Interactions Between Nitric Oxide and Lipid Oxidation Pathways: Implications for Vascular Disease

Valerie B. O’Donnell, Bruce A. Freeman

Abstract—Nitric oxide (NO) signaling pathways and lipid oxidation reactions are of central importance in both the maintenance of vascular homeostasis and the progression of vascular disease. Because both of these pathways involve free radical species that can also react together at extremely fast rates, convergent interactions between these pathways are expected. Biochemical and cell biology studies have defined multiple interactions of NO with oxidizing lipids that could lead to either vascular protection or potentiation of inflammatory vascular injury. For example, low levels of NO generated by endothelial nitric oxide synthase can terminate propagating lipid radicals and inhibit lipoxygenases, reactions that would be protective. Alternatively, if generated at elevated levels, for example, after inducible nitric oxide synthase expression in inflammation, NO can be converted to prooxidant species, such as peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂), that can potentiate inflammatory injury to vascular cells. Finally, both enzymatic and nonenzymatic lipid oxidation reactions can influence NO bioactivity by directly scavenging NO or altering the induction and catalytic activity of nitric oxide synthase enzymes. In this review, we summarize the biochemical interactions between NO and lipid oxidation reactions and discuss the recognized and potential roles of these reactions in the vasculature. (Circ Res. 2001;88:12-21.)

Key Words: eicosanoid signaling ■ lipid ■ nitric oxide ■ oxygen ■ free radical

The free radical species NO is an endogenously generated mediator of smooth muscle relaxation and inhibitor of platelet/leukocyte activation that is essential for maintenance of vascular homeostasis. In many vascular pathologies, altered NO generation rates, often coupled with accelerated NO removal through poorly understood pathways, leads to impaired NO signaling and secondary generation of toxic NO-derived species.¹–⁶ Reaction of NO with O₂⁻, yielding peroxynitrite (ONOO⁻), accounts for a major part of the accelerated NO removal²–⁶ but is not the only mechanism involved, because endothelium-derived relaxing factor (EDRF) activity is often incompletely restored by O₂⁻ scavengers.⁷–⁹ This suggests that the reaction of NO with other biochemical “sinks” can also account for enhanced rates of EDRF consumption. Further evidence for alterations in NO metabolism in vascular disease is provided by observations of...
the tyrosine oxidation/nitration product 3-nitrotyrosine (NO2-tyr) and elevations in inducible nitric oxide synthase (iNOS or NOS2) activity. For example, both NO2-tyr and NOS2 expression are consistently elevated clinically (transplant coronary artery disease, atherosclerotic lesions, cardiac allograft rejection, and myocardial inflammation) and in animal models of vascular disease (hypercholesterolemia-induced atherosclerosis, balloon-injured arteries, ischemic heart injury, and myocardial inflammation) (reviewed in Reference 10; see also References 11–17).

Increased lipid oxidation is a characteristic feature of inflammatory vascular diseases and has been suggested to sometimes play a causative role, although this has not been conclusively proven.

The candidate mechanisms that generate oxidized lipids in vivo are numerous and include metal-dependent Fenton oxidation, enzyme-catalyzed oxidation by lipoxygenase (LOX) or myeloperoxidase (MPO), metal-dependent Fenton oxidation, and reaction with hypochlorous acid (HOCl), cell-dependent oxidation, and myeloperoxidase (MPO), and via a caged radical pair (3).

The initial product, an organic peroxyxidinitrile (1), rapidly decomposes by several mechanisms including hydrolysis to form lipid hydroperoxides (2) and via a caged radical pair (3). The caged radical pair, nitrogen dioxide and alkoxyl radical, can either terminate after rearrangement (one possible rearrangement is shown (4) or dissociate and react further, eg, with additional molecules of nitric oxide (5).

Reactions of NO with Purified Lipids, Lipoproteins, and Membranes

NO potently inhibits lipid oxidation in a variety of in vitro model systems, including unsaturated free fatty acid emulsions, phosphatidylcholine liposomes or LDL oxidized by Cu2+, azo initiators of LOO- oxygenation, endothelial cells, or macrophages. This is primarily a consequence of NO reacting with lipid-derived radicals (eg, L·, LO·, and LOO·) via diffusion-limited rates (10⁹ to 10¹¹ [mol/L]⁻¹ s⁻¹) to terminate lipid peroxidation propagation reactions. During the reaction of LOO· with NO, two molecules of NO are consumed as the primary organic peroxyxidinitrile (LOONO) intermediate rapidly decomposes (τ₁/₂=0.2 to 0.6 second) to secondary radical species that reacts further. For example, LOONO can decompose to caged radicals [LO·NO₂⁻], which can either terminate after rearrangement of LO· to an epoxide, L(O)NO₂⁻, or dissociate and react with additional NO (Figure 1). Alternatively, LOONO can hydrolyze to LOOH and NO2·. The small molecular radius and hydrophobicity of NO facilitates partitioning into hydrophobic membrane compartments, thus enhancing the efficacy of NO to terminate lipid peroxidation.

The fast rate of reaction between LOO· and NO (2×10⁹ [mol/L]⁻¹ s⁻¹), compared with α-tocopherol (1 to 5×10⁸ [mol/L]⁻¹ s⁻¹), with rate depending on the alkyl chain length and charge characteristics of the LOO· species), allows NO to spare tocopherol during lipid peroxidation and predicts that steady-state NO concentrations of 30 nmol/L will outcompete endogenous α-tocopherol concentrations (20 μmol/L) for termination of LOO·. In addition, the reduction of...
topheroxyl radical by ascorbate is less effective at preventing lipid oxidation–induced tocopherol loss than the reaction of NO with LOO.\textsuperscript{52} These comparisons underscore the profound capacity of NO for antioxidant reactions in the vasculature. It is emphasized that these properties of NO can be manifested only if alternative prooxidative reactions (eg, NO reaction with $O_2^-$ to yield ONOO\textsuperscript{-}) do not predominate. NO-mediated termination of lipid radicals can also limit secondary lipid oxidation–mediated processes that are involved in vascular injury, including nuclear factor-\kappaB (NF-\kappaB) activation, the linkage of vascular cell adhesion molecule-1 (VCAM-1) gene expression with NF-\kappaB activation, lung injury secondary to intestinal ischemia, pulmonary epithelial cell oxygen injury, and the cytotoxicity of H$_2$O$_2$ and alkyl hydroperoxides.\textsuperscript{53–59} Finally, NO can undergo reactions with $O_2$ and/or $O_2^-$ to yield oxidizing and nitrating species that also cause NO-dependent cytotoxicity in vitro through inducing lipid oxidation.

**Peroxynitrite-Induced Lipid Oxidation and Nitration**

Peroxynitrite is unique as a lipid oxidant, because it mediates peroxidation of unsaturated fatty acids in the absence of transition metal catalysts.\textsuperscript{28} Peroxynitrite is more than two orders of magnitude more potent than $H_2O_2$ in catalyzing lipid oxidation in vitro and, in contrast to transition metal catalysts, mediates LDL oxidation even in the presence of lipophilic antioxidants.\textsuperscript{60} In vitro, ONOO\textsuperscript{-} oxidizes diverse classes of lipids (eg, purified fatty acids, neutral lipids and phospholipids, and lipophilic antioxidants and LDL lipids) forming conjugated diene, malondialdehyde, lipid peroxide, lipid hydroxide, F$_2$-isoprostane, and oxysterol products.\textsuperscript{29,44,50–63} In the case of LDL, this results in an LDL derivative recognizable by the macrophage scavenger receptor.\textsuperscript{64} In addition to oxidizing lipids, ONOO\textsuperscript{-} mediates linoleate and LDL cholesterol linoleate nitration to the derivative LNO$_2$.\textsuperscript{65} (also A. Bloodsworth and B.A. Freeman, unpublished data, 2001). This reaction proceeds via either hydrogen abstraction by NO$_2$ or addition mechanisms involving NO$_2$\textsuperscript{-} (Figure 2). A role for ONOO\textsuperscript{-} in initiating lipid oxidation in atherosclerosis has been suggested\textsuperscript{66}; however, this requires that the barrier of competing reactions with thiols be overcome, which are present intracellularly at concentrations of up to 10 mmol/L ($k=5.9\times10^4$ [mol/L]$^{-1}$s$^{-1}$ for ONOO\textsuperscript{-} reaction with cysteine).

**Reactions of the NO Metabolites NO$_2$ and NO$_2$Cl With Unsaturated Lipids**

Several reactive nitrogen species derived from NO oxidize and nitrate unsaturated fatty acids and their methyl/ethyl esters in vitro. Nitrogen dioxide will both oxidize and nitrate unsaturated lipids, with nitration occurring by hydrogen abstraction and addition reactions.\textsuperscript{28,65,67–74} These reactions result in formation of a complex mixture of products including nitrated lipid derivatives and alkyl nitrites, including those shown in Figure 3.\textsuperscript{75} Nitration of methyl linoleate and linolenate by NO$_2$ proceeds via initial hydrogen abstraction to form a carbon-centered alkyl radical, which at low oxygen tensions combines with NO$_2$ to form allylic nitro compounds (Figure 2). The yield of oxygen-containing lipid products (eg, LOOH, LOH, etc) formed by NO$_2$ oxidation thus depends on the concentration of O$_2$ that will facilitate peroxidation reactions.\textsuperscript{28} At high O$_2$ concentrations, for example, in lung lining fluid, NO$_2$ will predominantly mediate lipid oxidation. Conversely, at low O$_2$ tension (eg, within an inflamed hypoxic organ or microvessel), nitration reactions may preferentially occur. Somewhat analogous to NO, NO$_2$ can also react at diffusion-limited rates with peroxyl and alkoxyl radicals, leading to inhibition of peroxidation and formation of novel N-containing lipid derivatives.\textsuperscript{46,76}

The oxidation of nitrite (NO$_2$\textsuperscript{-}) by MPO-derived hypochlorous acid (HOCl) will yield nitryl chloride (NO$_2$Cl),\textsuperscript{77,78} which, in purified LDL, depletes $\beta$-carotene and $\alpha$-tocopherol, initiates lipid oxidation, forms 3-nitrotyrosine,\textsuperscript{27} and can yield an LDL particle similar to that found in foam cells. The direct oxidation of NO$_2$\textsuperscript{-} by MPO+$H_2O_2$ yields NO$_2$ and also oxidizes LDL lipids to a proatherogenic particle.\textsuperscript{79–81} Oxidation and nitration

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**Figure 2.** Mechanisms of peroxynitrite oxidation and nitration of unsaturated lipids. Peroxynitrite oxidizes lipids by hydrogen abstraction (1), probably via nitrogen dioxide formation. Nitration could then occur via either hydrogen abstraction (2) or an addition reaction of a nitrosonium-like intermediate (3). In the case of hydrogen abstraction, a conjugated diene product is expected. With addition reactions, rearrangement of the double bonds would not occur.

**Figure 3.** Nitrated lipids formed from nitrogen dioxide reaction with ethyl linoleate. Extensive reactions and rearrangements are possible during these reactions, with known products (see Reference 76 for details of formation pathways) including alkyl nitrites (LONO) and nitrolipids (LNO$_2$), shown.
of membrane lipids by MPO may be operative in atherogenesis, because products of MPO activity are found in vascular lesions; however, this has not been conclusively proven. Finally, acidification of NO$_2$ forms nitrous acid (HONO), which decomposes to nitrosating and nitrating species including N$_2$O$_3$; therefore, if these reactions are to occur in the blood, alkyl nitrite compounds are not stable and either hydrolyze to alcohols or lose HNO$_2$ via acid catalysis, forming the species shown. B, Nitrosation of lipid hydroperoxides by HONO forms nitroepoxy derivatives and likely proceeds via the LOONO intermediate shown in Figure 1. The two possible rearrangements are shown.

**Figure 4.** Products of nitrous acid reaction with ethyl linoleate and linoleate hydroperoxide. A, Nitrosation/nitration of ethyl linoleate by nitrous acid proceeds via similar pathways to nitrogen dioxide–dependent reactions. However, as a result of the low pH required for these reactions (typically <4.0), alkyl nitrite compounds are not stable and either hydrolyze to alcohols or lose HNO$_2$ via acid catalysis, forming the species shown. B, Nitrosation of lipid hydroperoxides by HONO forms nitroepoxy derivatives and likely proceeds via the LOONO intermediate shown in Figure 1. The two possible rearrangements are shown.

**NO-Derived Reactive Species Modulate the Activity and Expression of Lipid Oxidation Enzymes**

Enzymes such as LOX, prostaglandin endoperoxide H synthase (PGHS), and cytochrome P450 (CYP) that oxidize lipids to bioactive eicosanoids play critical signaling roles in the regulation of vascular cell function and inflammatory responses and are ubiquitously expressed by virtually all vascular cells under both physiological and inflammatory conditions (Figure 5). Generally, lipid oxidation by these enzymes involves formation of enzyme-bound radical intermediates, including lipid alkyl (L) and peroxy (LOO) radical species. Free peroxyl or alkyl radicals react with NO at diffusion-limited rates. Thus, reaction of NO with enzyme-bound lipid radicals will modulate rates of eicosanoid product formation and decrease bioavailable concentrations of NO, as discussed below. In addition to lipid radicals, these enzymes form several other intermediates that can react with NO during turnover, including amino acid or porphyrin radicals and various redox states of iron. In the next sections, the known interactions of NO with the various enzyme intermediates formed during catalysis are discussed.

**Prostaglandin Endoperoxide H Synthase (PGHS)**

Prostaglandins are generated via arachidonate oxygenation by PGHS, of which there are both constitutive (PGHS-1: stomach, gut, kidney, and platelets) and inducible (PGHS-2: fibroblasts and macrophages) isoforms. Under inflammatory conditions, both NOS2 and PGHS-2 expression is upregulated in tandem by proinflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor, indicating that high levels of both prostaglandin and NO will be produced in concert in vivo.

Reactive nitrogen species have multiple effects on PGHS activity. Purified PGHS-1 is not significantly inhibited by NO; however, in several cell types, including endothelial cells and platelets, NO highly stimulates prostaglandin production. In other cell types, NO suppresses lipopolysaccharide (LPS)-induced PGHS-2 expression, resulting in apparent enzyme inhibition. Finally, in NOS2 knockout mice, less urinary prostaglandin E$_2$ is found, although platelets from these animals generate more thromboxane B$_2$ in vitro.

NO can interact in several ways with PGHS, by forming an Fe-nitrosyl complex, acting as a peroxidase-reducing substrate, directly terminating the catalytic tyrosyl radical in the enzyme active site, and theoretically, by termination of the Fe-nitrosyl complex, acting as a peroxidase-reducing substrate, directly terminating the catalytic tyrosyl radical in the enzyme active site, and theoretically, by termination of the Fe-nitrosyl complex, acting as a peroxidase-reducing substrate, directly terminating the catalytic tyrosyl radical in the enzyme active site, and theoretically, by termination of the Fe-nitrosyl complex, acting as a peroxidase-reducing substrate, directly terminating the catalytic tyrosyl radical in the enzyme active site, and theoretically, by termination of the Fe-nitrosyl complex, acting as a peroxidase-reducing substrate, directly terminating the catalytic tyrosyl radical in the enzyme active site, and theoretically, by termination of the Fe-nitrosyl complex, acting 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Lipoxygenases (LOX)

Lipoxygenases are non-heme iron–containing enzymes that catalyze oxidation of arachidonate or linoleate to bioactive lipid hydroperoxides. In mammalian cells, at least three isoforms are known with the best characterized, 5-LOX, found mainly in leukocytes. 12-LOX isoforms are present in platelets and monocytes. 15-LOX is expressed in reticulocytes during maturation into erythrocytes, where it plays a central role in intracellular membrane degradation. In human monocytes, expression of 15-LOX is induced by IL-4 and IL-13.109,110 A role for 15-LOX in the initiation and progression of atherosclerosis has also been suggested by the observation of 15-LOX products at elevated levels in atherosclerotic lesions.21,22,34,111

Lipoxygenases contain a single non-heme iron that alternates between Fe2+ and Fe3+ during catalysis. Resting enzyme predominantly exists as the reduced form, requiring oxidation by hydroperoxides before dioxygenation can occur. Inhibition of LOX (soybean, rabbit and human 15-LOX, and human platelet 12-LOX) by NO has been reported39,112–115 and was proposed to result from formation of an Fe-nitrosyl complex with the ferrous enzyme. However, metal center reaction only occurs at high and nonphysiological NO concentrations,116–119 making this pathway of LOX inhibition unlikely. Rather, tissue LOX inhibition results from a termination reaction between NO and the enzyme-bound lipid peroxy radical (EredLOO•),43 which would be expected to occur at nanomolar concentrations of NO present in vivo. After this reaction, dissociation and hydrolysis of the organic peroxynitrite (LOONO) gives LOOH and NO−3 as products (Figure 7). Because the LOX catalytic cycle is not completed, reoxidation of the enzyme-bound iron is required.43 Thus, because NO reaction occurs after O2 insertion into the fatty acid substrate, the LOX product profile is unchanged, the rate of product generation is suppressed, and NO is consumed.

Catalytic NO Consumption by Lipid Oxidation Enzymes

PGHS and LOX catalyze NO consumption via reaction with intermediates formed during enzyme turnover, a reaction first confirmed using purified enzymes and isolated vascular cells, including platelets and monocytes43,103 (also M.J. Coffey and V.B. O’Donnell, unpublished data, 2001). In these cell models, the rates and amounts of NO consumed are high, relative to expected rates and amounts of NO generated, suggesting that these reactions might play a role in both physiological and pathological NO removal in vascular cells.
addition, isolated peritoneal macrophages from 12-LOX knockout mice display 50% less NO\textsubscript{3} + NO\textsubscript{2} generation after interferon-\gamma/LPS challenge.\textsuperscript{132} Finally, oxidized LDL can have opposing effects on NO bioactivity, either through lysolecithin-dependent impairment of endothelium-dependent arterial relaxation or by causing induction of NOS2.\textsuperscript{133,134}

**Conclusions: Implications for Vascular Disease**

NO and NO-derived reactive species interact with lipid oxidation pathways via multiple mechanisms in vitro that are only recently being revealed. Because both processes are central to vascular regulation, an understanding of the particular interactions that are involved in pathogenesis of vascular disease in vivo is important. A role for NO acting as an antioxidant in vivo by inhibiting proatherogenic lipid oxidation is suggested, because increasing NO bioactivity through l-arginine supplementation has been successful in attenuating vascular dysfunction in hypercholesterolemic rabbits.\textsuperscript{135–137} In humans, results have been mixed, with intravenous infusion of l-arginine acutely improving coronary vasodilation but having no effect on microvascular endothelial function in patients with hypercholesterolemia.\textsuperscript{138} An alternative successful strategy has been to lower steady-state concentrations of NO-inactivating reactive oxygen species in animal models, via supplementation with antioxidant enzymes and oxidant scavengers.\textsuperscript{139–144} Several isoforms of PGHS and LOX are upregulated in both clinical and experimental cases of vascular disease,\textsuperscript{21,34,145–151} with inhibition of these enzymes normalizing blood pressure in some cases.\textsuperscript{150,152–154} Our observations of catalytic NO consumption by PGHS and LOX indicate novel mechanisms by which these enzymes might contribute to blood pressure regulation, in addition to the generation of vasoactive prostanooids. These are the first demonstrations of controlled NO removal by regulated catalytic processes in mammalian cells. Recent studies using iNOS knockout mice demonstrated altered urinary prostaglandin E\textsubscript{2} levels, confirming direct interactions between NO signaling and PGHS pathways in vivo.\textsuperscript{99} Finally, the reactions of NO and NO-derived species (eg, NO\textsubscript{3}, or ONOO\textsuperscript{-}) with oxidizing lipids leads to generation of novel nitrated lipid derivatives. If formed in atherosclerotic lesions, these species will either act as novel NO donors or possess distinct signaling properties similar to eicosanoids (A. Bloodsworth, V.B. O’Donnell, and B.A. Freeman, unpublished data, 2001).

Currently, knowledge regarding interactions between lipid oxidation pathways and NO is mainly from in vitro and animal model studies. Although great progress has been made at that level, a challenge for the future is to more incisively define which reactions are involved in the maintenance of vascular homeostasis and the initiation and progression of clinical vascular diseases.

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References


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