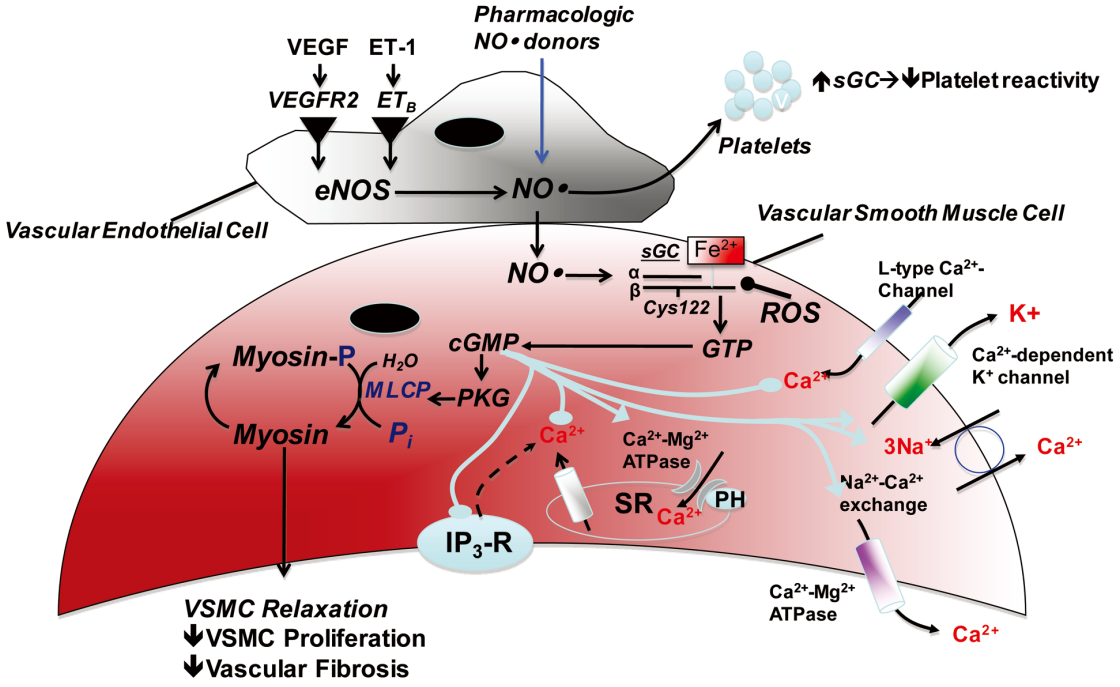

Figure 4. Pleiotropic effects of endothelial nitric oxide synthase-derived nitric oxide in vascular tissue. Endothelial nitric oxide synthase (eNOS) is the principal enzymatic source of nitric oxide (NO•) in vascular cells. Activation of eNOS occurs in response to acetylcholine, shear stress or via receptor-mediated activation by endothelin-1 (ET), vascular endothelial growth factor (VEGF), or bradykinin. NO derived from eNOS or pharmacological NO• donors diffuses across the platelet or vascular smooth muscle cell (VSMC) membrane, resulting in activation of the heterodimeric soluble guanylyl cyclase (sGC). Normal NO•-sGC signaling results in a robust increase in the synthesis of cGMP, which functions as a second messenger to stimulate protein kinase G (PKG)-dependent activation of myosin light chain phosphatase (MLCP) and promotes VSMC relaxation and inhibits platelet reactivity in platelets. Alternatively, cGMP may modulate VSMC relaxation by decreasing intracellular levels of Ca^{2+} via cGMP-induced inhibition of Ca^{2+} mobilization through L-type Ca^{2+} channels; through stimulation of the inositol triphosphate-3 receptor (IP_3-R); or by decreasing intracellular Ca^{2+} levels directly via activation of Ca^{2+}-dependent K^+ exchange, Na^{2+}–Ca^{2+} exchange, Ca^{2+}–Mg^{2+} ATPase(s), and phospholamban (PL) in the sarcoplasmic reticulum (SR). Collectively, these changes mediated by NO• modulate normal vascular tone by inducing blood vessel relaxation, inhibiting vascular cellular proliferation, and inhibiting vascular fibrosis. In turn, elevated levels of reactive oxygen species (ROS) may affect adversely normal eNOS-NO• signaling by oxidizing functional cysteines in the ET-B receptor, VEGF receptor (VEGFR2), eNOS, sGC, and/or PKG. Arrows and circular heads indicate agonist and inhibitory effects, respectively. PKG, cGMP-dependent protein kinase G-1α. (Adapted with modifications from Maron and Michel with permission.[172])

Functions of NO• in Vascular Tissue

NO• has been implicated in a number of processes that are integral to maintaining normal blood vessel structure and function.[48,49] Chief among these is cGMP-dependent protein kinase G-1α (PKG-1α)-dependent VSMC relaxation that occurs in response to activation of sGC by NO•. Low concentrations of NO• are sufficient to increase sGC activity ~30-fold, increasing cyclic guanosine monophosphate (cGMP), which activates PKG-1, leading to decreased intracellular calcium and vascular relaxation.[50] Alternatively, NO• may modulate vascular tone by targeting Ca^{2+}-dependent potassium channels directly to attenuate $[Ca^{2+}]_i$ flux.[51] NO• decreases vascular endothelial expression of adhesion molecules, such as ICAM-1,[52] P-selectin,[53] and β-1 integrin,[54] which are implicated in cellular migration, proliferation, atherogenesis, and arterial/venous thrombosis.[55] NO• exerts pleiotropic antithrombotic effects by (1) increasing sGC-derived cGMP to decrease the $[Ca^{2+}]_i$ flux that is required for activation of the platelet aggregation molecule glycoprotein IIb/IIIa,[56] (2) inhibiting thrombin,[57] (3) increasing synthesis of prostacyclin[58] and (4) increasing cAMP levels to induce phospholamban phosphorylation-mediated decreases in $[Ca^{2+}]_i$[59] (**Figure 4**).

NO• may also indirectly influence blood vessel tone by functioning as an antioxidant inhibiting the effects of ROS on vasodilatory cell signaling pathways; for example, through NO•-mediated upregulation heme-oxygenase-I (see later section on antioxidant enzymes) and SOD.[60] The dismutation of the strong oxidant superoxide $\bullet O_2^-$ to H_2O_2, which is a weaker oxidant, may also lead to activation of eNOS via the oxidation of redox-sensitive protein kinases that promote eNOS phosphorylation.[11] Alternatively, NO• may directly interact with $\bullet O_2^-$ to form $ONOO^-$, which may serve as an oxidant, leading to the nitration of protein tyrosine residues, or may react with cysteinyl thiols in glutathione, human serum albumin, or hemoglobin.[61] Additionally, NO• affects the redox status of cells via inhibition of mitochondrial oxidative phosphorylation, particularly

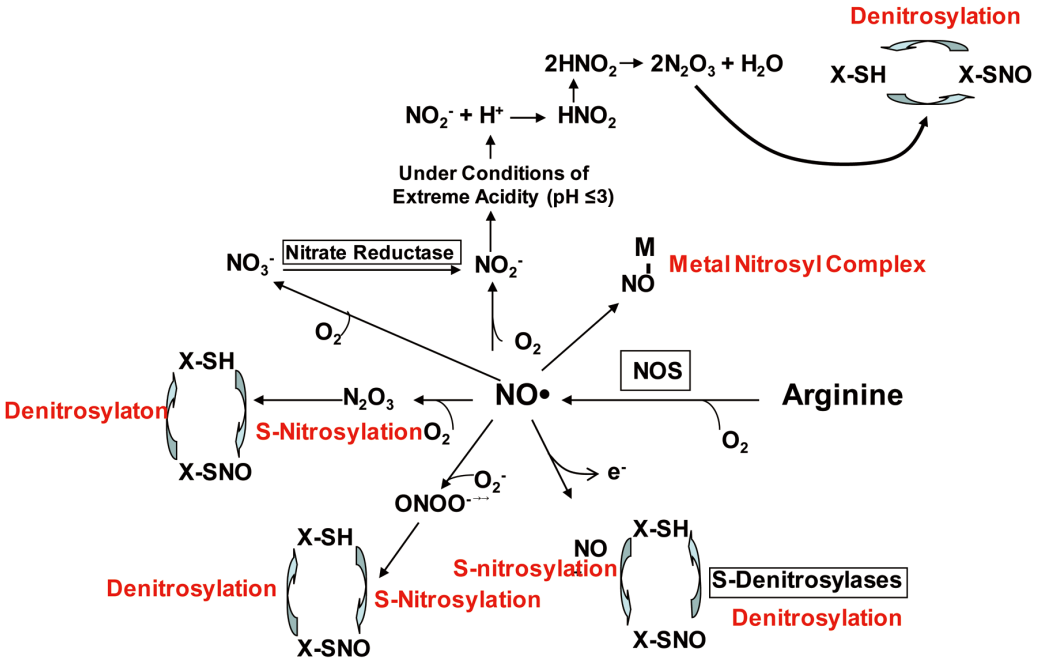

Figure 5. Biochemical mechanism(s) by which nitric oxide (NO•) serves as a nitrosylating agent in mammalian cells. Nitric oxide synthase (NOS)-derived NO• may be metabolized in the presence of diatomic oxygen (O_2) to form the nitrosylating agent dinitrogen trioxide (N_2O_3), nitrite (NO_2^-), or nitrate (NO_3^-). In turn, NO_3^- may be converted to NO_2^- in the presence of nitrate reductase; under conditions of extreme acidity, NO_2^- may undergo protonation to ultimately form N_2O_3 via the synthesis of nitrous acid (HNO_2). NO may also be oxidized (ie, consumed) to form peroxynitrite ($ONOO^-$) or may decompose spontaneously to form nitrosonium (NO^+), both of which may function as nitrosylating agents. (Adapted with modifications from Maron et al with permission.[65])

by interacting with the mitochondrial Complex IV a_3-Cu_B active site.[62]

Nitric Oxide and the Formation of *S*-Nitrosothiols

By functioning as a molecular adduct for NO•, the formation of *S*-nitrosothiols was originally proposed in order to account for the observed difference between the half-life of NO• in vitro (0.1 s) compared with its half-life in vivo (~6 s).[63] Early experiments studied the cysteine-rich structure of human serum albumin, which contains 17 disulfide bonds and a free cysteine at position Cys34 that has an anomalously low pKa, and is therefore highly reactive, to demonstrate that human plasma contains 7 μM *S*-nitrosothiols, of which approximately 80% are accounted for by *S*-nitroso-serum albumin.[64] The functional consequences of protein *S*-nitrosylation are recognized as being implicated in many aspects of cardiovascular (patho)physiology, including atherogenesis, regulation of NOS activity (see later), electrophysiologic signaling, myocyte contractility, formation of nitrosohemoglobin, regulation of mitochondrial bioenergetics, and the modulation of hypoxic pulmonary vasoconstriction (reviewed by Maron et al[65]).

Although the biochemistry of protein *S*-nitrosylation is complex, it is clear that NO• does not spontaneously bind to thiols under physiological conditions (Figure 5). *S*-nitrosylation occurs through liberation of the nitrosonium ion (NO^+) to covalently bind thiols during the metabolism of reactive nitrogen oxides such as N_2O_3, which is a strong nitrosating agent.[66] The predilection for NO^+ to participate in bonding with thiols depends on the relative nucleophilicity of thiol functionalities, with the thiolate ion ($R\text{-}S^-$) serving as a strong nucleophile and, thus biochemically favorable for interaction with NO^+ to form *S*-nitrosothiols.[67] Importantly, the distribution of nitrosylating agents within the intracellular compartments is indicative of each agent's biochemical properties. For example, the nitrosylating agent N_2O_3, which is lipophilic and known to form within protein-hydrophobic cores,[68] tends to localize to the lipid bilayer of cell membranes. This pattern of N_2O_3 subcellular distribution is consistent with that observed for other lipophilic nitrosylating agents and is a factor that favors the cell membrane as a site of *S*-nitrosylation.[13,19,69]

Role of eNOS and NO• Deficiencies in Human Disease

Deficient bioavailable/bioactive levels of NO• are associated with the development of important human vascular diseases, including myocardial infarction, stroke, essential hypertension, and pulmonary hypertension.[1] Analyses of genetically engineered animals deficient in eNOS expression ($eNOS^{-/-}$) provide support for the importance of eNOS-derived NO in vascular homeostasis. For example, $eNOS^{-/-}$ mice, which lack eNOS-derived NO•, develop numerous vascular disease states, including systemic hypertension, increased leukocyte-endothelium adhesion,[70] platelet aggregation and thrombosis,[71] atherogenesis,[72] and abnormal pulmonary vascular reactivity.[73]

Compensatory upregulation of the neuronal/inducible NOS isoforms has been proposed as a mechanism to account for detectable levels of bioavailable NO• in $eNOS^{-/-}$ mice. At low pH, dietary nitrite (NO_2^-) derived from nitrate (NO_3^-) synthesized by commensal bacteria may be converted to nitrous acid (HNO_2) and spontaneously form NO•[74] (Figure 5).

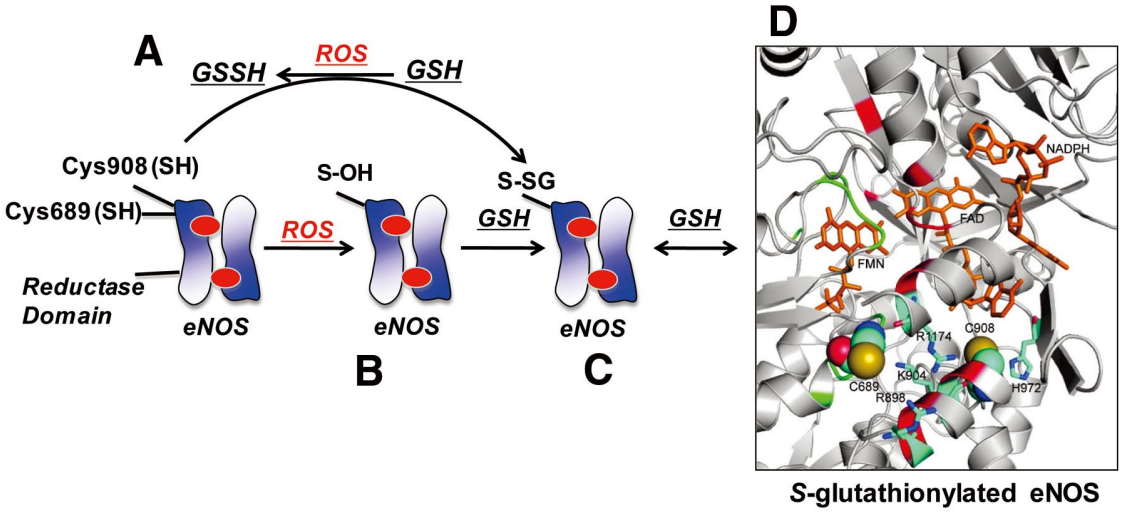

Figure 6. *S*-glutathionylation of endothelial nitric oxide synthase (eNOS). *S*-glutathionylation of eNOS reductase domain cysteinyl thiols Cys689 and/or Cys908 occurs via (**A**) thiol-disulfide exchange with oxidized glutathione (GSSG) or (**B**) oxidation to form protein thiyl radical(s) or cysteine sulfenic acid (S-OH) and subsequent conversion by reduced glutathione (GSH) to form (**C**) a mixed disulfide (R-SSG). The conversion of R-SSG to (**D**) *S*-nitrosylated eNOS is a reversible reaction catalyzed by GSH. The gold, green, red spheres in (**D**) represent the tripeptide glutathione whereas the blue sphere represents the putative cysteine(s) serving as *S*-glutathionylation sites. NADPH, nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide. (Adapted with modification from Zweier et al with permission.[84])

Redox Regulation of eNOS: Enzyme Post-Translational Modifications

Changes to the local redox potential may affect eNOS-dependent NO• synthesis in several ways. First, ROS, particularly $\bullet O_2^-$, may oxidize BH_4 to form BH_3, which is unable to serve as an eNOS cofactor.[75] As a result, eNOS "uncoupling" occurs, resulting in the preferential formation of $\bullet O_2^-$ (and a peroxyl radical of BH_4) (Figure 3A). eNOS uncoupling is also associated with decreased L-arginine and/or accumulation of methylarginines, which affects the balance of eNOS-derived $\bullet O_2^-$/NO• synthesis.[78] Increased ROS generation may consume NO•; for example, via formation of reactive nitrogen species (RNS) such as $ONOO^-$ or peroxynitrate (O_2NOO^-), synthesized from the interaction of $\bullet O_2^-$ and NO_2^-, which is a substrate for NO• synthesis. Additionally, protonation of $ONOO^-$ results in the formation of other RNS, including peroxynitrous acid (ONOOH), the conjugate acid of $ONOO^-$, which itself is homolyzed to form nitrogen dioxide (NO_2) and hydroxyl radical (OH•) or nitronium ion (NO_2^+) and hydroxide (OH^-) [$OH^\bullet + NO_2 \leftarrow HOONO \rightarrow OH^- + NO_2^+$].[79]

S-Glutathionylation

BH_4 supplementation in the presence of increased ROS modulates only partial restoration of eNOS-dependent NO• synthesis[30] activity, suggesting alternative mechanism(s) that link oxidant stress with enzyme activity. The redox status of specific eNOS-thiol(s) may influence the rate of L-arginine conversion to NO• by eNOS.[80] Differential effects of regulatory thiol(s) on enzyme activation have been described between other NOS isoforms.[81] Recently, 2 cysteine residues located within the eNOS reductase domain were identified as *S*-glutathionylation targets.[82] These thiols are believed to reside on the surface of the eNOS reductase domain. Molecular modeling suggests that these cysteines are likely to be surrounded by amino acid residues that permit nucleophilic attack by the thiolate anion.[83] *S*-glutathionylation has been demonstrated to induce eNOS uncoupling (Figure 6). In turn, site-directed mutagenesis substituting cysteine 908 for alanine abolished eNOS thiyl radical formation.[83] Oxidant stress generated through the *S*-glutathionylation reaction is not inhibited by competitive inhibitors of the NOS substrate L-arginine.[84] The relevance of this pathway has been studied in Wistar rats, in which *S*-glutathionylation is associated with impaired endothelium-dependent vasodilation.[85]

S-Nitrosylation of eNOS

In addition to *S*-glutathionylation, alternative thiol-dependent eNOS post-translational modifications have been studied to account for the inhibitory effect of NO• on enzyme activity. It has been established that *S*-nitrosylation is involved in the dynamic regulation of eNOS. Inhibition of basal eNOS activity is associated with tonic *S*-nitrosylation at the zinc-tetrathiolate cysteine residue Cys101/107.[34] eNOS stimulation with receptor-dependent agonists results in eNOS denitrosylation and increased eNOS-dependent NO• synthesis.[34] A shift from dimeric- to monomeric eNOS due to *S*-nitrosylation may influence enzyme function,[86] although it is not yet clear that dynamic regulation of eNOS dimer formation is a physiologically relevant mechanism for controlling eNOS activity. Other eNOS cysteine thiols may also undergo *S*-nitrosylation in vitro, although the functional consequences for cellular regulation have not been established.[87–89]

Hydrogen Peroxide-Mediated Redox Regulation of eNOS

The functional effects of H_2O_2 are linked to cell type, H_2O_2 concentration, and treatment conditions. For example, NOX-dependent synthesis of (pathophysiological) H_2O_2 concentra-

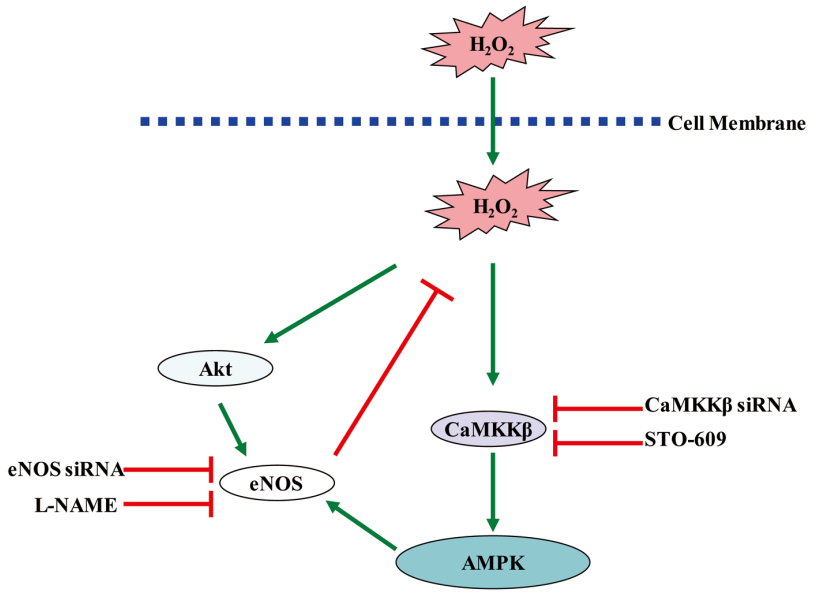

Figure 7. Model for eNOS regulation of CaMKKβ/AMPK pathways via H_2O_2. Genetic or pharmacological suppression of the eNOS-NO pathway increases intracellular H_2O_2 levels and thereby leads to CaMKKβ/AMPK-dependent phosphorylation of AMPK. In this model, H_2O_2 directly or indirectly activates CaMKKβ. The phosphorylated AMPK, in turn, phosphorylates and activates eNOS, representing a feedback mechanism controlling this pathway. AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase. (Adapted with modifications from Jin et al.[101])

tions in neutrophils is a central component of the immune response to pathogens.[90] Elevated levels of H_2O_2 derived from non-neutrophilic NOX isoforms in response to angiotensin II, endothelin-1, and aldosterone, among others,[91] are implicated in the development of systemic hypertension[92] and pulmonary hypertension[35] by inhibiting eNOS-dependent vasodilation. By contrast, physiological concentrations of H_2O_2 promote cell signaling processes, including normal cell proliferation,[93] migration, and survival,[94] as well as activation of eNOS.[11]

In addition to the effects on eNOS that derive from extra-endothelial H_2O_2 sources, endogenous H_2O_2 may also play a key role in eNOS signaling.[95] H_2O_2-dependent activation is NOS isoform-specific, as well as receptor-specific; for example, H_2O_2 activates eNOS but not nNOS in cardiac myocytes.[96] These changes in eNOS activity are dependent upon phosphorylation and activation of both the AMP-activated protein kinase and kinase Akt. Discrete receptor-dependent H_2O_2-modulated protein phosphorylation pathways lie upstream of eNOS, and reflect the differential roles of H_2O_2 in receptor pathways leading to eNOS phosphorylation and enzyme activation.

Redox Regulation of Signaling Pathways Upstream and Downstream of eNOS Activation

Upstream signaling intermediaries implicated in eNOS activation have been proposed as redox-sensitive targets include bradykinin[97,98] and caveolae.[99,100] Here, the mechanistic and functional consequences of perturbations to the intracellular redox status on Akt, the ET_B receptor, and vascular endothelial growth factor (VEGF) in the context of eNOS activation are discussed.

Akt- and AMPK-Dependent eNOS Phosphorylation

Whereas lower concentrations of H_2O_2 (<100 μmol/L) are associated with physiological cell signaling, higher H_2O_2 concentrations (≥500 μmol/L) may suppress eNOS phosphorylation and enzyme activation.[101,102] The AMP-activated protein kinase (AMPK)-stimulated phosphorylation of eNOS was found to be dependent upon H_2O_2-mediated phosphorylation of the upstream protein kinase CaMKKβ. Thus, H_2O_2-modulated protein phosphorylation pathways play a key role in the regulation of eNOS activation. In turn, eNOS activity may modulate cellular levels of H_2O_2. Studies using the H_2O_2 biosensor, HyPer,[103] showed that eNOS knockdown led to increased H_2O_2 and AMPK phosphorylation in endothelial cells.[101] Clearly, there is a close interplay between physiological levels of H_2O_2 and eNOS-dependent signaling pathways (**Figure 7**). Higher H_2O_2 concentrations appear to have broader effects on oxidant-modulated signaling pathways.[102] Higher H_2O_2 concentrations are associated with increased depletion of free thiols, possibly via the formation of higher oxidative cysteine intermediates and subsequently diminished eNOS phosphorylation. These data suggest a broader role for soluble as well as membrane-bound thiol oxidative post-translational modification(s) as a physiological regulatory mechanism for eNOS phosphorylation. Depletion of sulfhydryls by excessive oxidant stress may impair upstream signaling pathways required for normal regulation of eNOS. These observations underscore the important differences between physiological levels of H_2O_2 that are involved in normal cell signaling to eNOS and the pathological effects on eNOS of high concentrations of H_2O_2 that may arise as a consequence of oxidative stress.[103]

ET_B Receptor

The ET_B receptor is a heptahelical G-protein coupled receptor containing an intracellular cysteine-rich region near the N-terminus that is involved in normal ET_B signal transduction.[104,105] Recently, it has been demonstrated that exposure of cultured human pulmonary artery endothelial cells to oxidant stress results in the oxidative modification of these ET_B cysteinyl thiol(s), resulting in the formation of higher oxidative species of cysteine, including sulfenic acid (R-SOH) (**Figure 8**). Cells treated with pathophysiological concentrations of aldosterone akin to levels observed in animal models and in humans with pulmonary arterial hypertension induce NOX-4-dependent H_2O_2 generation in vascular cells.[35,92] Protein extracts from treated cells were immunoprecipitated using an anti-ET_B

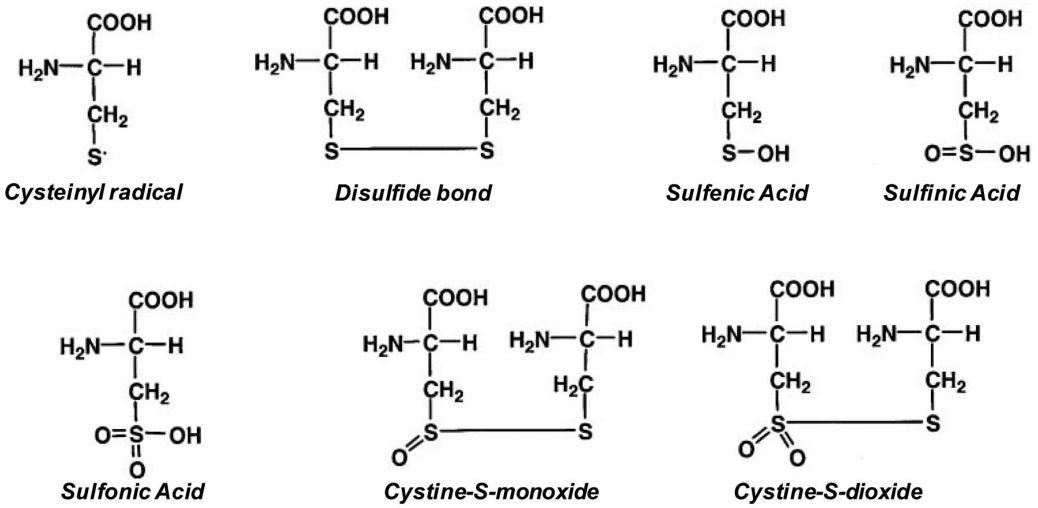

Figure 8. Higher oxidative intermediates of cysteine. Cysteine is a non-essential sulfur-containing amino acid. Reduced cysteine (R-SH) may undergo oxidation in the presence of oxidants, such as hydrogen peroxide, to form higher oxidative intermediates of cysteine. The conversion of reduced cysteine to cysteinyl radical, sulfenic acid or the disulfide form is reversible.

antibody specific to the region of ET_B containing the putative cysteines and immunoblotting was performed using an anti-sulfenic acid (R-SOH) antibody.[106,107] Site-directed mutagenesis substituting cysteine for alanine at ET_B Cys405 (C405A) inhibited disulfide bond formation in the presence oxidant stress and preserved ET_B-dependent activation of eNOS and $NO^{\bullet}$ synthesis, suggesting that ET_B Cys405 is a critical regulatory cysteine involved in ET_B-eNOS signaling.[35]

VEGF

The major vascular endothelial growth factor target receptor, VEGFR2, is a tyrosine kinase receptor that modulates various functional effects important to vascular biology.[108] Stimulation of VEGFR2 is associated with Akt-dependent phosphorylation of eNOS, whereas monoclonal antibody-mediated inhibition of VEGFR2 is a bona fide strategy by which VEGF-dependent cellular proliferation is inhibited in various forms of cancers.[109] The redox status of VEGFR2 has been studied with respect to eNOS activation. In an experimental model, the effect of cigarette smoke, which contains ROS and RNS,[110] was used to study redox regulation of VEGFR2-eNOS signaling. Cigarette smoke induced vascular ROS generation in mouse lungs, and exposed lung microvascular endothelial cells in culture showed downregulated VEGFR-2 and eNOS levels, and decreased VEGFR-2 phosphorylation.[111] Although RNS/ROS have been shown to interact directly with VEGFR-2 to modulate this effect through nitration of receptor tyrosine residues,[112] the mechanism by which this modulates receptor deactivation in vascular cells is not fully resolved.

Targets upstream of VEGFR-2 that modulate the inhibition of oxidant stress on VEGF-eNOS signaling have been identified. For example, inhibition of poly [ADP-ribose] polymerase (PARP-1), which is a DNA base repair enzyme activated in the presence of oxidant stress, is associated with attenuation of H_2O_2 or $ONOO^-$-mediated inhibition of VEGFR-2 phosphorylation in endothelial cells.[113] In human umbilical vein endothelial cells and rabbit aortas, inhibition of PARP-1 restores VEGF- and Akt- phosphorylation. PARP-1 inhibition restores eNOS activity and endothelial function in blood vessels of apolipoprotein E ($ApoE^{-/-}$) mice fed a high fat diet to induce atherosclerosis.[114]

sGC

sGC is a heterodimeric enzyme (α_1/β_1; α_2/β_1) that is activated by $NO^{\bullet}$ and results in the conversion of GTP to the second messenger cGMP, which is a potent stimulator of VSMC relaxation.[115] Normal $NO^{\bullet}$ sensing by sGC requires that the prosthetic heme ligand located near His105 on the β-subunit is reduced (Fe^{2+}). In contrast, ROS-mediated oxidation of heme to the ferric (Fe^{3+}) form impairs $NO^{\bullet}$-induced sGC activation and is implicated as a potential mechanism linking oxidant stress to the development of essential hypertension, pulmonary hypertension, stroke, and myocardial infarction.[116,117] Activation of heme-oxidized sGC is a contemporary pharmacotherapeutic target in these diseases. A family of novel synthetic compounds derived from 5-substituted-2-furaldehyde-hydrazone, which are believed to compete with oxidized heme for the enzyme-activating pocket of sGC, effectively activates heme-free sGC in vitro and is under study in ongoing clinical trials for the treatment of pulmonary hypertension.[118]

There is evolving evidence to suggest that sGC contains essential redox-sensitive cysteinyl thiols that participate in the regulation of $NO^{\bullet}$-sGC signaling.[31,119,120] The regulation of sGC by the redox status of functional cysteinyl thiol(s) and heme is interrelated and hierarchical: $NO^{\bullet}$-heme binding kinetics, and, thus, enzyme activity, are enhanced by $NO^{\bullet}$-binding to regulatory sGC thiols.[31,121]

PKG-1α

PKG-1α is the principal target of cGMP. Activation of the cGMP-PKG-1α axis stimulates the phosphorylation of numerous target proteins that is required for maintaining vascular tone and platelet function, as well as other key cellar processes such as cell division and nucleic acid synthesis. Regulation of PKG-1α dimerization involves Cys42 on each homodimer; oxidation of Cys42 results in activation of PKG-1α via

intermolecular disulfide bridge formation.[122] The change in PKG-1α due to disulfide bridge formation enhances its affinity for biological substrates.[123] Consistent with this are observations demonstrating that PKG-1 is activated by H_2O_2 or other thiol oxidants in an sGC-free system.[124,125] Genetically engineered mice containing a substitution cysteine with serine at position 42 of PKG-1α are incapable of PKG-1 dimerization and show decreased response to $NO^{\bullet}$ donors in vivo.[122]

Antioxidant Enzymes and the Cellular Redox Potential

Maintenance of the normal intracellular redox status relies upon endogenous antioxidant enzyme defenses. On the other hand, genetic or acquired antioxidant enzymopathies contribute to a disrupted cellular redox balance due to the depletion of intracellular reducing equivalents, which results in the accumulation of oxygen free radicals and their derivative oxidative species.

Glucose-6-Phosphate Dehydrogenase (G6PD)

G6PD catalyzes the first and rate-limiting step of the pentose phosphate pathway[126] in a reaction that requires $NADP^+$ and results in the conversion of G-6P to 6-phosphogluconate and the synthesis of NADPH. In turn, NADPH is (1) a key intracellular reducing equivalent by maintaining glutathione in the reduced form,[127] (2) a cofactor utilized by eNOS to generate $NO^{\bullet}$,[128] and (3) a key participant in the maintenance of normal BH_4 levels via the dihydrofolate reductase salvage pathway.[129] Long recognized as the primary mechanism by which to account for oxidative injury in erythrocytes,[130] impaired G6PD activity has also been linked to a swathe of other pathobiological processes, including diminished eNOS expression and activity that results in vascular endothelial dysfunction,[128] progressive myocardial dysfunction in response to ischemia-reperfusion injury,[131] pulmonary hypertension,[132] and renal dysfunction with albuminuria.[133] Endothelial cell migration and angiogenesis are also G6PD-dependent processes because enzyme deficiency is linked to diminished VEGF-dependent angiogenesis via decreased eNOS phosphorylation.[127] By contrast, 5-methyltetrahydrofolate is associated with improved endothelial function owing to its $ONOO^-$ scavenging effects, which, in human saphenous vein grafts is associated with an increase in the BH_4/superoxide ratio.[98]

Glutathione Peroxidase (Gpxs)

Gpxs are a family of 4 biologically relevant (GPx-1–4) selenocysteine-containing enzymes that utilize reduced glutathione, a low-molecular-weight tripeptide, to reduce H_2O_2 to water, and lipid hydroperoxides to their corresponding alcohols.[134] Detoxification of peroxides by Gpxs occurs via bidirectional second-order kinetics, and, thus is a saturation-limited process.[135] The reaction is enabled by the unique properties of selenium, which reacts with peroxide to convert selenol (R-SeH) to selenic acid (R-SeOH).[134,136,137] Next, 2 moles of glutathione (GSH) reduces R-SeOH to form initially the R-SG intermediate and, subsequently, oxidized glutathione (GSSG). Of the GPx isoforms, GPx-1 is the most abundant in mammals. Gpx-1 is present in all cells and localizes to the cytosolic, mitochondrial, and peroxisome cellular ultrastructures.[138–140] Deficiencies in GPx-1 are associated with increased vascular oxidant stress levels and endothelial dysfunction.[141] For example, in mice heterozygous for GPx-1 (GPx-$1^{+/-}$), mesenteric arterioles demonstrate increased vascular tone in response to (the eNOS activator) acetylcholine as compared with wild-type mice.[142] Interestingly, however, other reports have demonstrated that GPx-1 levels are elevated in rodent models of hypertension in vivo. Increased GPx-1 mRNA and protein expression levels are observed in phenylephrine pre-contracted aortic rings of spontaneously hypertensive rats, which is associated with impaired endothelium-dependent vascular relaxation despite attendant elevations in eNOS protein expression and activity level.[143] Authors of that study implicate consumption of $NO^{\bullet}$ by $\bullet O_2^-$ rather than via direct inhibition of eNOS per se as the mechanism by which to account for these findings. Collectively, these observations illustrate the complexities inherent in anticipating the (pathological) effect(s) of vascular oxidant stress on eNOS–$NO^{\bullet}$ signaling; viz., vessel type-specific properties (ie, systemic resistance or conduit vessels, pulmonary arterioles, etc) and other unpredictable factors that influence reaction kinetics upstream/downstream of eNOS complicate a priori predictions for determining the precise extent to which ROS affect eNOS-dependent signaling.

Glutathione Reductase (GR) and Thioredoxin Reductases

The NADPH-dependent enzyme GR reduces oxidized glutathione (GSSG) to reduced glutathione (GSH), which, in turn, is required for Gpx-dependent reduction of peroxides (as discussed above).[144,145] This is accomplished through the formation of a FAD-bound homodimer in GSR, which results in the generation of the $FADH^-$ anion that, in turn, reduces (or 'breaks') the GSSG Cys58-Cys63 disulfide bridge.[146] This allows for subsequent nucleophilic attack of the newly formed thiolate ion ($R\text{-}SH^-$) to generate a mixed disulfide bond, and, ultimately, 2 GSH molecules. Akin to GR, thioredoxin reductase (Trx) is also a flavin thiol-disulfide oxidoreductase. Trx maintains the intracellular thiol redox status by reducing targeting thiol disulfides through the reduction of oxidized thioredoxin, at the expense of NADPH-derived electrons.[147] The family of peroxiredoxins that catalyze the reduction of hydroperoxides belongs to the larger thioredoxin superfamily. The connections between subcellular localization of thiol-metabolizing proteins and physiological cell signaling is exemplified by a recent study in which the phosphorylation of peroxiredoxin-I resulted in the enzyme inactivation and local accumulation of H_2O_2.[17] These physiological roles of H_2O_2 must be contrasted with the effects of pathological levels of ROS, which may serve to deplete thiol-based antioxidants in vascular endothelial cells, associated with eNOS dysfunction, cellular senescence, and apoptosis.[29,148]

Because of the importance of ROS in modulating eNOS activity, the relationship between GR/Trx and eNOS is a target of contemporary scientific investigations. In cultured aortic endothelial cells, pharmacological or molecular inhibition (with siRNA) of GR, TrxR-1, or mitochondrial TrxR-2 is associated with diminished bioavailable $NO^{\bullet}$ because of a decrease in the ratio of reduced-to-oxidized glutathione, VEGF stimulation of the eNOS activator Akt, and eNOS phosphorylation at Ser1179.[29] Interestingly, in that study, TrxR-2, which is localized primarily to the mitochondrial matrix, but not TrxR-1 or GR inhibition, was associated with a robust increase in H_2O_2 generation that was not abrogated by BH_4 supplementation. This observation illustrates the differential downstream effects on phosphorylation signaling pathways of redox potential changes associated with Trx-1, TrxR-2, and/or GR, and raises speculation that a hierarchical order by which mitochondrial thiol oxidoreductases function is most likely among these enzymes in order to modulate eNOS activation.

Protein Disulfide Isomerase (PDI)

PDI is present on the cell surface and catalyzes the thio-disulfide exchange reaction, which, for example, facilitates via trans-*S*-nitrosylation the traversing of exogenous *S*-nitrosoproteins (ie, GSNO) into the intracellular compartment.[149,150] The functional effects of PDI on eNOS signaling remain elusive, but probably involve interactions with metal binding proteins. For example, metallothionein is a small (7kDa) cysteine-rich protein that functions as an antioxidant and intracellular buffer against the toxic effects of non-essential metals.[151] In the setting of (1) PDI-facilitated entry of $NO^{\bullet}$ into the intracellular compartment or (2) rapid increases in $[Ca^{2+}]_i$ flux resulting in increased levels of bioavailable $NO^{\bullet}$, metallothionein is targeted by $NO^{\bullet}$ (and Fe^{2+}) to generate iron nitrosyl thiolates as detected by electron spin resonance (with unique *g* value signaling of 2.013 and 2.039, respectively).[152] This is postulated to occur via *S*-transnitrosation that induces the release of metallothionein-containing metals, including cadmium and zinc.[151] Overall, these findings provide a potential mechanism by which to account for the protective effects of metallothionein against eNOS-derived nitrosative stress in some vascular (and non-vascular) cell lines.[153–155]

Heme Oxygenase

Heme oxygenase is a 32-kDa enzyme that exists as inducible (HO-1) and constitutive isoforms (HO-2).[156] Heme oxygenase is the first and rate-limiting step in the metabolism of heme into equimolar concentrations of carbon monoxide, Fe^{2+}, and biliverdin[157] in a reaction that requires molecular oxygen and NADPH. Free intracellular Fe^{2+} upregulates the expression of heavy-chain ferritin and Fe^{2+}-dependent channels,[158] whereas biliverdin is subsequently reduced by biliverdin reductase to form billirubin, a peroxyl radical scavenger. Thus, it follows that HO-1 is a key molecular player in the prevention of lipid peroxidation.[159] Bilirubin also exerts vascular-protective effects by attenuating apoptosis and cellular proliferation.[160] In support of this claim are investigations involving translational animal models of atherosclerosis, which have linked deficient HO-1 activation with atherogenesis. For example, in a vulnerable plaque model using $ApoE^{-/-}$ mice, increased HO-1 expression positively associates with atherosclerotic plaque features linked to increased plaque vulnerability, including elevated lipid and macrophage content. Similar findings have also been reported for human carotid endarterectomy samples, in which HO-1 expression levels correlate with thrombogenicity and erythrocyte extravasation, both of which are markers associated with plaque rupture.[161]

The relationship between vascular oxidant stress, HO-1, and NOS activation has also been studied. Durante et al demonstrated that cytokine-mediated increases in $NO^{\bullet}$ result in L-NAME-inhibitable upregulation of HO-1 protein expression levels in VSMC.[160] Similar results have been observed in cultured vascular endothelial cells, in which treatment with the exogenous $NO^{\bullet}$ donors sodium nitroprusside, *S*-nitroso-N-acetylpenicillamine, and 3-morpholinosydnonimine upregulates HO-1.[162] The functional effects of $NO^{\bullet}$-dependent HO-1 activation include sustained cell viability in the presence of pathological concentrations of oxidant stress (eg, H_2O_2 250 μmol/L).[163] Moreover, by decreasing ROS levels, upregulation of HO-1 is, in turn, associated with restored eNOS activation despite conditions of increased oxidant stress. In a study of diabetic rats, low constitutive levels of eNOS expression in the aorta could be enhanced by pharmacologic stimulation of HO-1 using cobalt protoporphyrin.[164]

The redox-sensitive regulation of $NO^{\bullet}$–HO-1 signaling appears to depend, in part, on local thiol concentration levels. For example, SNAP- and hypoxia-dependent upregulation of HO-1 in vascular endothelial and smooth muscle cells, respectively, is attenuated in the presence of the glutathione precursor N-acetylcysteine,[165,166] although the biological importance of this association is not fully resolved.

Conclusions

Discoveries over the previous decade have enhanced our understanding of the redox-dependent mechanisms involved in the regulation of eNOS-dependent signaling pathways in discrete subcellular compartments, both in the context of physiological oxidant signaling pathways and in disease states. The identification of subcellular targets of ROS, as well as of functional redox-sensitive cysteinyl thiols in eNOS and its signaling partners, has expanded the number of potential future treatment targets to modify cardiovascular diseases associated with impaired eNOS function. Because of the biologically diverse enzymatic sources of ROS functioning, both in normal tissues and in pathological oxidant stress, it is likely that therapies aiming to preserve eNOS signaling will require the identification of site-specific therapies rather than nonspecific antioxidant treatments alone. Efforts to identify druggable eNOS sites are ongoing; for example, systematic enrichment of ligands by exponential enrichment (SELEX) technology[167] may represent a promising technology implemented to synthesize an eNOS site-selective aptamer against particular redox-sensitive cysteinyl thiol(s) in order to preserve eNOS function despite an unfavorable local redox milieu. The success of these endeavors, however, is likely to hinge on a more comprehensive understanding of the underpinnings of the redox-sensitive mechanisms that regulate the eNOS-$NO^{\bullet}$ signaling axis both in normal vascular physiological signaling pathways and in disease states.

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Disclosures

None.

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