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Forum on Nitric Oxide: Chemical Events in Toxicity

Nitric Oxide Regulation of Tissue Free Radical Injury

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Introduction

Nitric oxide (•NO, nitrogen monoxide)[1] exerts potent actions in the regulation of cell function and tissue viability. These properties extend beyond the recognized ability of •NO to mediate signal transduction via stimulation of guanylate cyclase-mediated cGMP synthesis. Chemical reaction systems, cell and animal models, and clinical studies have recently revealed an ability of •NO to modulate reactions and pathologic processes long associated with the excess production and biological effects of reactive oxygen species. The focus of this review will be to discuss the observed prooxidant and antioxidant reactions of •NO in the context of what is currently understood about the target molecule reactivities of this free radical species. Since the accompanying reviews in this series concentrate on the potentially toxic mechanisms of •NO, we will focus on those interactions that lie at the crossroads between cytotoxicity (*1*, *2*) and cytoprotection, building a case from the literature and our own recent observations that the cytoprotective effects of •NO can often be ascribed to its antioxidant properties and its ability to redirect the reactivity of partially reduced oxygen species (Figure 1).

Free radicals play an essential role in many important metabolic processes which require the transfer of single electrons. Rarely are such free radicals released from the active site of enzymes, unless stabilized by delocalization of the unpaired electron. Those free radicals formed outside such protective environments may be toxic in two ways. First, they can interact with metal or organic redox centers and promote irreversible oxidation reactions outside normal catalytic cycles and inactivate the target metabolic process. Second, free radicals have the capacity to initiate reactions which then become self sustaining through the regeneration of propagating radicals. In either case, this can result in deleterious effects on the cell. The most effective protection against these processes is to terminate the radicals that sustain propagation or scavenge the initiating radical. A number of such antioxidant protective systems have been identified in the cell, and in this review, we exemplify the hypothesis that •NO also plays a key role in these processes. Many of the same chemical and physical properties of NO which support its antioxidant actions also allow it to mediate cytotoxic effects. Thus, there is no conceptual flaw in holding the view that the superoxide anion ($O_2^{\bullet -}$) and metalloprotein reactions of •NO lead to prooxidant or pathologic events, since there is already a solid foundation of experimental support for this occurrence. Rather, we emphasize herein that an important area of investigation beckons on the horizons of free radical biology and medicine—namely, defining the biochemical and physiological conditions which regulate the manifestation of tissue-protective versus toxic reactions of •NO.

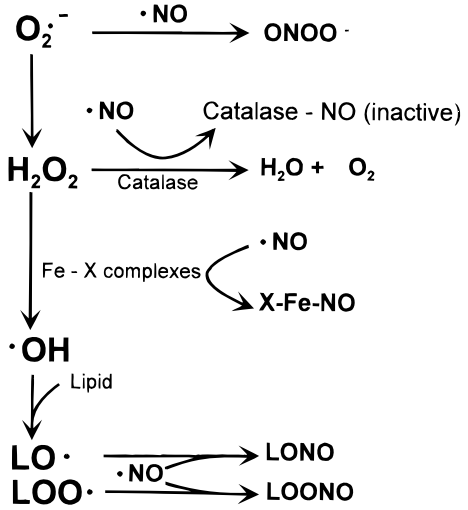

Figure 1. Key sites of nitric oxide action on pathways of reaction of partially reduced oxygen species.

Nitric Oxide: A Free Radical Signal Transducing Agent

Nitric oxide is an endogenously-synthesized free radical first characterized as a non-eicosanoid component of endothelial-derived relaxation factor (EDRF) (*3*). Nitric oxide is produced by a variety of mammalian cells including vascular endothelium, neurons, smooth muscle

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[1]Abbreviations: •NO = nitrogen monoxide; $O_2^{\bullet -}$ = superoxide anion; EDRF = endothelial-derived relaxation factor; cGMP = guanosine 3′,5′-monophosphate; NOS = nitric oxide synthases; cNOS = constitutive nitric oxide synthase; iNOS = inducible nitric oxide synthase; LO• = lipid alkoxyl radical; LOO• = lipid peroxyl radical; $ONOO^-$ = peroxynitrite anion; SOD = superoxide dismutase; ONOOH = peroxynitrous acid; •OH = hydroxyl radical; NO_2^+ = nitronium cation; H_2O_2 = hydrogen peroxide; LDL = low density lipoprotein; SIN-1 = 1,3-morpholinosydnonimine hydrochloride; TBA = 2-thiobarbituric acid; NO_3^- = nitrate; ROOH = alkyl hydroperoxide; SLO = soybean lipoxygenase; ESR = electron spin resonance; LOOH = organic hydroperoxide; NO_2^- = nitrite; ICAM-1 = intercellular cell adhesion molecule-1; mmLDL = minimally modified low density lipoprotein; LDLox = oxidized low density lipoprotein; VCAM-1 = vascular cell adhesion molecule-1; $\bullet NO_2$ = nitrogen dioxide.

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cells, macrophages, neutrophils, platelets, and pulmonary epithelium (*4*). The physiological actions of •NO range from mediating vasodilation, neurotransmission, inhibition of platelet adherence/aggregation, and the macrophage and neutrophil killing of pathogens. Many if not all of these effects are mediated by the activation of soluble guanylate cyclase, synthesis of cyclic guanosine 3′,5′-monophosphate (cGMP), and the activation of a family of cGMP kinases (*5*). Nitric oxide is synthesized by the oxidative deamidation of L-arginine to L-citrulline in the presence of the cofactors NADPH, (6*R*)-5,6,7,8-tetrahydrobiopterin, flavin adenine dinucleotide, and iron−protoporphyrin IX. The family of three enzymes responsible for the synthesis of •NO, nitric oxide synthases (NOS), require calmodulin binding for activity and are homologous to cytochrome P450 reductase. Both constitutively expressed (cNOS) and inducible (iNOS) forms of NOS are reported (*6*, *7*). The inducible nitric oxide synthases are transcriptionally regulated by cytokines and redox-sensitive transcriptional factors. Bacterial and parasitic antigens, which potently induce the expression of cytokines, also lead to induction of iNOS gene expression (*8*, *9*).

Nitric oxide has proven to be a ubiquitous signal transduction molecule and mediator of tissue injury because of its chemical properties, which include (a) relatively low reactivity for a free radical species, resulting in a biological half-life in the range of seconds, (b) charge neutrality, (c) a smalI molecular radius, (d) hydrophobicity, allowing facile transmembrane diffusion, (e) selective reactivity with heme, iron−sulfur proteins, and (f) facile reaction with molecular oxygen and oxygen-derived free radical species (e.g., $O_2^{\bullet-}$ and organic-derived free radicals). The latter reactivity of •NO provides tissues with a nonenzymatic method for modulating the local concentration of •NO and leads to many of the toxic and cytoprotective actions of •NO. Because of the transient nature of most free radical species and their often broad range of reactivities, it thus becomes challenging to define the mechanisms of tissue injury in pathologic events including a role for oxidant stress, when a diverse spectrum of reactive species is produced.

Target Molecule Reactions of Nitric Oxide

Nitric oxide is unique as a signal transduction mediator because its action principally depends on direct binding to the metal ion in metalloproteins and with partially reduced oxygen species, rather than the more prevalent structural (ionic and hydrophobic) interactions which are characteristic of signal transduction mediator−biological target reactions. The principal metalloprotein target of •NO, the sixth coordination position of the heme iron of guanylate cyclase, results in responder cell stimulation of synthesis of the secondary mediator, cGMP. This results in activation of cGMP kinases and the activation of membrane ion channels. A well-characterized example of this of this process is the •NO-dependent relaxation of smooth muscle due to the lowering of intracellular Ca^{2+} (*5*). During the initial description of endothelial-dependent relaxation, now known to be due to •NO, it was noted that strategies which enhanced tissue rates of $O_2^{\bullet-}$ production inhibited EDRF action and, conversely, inhibition of $O_2^{\bullet-}$ production/reactions enhanced EDRF activity (*10*). This revealed that oxygen radicals can serve critical roles as modulators of the biological reactions of •NO. We now know that •NO reacts with radical species including $O_2^{\bullet-}$ and lipid peroxyl radicals (LOO•) at almost diffusion-limited rate constants (*11*, *12*). One circumstance where this may occur is in inflammation.

Nitric oxide has been recognized as a key macrophage-derived effector molecule, with its cytotoxic reactions defending the host against bacteria, tumor cells, and parasites (*4*, *13*). Also, excess endogenous tissue •NO production may lead to pathological responses occurring during such diverse events as allograft transplant rejection, tissue ischemia-reperfusion phenomena, excitatory amino acid-induced brain injury, and immune complex-stimulated pulmonary edema (*14*−*16*). Metal- and thiol-containing proteins serve as major target sites for •NO reaction. The toxicity of •NO has principally been attributed to direct •NO reaction with thiol and iron−sulfur-containing mitochondrial enzymes (*17*) and the inhibition of DNA synthesis via inactivation of the non-heme iron-containing enzyme ribonucleotide reductase (*18*). Nitric oxide also mediates inhibition of mitochondrial cytochrome *c* oxidase (*19*−*22*) and deenergizes mitochondria at low •NO and oxygen concentrations (*20*). Thiol-containing enzymes are also critical targets for •NO, via as yet poorly defined pathways, since •NO does not directly react with sulfhydryls to yield *S*-nitrosothiols (*23*). The active site thiol of glyceraldehyde-3-phosphate dehydrogenase is subject to •NO-, •NO donor-, and $ONOO^-$-dependent reactions which often result in ADP-ribosylation and enzyme inhibition (*24*, *25*). The occurrence and significance of an interaction of •NO with thiols in biological systems is still an open question. Given the prevalence of thiol-containing proteins in the cell and the specificity of the reactions of •NO, selective modification of the chemical properties of a protein thiol are required to exert a specific and physiological effect. One possible example of this process is the •NO-dependent inactivation of the low M_r phosphotyrosine protein phosphatase family, sulfhydryl-containing enzymes involved in signal transduction, e.g., transmission of mitotic signals at different levels of the mitotic activation cascade (*26*).

Heme proteins such as guanylate cyclase are activated by low •NO concentrations (*27*), while cytochrome P450 (*28*) and other non-heme iron proteins such as aconitase (*17*), lipoxygenase (*29*), and xanthine oxidase (*30*) are inhibited by •NO, albeit minimally or from much greater concentrations of •NO than biologically achievable. Since tissue •NO concentrations are low, reaching a maximum of ∼1 μM during acute events (reperfusion of ischemic organs or inflammation; *31*, *32*), it is important to note that significant reactivity with non-heme iron, iron−sulfur complex, and thiol-containing proteins often requires high concentrations of •NO, •NO-generating agents, or the reactant. This point was recently made by the observation that •NO has minimal direct inhibitory action toward aconitase isoenzymes, critically dependent on cubane iron−sulfur centers (4Fe-4S) for catalytic activity (*33*). Aconitase was long thought to be a key toxic target molecule reaction during monocyte •NO-mediated host defense processes. This observation is consistent with the hypothesis that •NO must first react with monocyte or target cell-derived $O_2^{\bullet-}$ to yield $ONOO^-$, which then reacts with and inhibits aconitase at a much greater rate. These pivotal reports reaffirm that when a diverse spectrum of reactive species are being produced, relative rates of production of individual reactants, the chemical nature of nearby target molecules, and local concentrations of antioxidant defenses will profoundly affect outcome.

Nitric Oxide Reaction with Superoxide

A critical reaction that •NO undergoes in oxygenated biologic media is direct bimolecular reaction with $O_2^{\bullet-}$, yielding peroxynitrite ($ONOO^-$) at almost diffusion-limited rates (6.7×10^9 mol^{-1} s^{-1}; *11*). This rate constant is ~3.5 times faster than the enzymatic disproportionation of $O_2^{\bullet-}$ catalyzed by superoxide dismutases (SOD) at neutral pH ($k_{SOD} = 2 \times 10^9$ mol^{-1} s^{-1}). Thus, $ONOO^-$ formation represents a major potential pathway of •NO reactivity which depends on both rates of tissue •NO and $O_2^{\bullet-}$ production and scavenging (e.g., local superoxide dismutase and oxyhemoglobin concentrations). Peroxynitrite has a half-life of <1 s under physiological conditions, due to proton-catalyzed decomposition of peroxynitrous acid (ONOOH) and competing target molecule reactions of ONOOH (*23*, *34*, *35*). Nitric oxide will potentiate many aspects of $O_2^{\bullet-}$-mediated tissue damage via $ONOO^-$ formation. To date, it has been shown that $ONOO^-$ is a potent oxidant capable of (a) directly oxidizing protein and nonprotein sulfhydryls (*35*, *36*), (b) protonating to ONOOH, which exhibits both unique and hydroxyl radical (•OH)-like reactions via metal-independent mechanisms (*34*, *35*, *37*−*40*), and (c) reaction with metal centers to yield a species with the reactivity of nitronium cation (NO_2^+), an oxidizing and nitrating intermediate (*41*). It is noteworthy that the mechanisms and extents of $ONOO^-$ reaction will be strongly influenced by CO_2/H_2CO_3, which is typically 25 mM in biological tissues and can significantly exceed this concentration during pathologic processes (*42*−*44*).

Nitric oxide can potentiate $O_2^{\bullet-}$-mediated tissue damage and leads to $ONOO^-$ formation, representing a major potential pathway of •NO reactivity. In many instances, it is also becoming apparent that $ONOO^-$ serves as a mediator in oxidative actions originally attributed to •NO or other oxygen-derived species, as noted for aconitase inhibition (*33*). Peroxynitrite is now being revealed as a key contributing reactive species in pathological events associated with stimulation of tissue production of •NO, e.g., systemic hypotension, inhibition of intermediary metabolism, ischemia-reperfusion injury, immune complex-stimulated pulmonary edema, cytokine-induced oxidant lung injury, and inflammatory cell-mediated pathogen killing/host injury (*14*, *16*, *45*−*49*). There is growing evidence that •NO-mediated production of $ONOO^-$ readily occurs *in vivo*, underscoring the importance of understanding the target molecule reactions occurring during the coordinated production of oxygen- and nitrogen-containing reactive species (*47*, *50*−*52*).

Antioxidant Reactions of Nitric Oxide

Since the reaction of •NO with $O_2^{\bullet-}$ yields the potent oxidant $ONOO^-$, from a purely chemical point of view it would follow that (a) an even broader array of target molecules would become susceptible to the toxic effects of reactive oxygen species when •NO is present and (b) •NO will potentiate the toxicity of reactive oxygen species. While this is sometimes the case, it is evident that •NO also exerts direct or indirect antioxidant actions in biological systems subjected to concomitant oxidant stress from excess production of reactive oxygen species. The following sections develop these concepts in more detail.

Nitric Oxide Reaction with Lipid Epoxyallylic and Peroxyl Radicals. Nitric oxide has been observed to play a critical role in regulating lipid oxidation induced by reactive oxygen and nitrogen species ($O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), •OH, and $ONOO^-$) and activated reticuloendothelial cells (*44*, *53*−*55*). Nitric oxide (in some conditions) will stimulate $O_2^{\bullet-}$-induced lipid and lipoprotein oxidation and under other conditions mediate protective reactions in membranes by inhibiting $O_2^{\bullet-}$- and $ONOO^-$-induced lipid oxidation. The latter actions require higher (but still biologically relevant) rates of •NO production. The prooxidant versus antioxidant outcome of lipid oxidation reactions sensitive to •NO regulation are critically dependent on relative concentrations of individual reactive species (*53*−*55*).

Nitric oxide has been reported to have contrasting effects on low density lipoprotein (LDL) oxidation. For both macrophage and endothelial cell model systems, increased rates of cell •NO production via cytokine-mediated stimulation of inducible macrophage nitric oxide synthase gene expression and activity or exogenous addition of •NO have been shown to inhibit cell and $O_2^{\bullet-}$-mediated lipoprotein oxidation (*54*, *56*−*59*). Alternatively, *in vitro* systems examining the oxidation of LDL by $O_2^{\bullet-}$ show that inhibitors of •NO synthesis will enhance cell-mediated lipoprotein oxidation. In contrast to these examples, the simultaneous production of •NO and $O_2^{\bullet-}$ by 1,3-morpholinosydnonimine-HCl (SIN-1) or the direct addition of $ONOO^-$ has been shown to oxidize lipoproteins to potentially atherogenic forms (*44*, *51*, *60*−*62*). Peroxynitrite-dependent tyrosine nitration reactions in areas of atherosclerotic vessel lipid deposition has also been shown to occur during both early and chronic stages of atherosclerotic disease (*50*). In spite of observations suggesting a proatherogenic role for •NO, dietary L-arginine and other strategies for enhancement of vessel wall •NO synthesis have been shown to be antiatherogenic in this vascular disease long-associated with excess production and reactions of reactive oxygen species. Several factors may account for this phenomenon.

Nitric oxide not only stimulates $O_2^{\bullet-}$-induced lipid and lipoprotein oxidation via $ONOO^-$ production, but will also inhibit $O_2^{\bullet-}$- and $ONOO^-$-induced lipid oxidation at slightly higher rates of •NO production (*44*, *53*, *54*). The prooxidant versus antioxidant outcome of these reactions which are sensitive to •NO regulation is extremely dependent on relative concentrations of individual reactive species. For example, the continuous infusion of •NO at various rates into liposome suspensions exposed to xanthine oxidase first stimulated and then inhibited formation of 2-thiobarbituric acid (TBA)-reactive products at greater rates of •NO infusion (*53*). Nitric oxide only stimulated $O_2^{\bullet-}$-dependent lipid peroxidation when production rates of •NO were less than or equivalent to rates of $O_2^{\bullet-}$ production (*53*). Thus, there is a dynamic competition between $O_2^{\bullet-}$ and lipid radicals for reaction with •NO. When available for reaction with lipid radicals, •NO can act as an inhibitor of chain propagation reactions via radical−radical reaction with at least lipid peroxyl radicals at near diffusion-limited rates (for LOO•, $k = 1.3 \times 10^9$ M^{-1} s^{-1}; *12*). More investigation is required to understand the interaction of •NO with lipid epoxyallylic radicals, the predominant species to which lipid alkoxyl radical (LO•) rearranges following cyclization (*63*).

The LDL particle consists of an apolar core of cholesteryl esters and triglycerides, surrounded by a monolayer of phospholipids, unesterified cholesterol, and one molecule of apolipoprotein B-100, with cholesteryl esters the most abundant lipid class found in LDL and cholesteryl linoleate the principal oxidizable lipid (*64*). Nitric oxide

significantly concentrates in lipophilic cell compartments, with an 1-octanol/water partition coefficient of 6−8:1. This solvation property will further enhance the ability of •NO to regulate oxidant-induced lipid oxidation. Indeed, we observed that •NO inhibited cholesteryl linoleate oxidation in LDL in a dose-dependent manner, with the concomitant formation of nitrogen-containing lipid adducts (*65*). In addition, analysis of atherosclerotic human vessel lipid extracts by liquid chromatography−mass spectroscopic analysis showed that cholesteryl linoleate oxidation products represented more than 85% of the total cholesteryl linoleate fraction of atherosclerotic vessels. At least 25% of the luminal cell and plaque cholesteryl linoleate fraction of atherosclerotic vessels consisted of nitrogen-containing oxidized lipid derivatives, with the derivative found on esterified linoleic acid but not cholesterol (*65*). It is important to note that the products of •NO termination of lipid radical species are unstable and may mediate a different spectrum of as yet undefined target molecule and pathologic reactions.

Nitric Oxide−α-Tocopherol Interactions in Lipid Oxidation. α-Tocopherol, a lipophilic chain-breaking antioxidant in biological membranes and lipoproteins, acts by donating hydrogen atoms to chain-propagating peroxyl radical species (LOO•) to form the corresponding hydroperoxide (*66*). Since the reaction of LOO• with α-tocopherol occurs at a rate 3 orders of magnitude less than for the reaction of LOO• with •NO, •NO could act more readily than or in concert with α-tocopherol, lycopene, retinyl derivatives, and *β*-carotene as an antioxidant defense against oxygen radical and lipoxygenase-derived oxidized lipid species. Nitric oxide crosses cell membranes and can concentrate in lipophilic milieu by virtue of its low molecular mass, volatility, free radical nature, and high lipid partition coefficient. Based on comparison of relative rate constants, it is predicted that the termination of LOO• by •NO will be significantly more facile than both the reaction of LOO• with α-tocopherol ($k = 2.5 \times 10^6$ M^{-1} s^{-1}) and the initiation of secondary peroxidation propagation reactions by LOO• with vicinal unsaturated lipids ($k = 30-200$ M^{-1} s^{-1}).

In support of this argument, introduction of •NO into lipid oxidation systems containing α-tocopherol results in preferential reaction of •NO with lipid-derived radical species and prevents oxidation of α-tocopherol (*57*, *67*). One mechanism explaining the protection of α-tocopherol from oxidation by oxidizing lipids, until •NO falls to a limiting concentration, can be the preferential reaction of •NO with LO• and LOO• at significantly greater rates than α-tocopherol to yield nitrogen-containing radical−radical termination products (*53*, *54*). Another mechanism can be the direct reduction of α-tocopheroxyl radical (and possibly further oxidation states of α-tocopherol) by •NO, thus regenerating reduced α-tocopherol from that oxidized in antioxidant reactions and limiting the net extent of apparent α-tocopherol oxidation (Figure 2; *67*). Nitric oxide is thermodynamically capable of inhibiting accumulation of α-tocopherol oxidation products via one electron reduction of α-tocopheroxyl radical, with $\Delta G^{\circ\prime} = -5$ kcal/mol (*67*). Because lipid radicals in the lipophilic milieu do not readily partition into the bulk aqueous medium, we postulate that •NO can act as a reductant of α-tocopherol in membrane and hydrophobic lipoprotein compartments, where reducing equivalents are not readily transferred from water-soluble reductants (e.g., ascorbate, thiols). The mobility of α-tocopherol in the lateral plane of the membrane and its exact positioning in the membrane may restrict its antioxidant actions, in part explaining why •NO can be much more facile at terminating lipid peroxyl radical species. Thus, because of a high reactivity with other radical species, a relatively lower reactivity of lipid radical−•NO termination products, and an ability of •NO to readily traverse membranes and lipoproteins, •NO can effectively terminate radical species throughout all aspects of membrane and lipoprotein microenvironments. This can help maintain other tissue antioxidant defenses as well, during periods of oxidant stress.

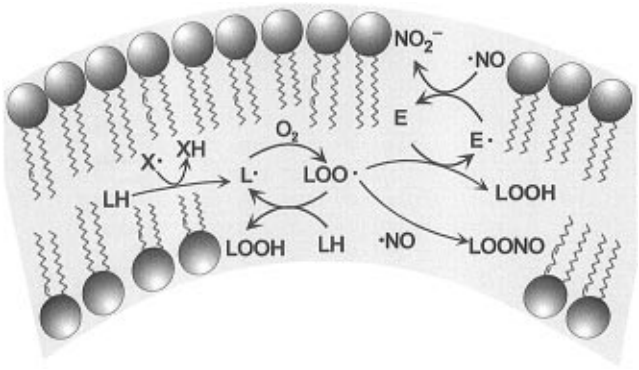

Figure 2. Nitric oxide inhibition of membrane and lipoprotein oxidation. Nitric oxide can impact on multiple aspects of lipid oxidation, ranging from termination of lipid radicals to preservation of lipophilic antioxidants.

Nitric Oxide Reactions with Metals. Nitric oxide can react with metal centers in proteins including heme iron, iron−sulfur clusters, and copper. Examples are the activation of soluble guanylate cyclase, a heme-containing enzyme, via the formation of an iron−nitrosyl complex (*68*). The reversible binding of •NO to the iron of reduced hemoproteins (i.e., cytochromes P450; *69*) is a well-established interaction that results in the formation of •NO−heme. It has been postulated that •NO can exert a protective role toward metal complex and metalloprotein-catalyzed lipid oxidation, via formation of catalytically inactive metal iron−nitrosyl complexes, thereby modulating the prooxidant effects of iron and other transition metals (*70*). Iron−nitrosyl complexes were also detected in mammalian ferritin (*71*), transferrin (*72*), myoglobin, and hemoglobin, albeit in the presence of high concentrations of •NO (*73*). It is important to note that the rate of •NO reaction with most metal centers is significantly slower than for the almost diffusion-limited reaction of •NO with either $O_2^{\bullet -}$ or LO• and LOO•, species critical for propagation of radical chain reactions (*11*, *12*). It should also be noted that •NO can exert prooxidant effects with transition metals as well, by reducing ferric iron complexes. This can induce the release of bound iron and indirectly substitute for other reductants in the Haber−Weiss reaction-mediated production of •OH from H_2O_2. From these and the below experimental observations, there is solid support for the view that modulation of radical reactions following •NO−metal complex formation (aside from binding to guanylate cyclase) often does not explain many oxidant-protective actions of •NO.

A principal modulatory event which regulates •NO signal transducing activity is the reaction of •NO with oxyferrohemoglobin (*73*). This reaction is aided by the extremely high concentration of red cell hemoglobin in the vascular compartment (~25 mM), assuring some tendency for both unidirectional signaling actions of •NO and its scavenging in the vascular lumen. The

reaction of •NO with oxyhemoglobin and oxymyoglobin results in the formation of nitrate (NO_3^-) and ferric ion, through the initial formation of $ONOO^-$ (*74*). In fact, the conversion of oxyhemoglobin or oxymyoglobin by •NO to methemoglobin or metmyoglobin serves as the basis of an often utilized spectroscopic assay for •NO. Because both myoglobin and hemoglobin react with alkyl hydroperoxides (ROOH) to yield several redox-active species, as well as a number of free radical intermediates, •NO may in some instances function via another protective antioxidant mechanism by reducing oxoferryl myoglobin/hemoglobin species formed *in vivo* (*73*).

Structural−functional studies of the catalytic site of lipoxygenase (SLO) reveal formation of a ferrous−nitrosyl complex following enzyme exposure to •NO. From this •NO−SLO interaction, it was proposed that •NO inhibits SLO-dependent lipid oxidation via direct enzyme inactivation (*29*). In these studies, extremely high and nonbiological •NO concentrations were employed under often anaerobic conditions, yielding predictable formation of ferrous−nitrosyl complexes and catalytic inhibition of SLO. This event was reproduced, again using high and extended rates of •NO production (*54*). However, at μM min^{-1} rates of •NO production or less, •NO only minimally inhibits lipoxygenase catalytic activity, if at all. Since the extent of inhibition of linoleic acid oxidation by SLO was similar for both •NO and lipophilic chain-breaking antioxidants, it was concluded that inhibition of SLO-induced lipid peroxidation •NO was actually due to inhibition of lipid radical chain propagation reactions, rather than direct inhibition of SLO catalytic activity. Finally, at μM min^{-1} rates of •NO production, no evidence of •NO reaction with either Fe-EDTA or the active site of SLO was detectable by electron spin resonance (ESR) analysis (*53*, *54*). From all of the above, it is concluded that the inhibitory effect of •NO toward oxygen radical or SLO-dependent oxidation of multiple lipid and lipoprotein targets, as determined by multiple criteria, was due to termination of lipid radical chain propagation reactions rather than •NO reaction with transition metals.

Nitric Oxide Detoxification of Peroxynitrite-Mediated Reactions. Peroxynitrite-mediated lipid peroxidation occurs predominantly by an iron-independent mechanism and can be inhibited by •NO derived from *S*-nitrosothiols or direct introduction of dissolved •NO gas into reaction systems, yielding nitrogen-containing oxidized lipid adducts (*37*, *53*). Nitric oxide may also inhibit $ONOO^-$-induced oxidant injury by directly reacting with the trans form of $ONOO^-$ via one of two possible mechanisms to yield less reactive products. This possibility deserves further investigation in both chemical and more biologically-oriented model systems.

Of the several functional groups in the biological milieu which can react with $ONOO^-$, thiols and carbohydrates are of particular interest. While $ONOO^-$ can irreversibly oxidize thiols to higher oxidation states and degrade sugars to aldehydic products, it can also form *S*-nitrosothiols and oxidize carbohydrates to organic nitrates/nitrites, which can subsequently react as •NO donors (*35*, *75*−*79*). Although, the yield of this reaction is low, the potency of •NO as an activator of guanylate cyclase is such that when isolated vascular tissues are exposed to $ONOO^-$, vasorelaxation occurs. Thus, •NO could protect against peroxynitrite-dependent cytotoxicity both through stimulation of tissue cGMP levels or via other mechanisms noted herein.

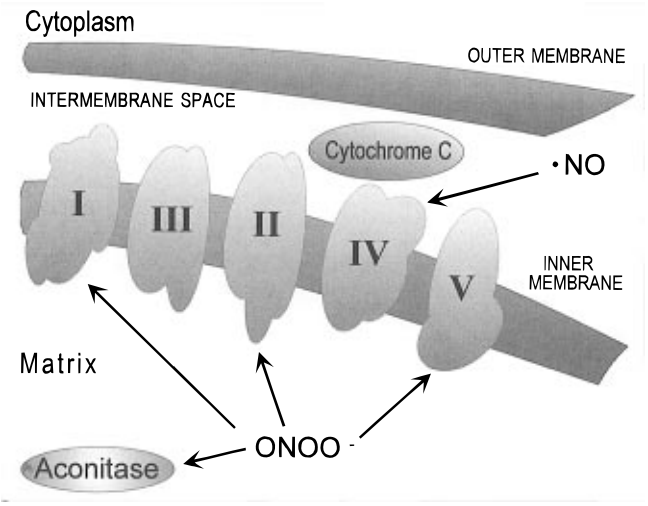

Figure 3. Sites of inhibition of nitric oxide and peroxynitrite in mitochondria. The mitochondrial respiratory chain is depicted with respiratory complexes I−V embedded in the inner membrane, aconitase in the matrix, and cytochrome *c* (c) in the intermembrane space.

Nitric Oxide Modulation of Mitochondrial Respiration and Oxidant Production

It has long been postulated that one principal mechanism of •NO-induced tissue damage includes direct inhibition of mitochondrial respiration, thought to be particularly significant in the macrophage-dependent killing of tumor cells (Figure 3; *13* and *17*). The targets susceptible to •NO-dependent inhibition in these studies have the characteristic that they contain FeS centers as integral components of electron transfer including complex I of the respiratory chain and aconitase, a key enzyme in the tricarboxylic acid cycle resident in the matrix of the mitochondrion (*17*, *33*). Most of these studies were conducted in cell cultures, and only recently have the direct effects of NO on mitochondrial function been investigated. In isolated mitochondria, •NO has little or no direct effect on components of the respiratory chain containing FeS centers but does inhibit cytochrome *c* oxidase, the terminal member of the respiratory chain (*19*, *22*). Investigation of the mechanism of inhibition of cytochrome *c* oxidase by •NO has revealed that it is a more potent inhibitor of this enzyme than the binding constant for the heme in the oxygen binding site, heme a_3, would predict (*80*). This has led to the suggestion that •NO may first bind to reduced CuB in the oxygen binding site before forming an adduct with cytochrome a_3.

At lower concentrations, such as those which prevail in the normal vasculature, the high affinity of •NO for cytochrome *c* oxidase predicts that it may be an effective inhibitor of cellular respiration under physiological concentrations of both •NO and oxygen. Direct evidence for such an effect has been reported for both *in vivo* and *in vitro* studies (*20*, *81*). What is the function of this interaction? It is not obvious whether the reversible binding of •NO with the respiratory chain is of no physiological significance, like the reaction of •NO with oxyhemoglobin, or that •NO may play a role in modulating oxygen sensing functions. Since •NO and oxygen compete for the same binding site on cytochrome *c* oxidase, it is likely that •NO modulation of mitochondrial respiration will be a function of the relative concentration of •NO and oxygen, as well as the position of the mitochondrion relative to the concentration gradients of each species. The recent finding that •NO synthase is

present within the mitochondrion suggests activation and synthesis may be under the control of the pathways which coordinate energy demand in the cell through controlling the concentration of Ca^{2+} in the matrix (*82*). Flooding the cell with •NO at low oxygen tension will overide the dependence on such intracellular gradients, synchronously depolarize a large proportion of mitochondria in a cell, and thus lead to increased cytosolic Ca^{2+} and depressed ATP synthesis. Ultimately this may be cytotoxic (*83*, *84*).

Inhibition of cytochrome *c* oxidase was not reported in the earlier studies of the effects of •NO on mitochondria in cells. What is the explanation for this discrepancy? The lack of inhibition is perhaps easiest to explain since it is reversible, is dependent on the enzyme being partially reduced, and is competitive with oxygen (*80*). It could not therefore be expected to persist and remain measurable after •NO has been removed from the system or the mitochondria isolated. The lack of an effect of •NO on the other respiratory complexes in isolated mitochondria, in spite of *in vitro* and *in vivo* evidence to the contrary, is more difficult to explain, and remains unresolved. One possibility is that superoxide ($O_2^{\bullet-}$) production, a byproduct of normal respiratory metabolism by mitochondrial membranes, is enhanced by inhibition of cytochrome *c* oxidase, and this event alone is sufficient to damage FeS centers. Direct addition of $ONOO^-$ to cells is reported to inhibit mitochondrial function (*48*), but it is unlikely that $ONOO^-$ formed in the extracellular environment can reach a mitochondrion within a cell given the myriad targets with which it could react (*85*). However, this does not preclude local generation and deleterious effects at the mitochondrial inner membrane itself (*48*).

In neuronal cells, an irreversible inhibition of cytochrome *c* oxidase has been detected following prolonged exposure to •NO (*84*). Since this may be reversed by the water-soluble analog trolox, it implies a mechanism other than a direct effect of •NO which may include the secondary formation of $ONOO^-$ (*86*) or the initiation of lipid peroxidation by oxidants formed by mitochondrial respiratory complexes. Since trolox scavenges both $ONOO^-$ and peroxyl radicals, this probe cannot distinguish between these two mechanisms. The distinct effects of NO and $ONOO^-$ on mitochondrial respiration are shown in Figure 3. In summary, this intracellular organelle is affected not only by the balance between •NO and $O_2^{\bullet-}$ in determining a pathologic or physiological outcome during oxidant stress, but by that between •NO and oxygen as well.

In Vitro Cell Models

The concept of •NO-mediated cytoprotection from oxidative stress was affirmed by the seminal observation that rodent lung fibroblasts (V79 cells) and dopaminergic mesencephalic neuronal cells were protected from toxicity induced by either purine plus xanthine oxidase or addition of H_2O_2 to culture medium, when •NO was introduced by release from a series of compounds known as NONOates (*87*−*89*). Subsequently, it was observed that V79 cells also became more resistant to the toxicity of organic hydroperoxides (LOOH) added to culture medium if chemical sources of •NO were present during LOOH exposure (*90*). More recent data confirm these observations and add some critical points. First, endogenous cell •NO production is also cytoprotective toward exogenously- and endogenously-generated reactive oxygen species added to cultures of pulmonary epithelial cells (*91*). Second, if rates of •NO production in the culture medium are less than for $O_2^{\bullet-}$, then $ONOO^-$ production will be favored and cause •NO to increase, not decrease, oxidant injury induced by xanthine oxidase-derived $O_2^{\bullet-}$ and its secondary dismutation and metal reaction products. Third, low rates of •NO production ($\sim$1 μM min^{-1}) can enhance vascular endothelial cell injury induced by H_2O_2 generated by addition of glucose plus glucose oxidase to culture medium (*55*). It was documented that cell catalase activity, inhibitable by •NO (*92*), was significantly inhibited and that H_2O_2 accumulated to higher concentrations in the medium of •NO-exposed cells (*55*). In aggregate, these studies all suggest that one important cytoprotective action of •NO is to inhibit lipid oxidative processes and that, in certain conditions, it can enhance oxidant injury.

These above points emphasize the ability of nitric oxide to redirect reactive oxygen species through other oxidative pathways (Figure 1). This was recently exemplified by observation of the increased cytotoxicity of SIN-1, a source of •NO, $O_2^{\bullet-}$, and $ONOO^-$, in the presence of SOD (*93*). When SIN-1, which has minimal cell permeability, was added to cell monolayers, the production of $ONOO^-$ was favored, which can either react with cellular targets or undergo proton-catalyzed decomposition to nitrite (NO_2^-) and NO_3^-. In the presence of SOD, dismutation of $O_2^{\bullet-}$ to H_2O_2 was favored, leading to, in this case, enhanced cytotoxicity. Another redirection of oxidative pathways can occur if significant oxidation of •NO to NO_2^- has occurred in a biological compartment. Heme iron reaction with hydroperoxides will form high valent iron complexes which can react with NO_2^- to yield $^{\bullet}NO_2$:

$$Fe^{II} + LOOH \rightarrow Fe^{IV}O + LOH \quad (1)$$

$$Fe^{IV}O + NO_2^- \rightarrow {}^{\bullet}NO_2 + Fe^{III} \quad (2)$$

Investigation of the effects of •NO on neutrophil and macrophage $O_2^{\bullet-}$ production have produced some important results, as well as yielding some highly questionable data, mostly a result of artifacts in $O_2^{\bullet-}$ quantitation in the presence of •NO. The main problem with many of these studies (*94*−*96*) is that introduction of exogenous •NO or enhancement of rates of endogenous •NO production in test systems relying on cytochrome *c*-mediated $O_2^{\bullet-}$ detection creates a number of analytical pitfalls. First, when cell-derived $O_2^{\bullet-}$ reacts with •NO, its production is not necessarily inhibited, rather it reacts to form $ONOO^-$ which will not mediate cytochrome *c* reduction. Since the rate constant for •NO reaction with $O_2^{\bullet-}$ is 3.5 times greater than for SOD scavenging of $O_2^{\bullet-}$ and SOD is not only membrane-impermeable but is electrostatically repelled at pH 7.4, it will be difficult to test the specificity of cytochrome *c* reduction by $O_2^{\bullet-}$ versus direct enzymatic electron transfer reactions, which can both reduce or oxidize cytochrome *c*. Second, when $ONOO^-$ is produced, it will cause reoxidation of ferrocytochrome *c* to ferricytochrome *c*, leading to an underestimation of rates of $O_2^{\bullet-}$ production (*97*). Third, solvation of high concentrations of •NO gas into test systems, in addition to being nonphysiologic, will render solutions partially anaerobic and inhibit partial reduction of oxygen by cellular reductases.

The final issue is one of semantics. It is a poor reflection of our current understanding of the reaction

of •NO with $O_2^{\bullet-}$ to term this event "free radical scavenging", since a generally more potent oxidant is formed from this reaction. Rather, this reaction of •NO with $O_2^{\bullet-}$ more appropriately represents redirection of the reactivity of $O_2^{\bullet-}$ to other oxidative pathways, which may be more or less acutely and chronically cytotoxic. The same can be said for •NO reaction with lipid radical species. In spite of the above qualifications, it remains that •NO production will modulate the inflammatory and oxidant-producing potential of both tissue and reticuloendothelial cells. For example, stimulation of either •NO or $O_2^{\bullet-}$ production by cultured vascular endothelial cells, isolated peripheral blood neutrophils, or alveolar macrophages obtained by pulmonary lavage concomitantly increases rates of cell $ONOO^-$ production. This will in turn enhance $ONOO^-$-dependent reactions, including lipid and sulfhydryl oxidation, as well as tyrosine and tryptophan nitration reactions at inflammatory foci (*47*, *52*, *98*−*100*). It will be important to place these reactions in the context of the cell adhesion, migration, proliferation, and gene expression influences which •NO exerts in addition to its radical termination properties, to gain an overall view of cytotoxic versus protective consequences of •NO production during oxidant stress.

Inflammation and •NO

A number of model systems for inflammation, vascular disease (atherogenesis, restenosis following angioplasty), and surgical problems (ischemia-reperfusion injury, graft reanastomosis) that include a pathogenic role for oxidant injury indicate that either endogenous •NO biosynthesis or exogenous supplementation with sources of •NO inhibit oxidant-dependent damage at both molecular and tissue functional levels. Many if not all of these studies have inflammatory injury as a common denominator.

In the initial stages of inflammation, O_2^- synthesis is stimulated and appears to exceed the rate of •NO formation which may be necessary to remove the inhibitory effects of •NO on the recruitment of inflammatory cells (*101*). Later in the process, nitric oxide synthases are induced which are capable of generating more •NO than O_2^- (*102*). Ultimately, the resolution of inflammation requires the restoration of the relative rates of •NO and O_2^- formation which prevailed before the process started. Failure to normalize may be an important factor predisposing to chronic inflammation, and this may occur when detoxification pathways become overwhelmed. Atherosclerosis is one example where this phenemenon occurs (Figure 4). The changes which occur during atherosclerosis include loss of the control of vascular tone, an •NO-dependent event (*51*, *60*, *103*). This response to hypercholesterolemia can be reversed by vessel wall delivery of SOD, suggesting that it arises as a consequence of a shift in the balance between NO and $O_2^{\bullet-}$ toward the latter. As predicted from this hypothesis, increasing the availability of the substrate L-arginine for •NO synthesis will restore vascular function, while inhibiting •NO synthesis is pro-atherogenic (*104*−*106*).

Another early event in the atherosclerotic process is the chemical transformation of LDL through the initiation of oxidation. Probably in an attempt at host protection, oxidized LDL is taken up by macrophages, resulting in lipid-laden foam cells. These cells then become part of the problem, because of the effect of their secretory products on other cells in the lesion and the release of prooxidative enzymes such as 15-lipoxygenase. The

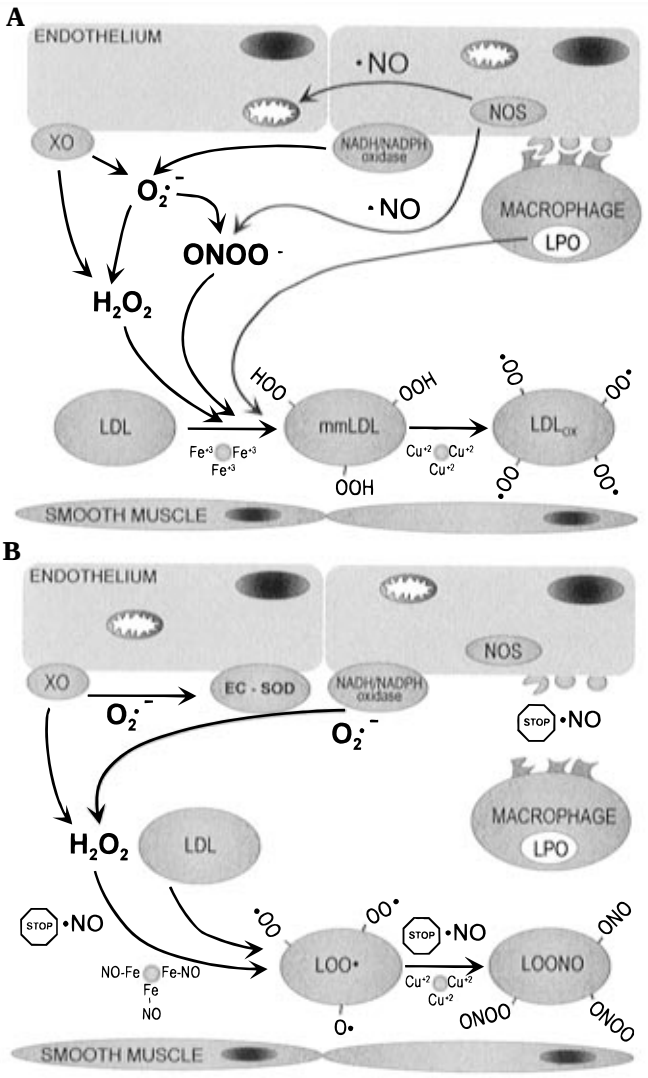

Figure 4. Prooxidant and antioxidant reactions of nitric oxide in the vasculature. Panel A (upper panel) depicts the subendothelial region of the artery wall under conditions where •NO contributes to oxidative stress. High concentrations of •NO can diffuse across the endothelial cell membrane and inhibit mitochondrial respiration, leading to ATP breakdown and elevation in available purine substrate for xanthine oxidase. The $O_2^{\bullet-}$ formed from this reaction can react with •NO to form peroxynitrite ($ONOO^-$), leading to the insertion of lipid peroxides into low density lipoprotein (LDL) and formation of minimilly modified LDL (mmLDL). Lipid peroxides can also be inserted in LDL by the enzymatic action of macrophages containing 15-lipoxygenase. In the presence of transition metals such as iron in heme proteins or Cu derived from ceruloplasmin, peroxides will decompose to ultimately yield peroxyl radicals (LOO•) and oxidized LDL. Panel B (lower panel) depicts mechanisms underlying the anti-atherogenic effects of •NO. For this to occur, it is hypothesized local concentrations of •NO exceed $O_2^{\bullet-}$. Because of its concentration in the interstitial matrix, extracellular SOD (ECSOD) competes effectively with •NO for $O_2^{\bullet-}$. Nitric oxide also inhibits adherence and recruitment of monocytes and macrophages into the artery wall. Antioxidant effects of •NO are also shown, including its ability to terminate lipid peroxidation reactions.

balance between •NO and $O_2^{\bullet-}$ production in the artery wall may also play a role in the oxidation of LDL (*53*, *54*, *61*, *62*). Peroxynitrite oxidizes LDL, causes a rapid depletion of several antioxidants (ascorbate, urate, protein thiols, and ubiquinol), and releases copper ions from the plasma protein ceruloplasmin (*43*). Copper ions are powerful catalysts of LDL oxidation which have been detected in advanced human atherosclerotic lesions. Consistent with formation of $ONOO^-$ in the vasculature,

immunoreactive material which cross-reacts with an antibody recognizing nitrotyrosine has been found in human atherosclerotic lesions (*50*).

The protective effects of •NO toward *in vivo* models of reperfusion injury, when •NO is administered as a bolus of an •NO-donating drug, are often ascribed to •NO inhibition of inflammatory cell margination and function (*107*−*111*). In some of these models, inhibition of endogenous cell •NO synthesis enhanced injury as well. Mechanisms underlying this •NO-mediated phenomenon include both acute events and more delayed processes involving regulation of integrin gene expression (discussed in a later section of this review). Acutely, nitric oxide administration to reperfused ischemic tissues will result in stimulation of vessel wall and circulating platelet and neutrophil cGMP levels. This will result in increased blood flow and oxygen delivery to tissues, as well as an alteration in shear forces on the vessel wall, critical for regulating inflammatory cell−vessel wall interactions and secondary gene expression events (*112*). The translocation of P-selectin to the platelet surface or the function of P-selectin becomes inhibited by •NO as well, resulting in attenuation of platelet−neutrophil aggregation and secondary "downstream" inflammatory cell-derived oxidant stress to the vasculature. Isolated organ preparations treated with inhibitors of •NO synthesis showed increased vessel wall neutrophil adhesion and margination which was inhibited by anti-CD18, the β-subunit of the integrin receptor for intercellular cell adhesion molecule-1 (ICAM-1) on neutrophils. Mast cell degranulation is also inhibited by •NO, which will limit the release of other proinflammatory mediators such as histamine and platelet activating factor (*110*). *In vitro* studies also show that neutrophil leukotriene B_4 production, degranulation, and chemotaxis, all properties regulated in part by cGMP, are inhibited by •NO donors (*113*) albeit at high concentrations and without documentation of rates of •NO delivery to cells.

A number of studies, sometimes in the absence of reliable biochemical support, claim that a rapid fall in •NO production occurs upon reperfusion of ischemic tissues, either due to inhibition of synthesis or •NO "inactivation" by $O_2^{\bullet -}$ (*114*). It is becoming apparent that •NO synthesis can be either inhibited or stimulated during ischemia-reperfusion phenomena and that relative concentrations or rates of production of tissue •NO and $O_2^{\bullet -}$ will dramatically affect outcome (e.g., •NO-mediated tissue protection or acceleration of injury; *14*, *115*). For these reasons, greater insight can be gained by studying a range of nitric oxide generation rates and oxygen and antioxidant concentrations when measuring cell or organ responses to ischemia-reperfusion and other forms of oxidant stress. This is due to the distinct possibility that contrary responses can be observed, depending on which reaction pathways predominate.

An exciting aspect of •NO-mediated protection of ischemic tissues is the observation that both lung and heart preservation for transplantation can be enhanced by •NO. This occurs for both heterotopic and orthotopic transplantation models (*111*, *116*), which showed improved vascular function, tissue viability, and decreased graft rejection following treatment with •NO donors or other strategies which increased tissue cGMP levels. Vascular disease is often the cause for cardiovascular surgical procedures, which may also be rendered safer through the protective actions of •NO during reperfusion injury. It is interesting to note that both animal model and clinical studies are showing that chronic administration of L-arginine improves endothelial dependent relaxation, decreases inflammatory cell accumulation at the vessel wall, and reduces intimal hyperplasia, all hallmarks of atherosclerotic disease (*117*−*119*). Furthermore, balloon angioplasty is often used to treat atherosclerotic vasoocclusive problems. Both administration of •NO donors as well as transfection of constitutive nitric oxide synthase to balloon-injured vessels reduces intimal cell hyperplasia, often the cause for repeat angioplasty, aortocoronary bypass graft surgery, or myocardial infarction (*120*).

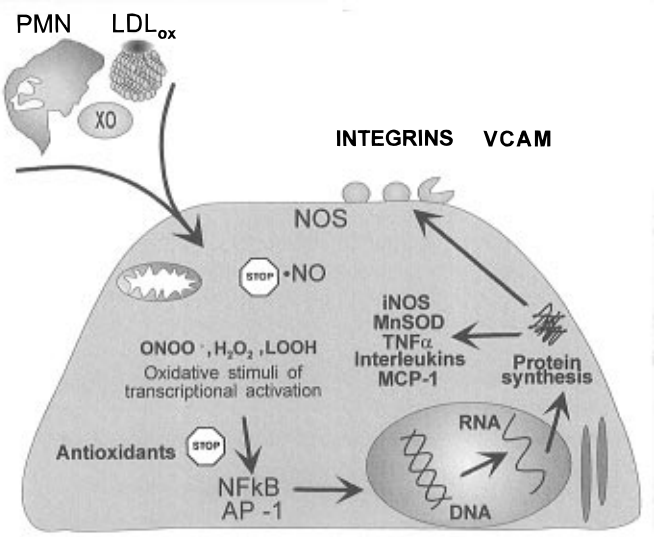

Figure 5. Nitric oxide inhibition of gene expression of proinflammatory genes. Nitric oxide can inhibit expression of proinflammatory genes by impacting on the oxidative signal, causing activation of transcriptional factors, as well as by directly affecting the induction and stability of transcription factors themselves.

Nitric Oxide Regulation of Redox-Sensitive Gene Expression

Oxidative signals play critical roles in the activation of gene expression of key proinflammatory mediators. In particular, the redox-sensitive modulation of the expression and function of vascular genes is being revealed to play a central role in the initiation and propagation of atherosclerosis (*121*). This process involves regulatory events that are mediated by oxidative reactions in at least three key points, all of which can be modulated by •NO (Figure 5). First, the oxidative modification of LDL by reactive oxygen species and enzymes such as lipoxygenase will generate novel epitopes on LDL, yielding minimally modified (mmLDL) and further oxidized species of LDL (LDLox). These oxidized forms of LDL are recognized by macrophage scavenger receptors and concentrate both intracellularly and at sites of macrophage accumulation (*122*). As mentioned previously, •NO can both stimulate as well as inhibit many of these lipid oxidative events. A second critical oxidative event in atherogenesis is the activation of proinflammatory gene transcription by oxidized lipids (*121*, *123*). Specifically, fatty acid hydroperoxides found in oxidized LDL (e.g., 13-hydroperoxyoctadecadienoic acid) activate (at least) NFκB, with this transcription factor then mediating gene expression of an integrin termed vascular cell adhesion molecule-1 (VCAM-1). Similarly, cytokines such as interleukin-1α, interleukin-1β, and tumor necrosis factor-α, proposed to also activate NFκB via stimulation of

cellular production of reactive oxygen species, induce gene expression of VCAM-1 via redox-sensitive mechanisms (*124*). In both oxidized lipid and cytokine-mediated activation of VCAM-1 gene expression, •NO and other inhibitors of either lipid peroxyl radical formation or reaction serve an inhibitory role to both oxidative activation of NFκB and the ultimate gene expression of VCAM-1 (*121, 124*). Additionally, •NO indirectly inhibits NFκB-mediated gene expression by inducing the expression of and stabilizing the inhibitory protein for NFκB, IκBα (*125*). The third and final oxidant-protective event that •NO mediates in vascular injury mechanisms is a consequence of •NO-mediated inhibition of integrin gene expression. When inflammatory cells bind to target cells via receptor−ligand interactions requiring integrins, the target cell is drawn into a closer range of attack for not only reactive oxygen species, but also effector cell-derived cytokines and proteases which can exert cytotoxic effects as well. Numerous studies in diverse model systems have shown that both antibody blocking of integrins and inhibition of integrin gene expression profoundly inhibit inflammatory cell-induced injury (*126*). To summarize, •NO can often be vasoprotective because it inhibits lipid oxidation, the oxidative activation of gene transcriptional factors, and the extent of oxidative injury induced by inflammatory cells.

Pulmonary Disease and Tissue Responses to •NO

Inhaled •NO, at concentrations similar to those produced *in vivo*, has been clinically administered in the gas phase for up to several weeks when treating pulmonary hypertension, providing an informative test system for the toxicological properties of •NO (*127*). This use of inhaled •NO as a selective pulmonary vasodilator is possible because it will react with oxyhemoglobin before reaching the systemic circulation. Inhaled •NO is frequently administered in the presence of hyperoxic gas mixtures, since many of the pulmonary pathologies indicating use of •NO as a pulmonary vasodilator involve impaired pulmonary gas exchange and arterial hypoxemia. The lungs of those receiving inhaled •NO are also suffering from oxidative stress due to active inflammatory processes and exposure to hyperoxia, known to stimulate lung tissue production of reactive oxygen species at rates directly proportional to oxygen concentration (*128*). Hyperoxia will also increase rates of gas phase oxidation of •NO to nitrogen dioxide ($^{\bullet}NO_2$), N_2O_3, and N_2O_4.

The first indication that •NO was protective in oxidant-induced lung injury came from the observation that lungs, perfused *ex vivo* with purine plus xanthine oxidase as a source of $O_2^{\bullet-}$, H_2O_2, and •OH, showed protection from oxidant-induced increases in vascular resistance and reduced injury to alveolar−capillary barrier function upon ventilation with 90−120 ppm •NO (*129*). *In vivo* studies of rats exposed to 100% oxygen plus ∼50 ppm •NO showed an •NO-dependent increase in survival time to the normally lethal hyperoxia, confirming the isolated lung observation (*91*). A clinical study of patients suffering from adult respiratory distress syndrome, long associated with inflammatory cell and oxidant stress-mediated lung injury, showed that inhalation of 18 ppm •NO for 4 days resulted in decreased indices of pulmonary lavage neutrophil activation (H_2O_2 production) and indices of inflammation (β_2 integrin CD11b/CD18 expression and lavage IL-6 and IL-8 content; *130*).

While these observations encourage that •NO can act in an oxidant-inhibitory manner in acute lung injury, the caveat should be added that more subtle toxic reactions can be occurring at the same time, including inhibition of mitochondrial respiration, damage to alveolar interstitial components (*131*), inhibition of surfactant function (*132*), formation of peroxynitrite and nitrated aromatic acid derivatives (*133*), and above 40 ppm, significant formation of methemoglobin, a particular risk for methemoglobin reductase-deficient infants (*134*). The clinical experiences of critical care physicians studying this therapeutic modality also suggest that it is sometimes difficult to "wean" patients from the •NO inhaled with other ventilator air/oxygen blends, in terms of the patient being able to autoregulate pulmonary blood flow. This observation provides possible *in vivo* evidence for the ability of •NO to inhibit endogenous nitric oxide synthases by coordination at the catalytic heme iron of the enzyme (*135*). In spite of these qualifiers, however, these data indicate that •NO can exert oxidant-protective effects.

Summary

We have presented evidence from a broad range of chemical, cell biological, and *in vivo* studies showing that •NO can mediate tissue-protective reactions during oxidant stress, as well as toxic and tissue prooxidant effects. One predominant factor that has been identified which influences •NO being protective versus toxic is the relative rates of production and concentrations of •NO and the more "traditional" family of reactive oxygen species, including $O_2^{\bullet-}$, H_2O_2, •OH, LO•, LOO•, and high valency complexes of iron. Also, since so many anti-neutrophil actions of •NO have been described, it is likely that •NO will serve a protective role in acute inflammatory reactions. One issue is certain—many new truths remain to be revealed, as we continue to develop our understanding of the toxicology of reactive oxygen- and nitrogen-containing species.

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