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 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.18–24 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), reaction with hypochlorous acid (HOCl), cell-dependent oxidation via a diversity of O2− and H2O2-generating oxidases, and, finally, oxidation by NO-derived reactive species (eg, NO2, nitryl chloride [NO2C], and ONOO−).25–32 In particular, support for a pathogenic role of LOX-catalyzed lipid oxidation in vivo in atherogenesis includes the observations that functional 15-LOX and its products are present in human and rabbit lesions,21,33,34 disruption of the mouse 12/15-LOX gene diminishes atherosclerosis in apoE-deficient mice, and inhibition of 15-LOX prevents development of atherosclerosis in cholesterol-fed rabbits.35,36 In contrast, targeted overexpression of rabbit macrophage 15-LOX prevents diet-induced atherosclerosis.37

In the vasculature, nitric oxide (NO) and lipid oxidation signaling pathways can potentially interact at several levels. Because of the diversity of the biochemical pathways involved, an understanding of how these processes might impact on vascular homeostasis is important. In this review, we summarize current knowledge of how lipid oxidation pathways and NO-derived species interact at a chemical and cellular level and describe what is known about how these interactions might influence disease progression.

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− formation, ONOO−, endothelial cells, or macrophages.30,31,38–42 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.38,40,43–50 During the reaction of LOO⋅ with NO, two molecules of NO are consumed as the primary organic peroxy nitrite (LOONO) intermediate rapidly decomposes (t1/2 = 0.2 to 0.6 second) to secondary radical species that react further.38 For example, LOONO can decompose to caged radicals [LO⋅NO2⋅], which can either terminate after rearrangement of LOO⋅ to an epoxide, L(O)NO2⋅, or dissociate and react with additional molecules of nitric oxide (Figure 1).

Figure 1. Fate of the termination product of NO and lipid peroxyl radicals. The initial product, an organic peroxy nitrite (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).

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topheroxyl radical by ascorbate is less effective at preventing lipid oxidation–induced tocopherol loss than the reaction of NO with LOO⁻.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 O₂⁻ to yield ONOO⁻) 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-KB activation, the linkage of vascular cell adhesion molecule-1 (VCAM-1) gene expression with NF-κB activation, lung injury secondary to intestinal ischemia, pulmonary epithelial cell oxygen injury, and the cytotoxicity of H₂O₂ and alkyl hydroperoxides.⁵³–⁵⁹ Finally, NO can undergo reactions with O₂ and/or O₂⁻ 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.⁶⁰ Peroxynitrite is more than two orders of magnitude more potent than H₂O₂ in catalyzing lipid oxidation in vitro and, in contrast to transition metal catalysts, mediates LDL oxidation even in the presence of lipophilic antioxidants.⁶⁰ In vitro, ONOO⁻ 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, F₂-isoprostane, and oxysterol products.⁴⁶,⁷⁶ These comparisons underscore the potential for ONOO⁻ in initiating lipid oxidation in atherosclerosis. The oxidation of nitrite (NO₂⁻) by MPO-derived hypochlorous acid (HOCl) will yield nitryl chloride (NO₂Cl),⁷⁷,⁷⁸ which, in purified LDL, depletes β-carotene and α-tocopherol, initiates lipid oxidation, forms 3-nitrotyrosine,⁷⁷ and can yield an LDL particle similar to that found in foam cells. The direct oxidation of NO₂⁻ by MPO+H₂O₂ yields NO₃ and also oxidizes LDL lipids to a proatherogenic particle.⁷⁹–⁸¹ Oxidation and nitration

**Reactions of the NO Metabolites NO₂ and NO₂Cl 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 nitrating occurring by hydrogen abstraction and addition reactions.⁶⁰,⁶⁵,⁶⁷–⁷⁴ These reactions result in formation of a complex mixture of products including nitrated lipid derivatives and alkynitrates, including those shown in Figure 3. Nitration of methyl linoleate and linolenate by NO₂ proceeds via initial hydrogen abstraction to form a carbon-centered alkyl radical, which at low oxygen tensions combines with NO₂ to form allylic nitro compounds (Figure 2). The yield of oxygen-containing lipid products (eg, LOOH, LOH, etc) formed by NO oxidation thus depends on the concentration of O₂ that will facilitate peroxidation reactions.⁶⁸ At high O₂ concentrations, for example, in lung lining fluid, NO₂ will predominantly mediate lipid oxidation. Conversely, at low O₂ tension (eg, within an inflamed hypoxic organ or microvessel), nitration reactions may preferentially occur. Somewhat analogous to NO, NO₃ 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.⁴⁶,⁷⁶

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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 forms nitrous acid (HONO), which decomposes to nitrosating and nitrating species including N₂O₃ at pH < 4; therefore, if these reactions are to occur in the vasculature, they will require acidic microenvironments, for example, in the phagolysosomes of neutrophils or macrophages.

**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 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 peroxyl (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 E₂ is found, although platelets from these animals generate more thromboxane B₂ 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 enzyme-bound lipid radicals (Figure 6). Formation of the nitrosyl complex of PGHS by NO is favored for the ferrous form, whereas the K₅₄ for formation of the ferric NO complex is very high, being 0.92 mmol/L for NO. Because the ferrous enzyme is not involved in enzyme catalysis and nonbiological (mmol/L) concentrations of NO would be required for significant metal center reaction to occur, nitrosyl complex formation is unlikely to be a mechanism of PGHS activity modulation by NO in vivo. Although termination of tyrosyl radicals by NO proceeds at essentially diffusion-limited rates, it is intriguing that PGHS is not more readily inhibited in vitro by NO. Possible explanations are that NO-tyrosyl radical reactions are readily reversible, or by acting as a peroxidase-reducing substrate, NO alternatively contributes to enzyme activation. Peroxynitrite is also an
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 predominately 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 reported 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, making this pathway of LOX inhibition unlikely. Rather, tissue LOX inhibition results from a termination reaction between NO and the enzyme-bound lipid peroxyl radical (E red LOOH), which would be expected to occur at nanomolar concentrations of NO present in vivo. After this reaction, dissociation and hydrolysis of the organic peroxyxynitrite (LOONO) gives LOOH and NO as products (Figure 7). Because the LOX catalytic cycle is not completed, reoxidation of the enzyme-bound iron is required. 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 monocytes (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.

**Figure 6.** Potential sites of NO interaction with PGHS. The cyclooxygenase and peroxidase catalytic cycles of PGHS are shown. Tyr indicates tyrosine; LOOH, lipid hydroperoxide; LOH, lipid hydroxide; AA, arachidonate; and NO2-Tyr, nitrotyrosine.

**Figure 7.** Reaction of NO with enzyme-bound lipid peroxyl radicals formed during lipoxygenase turnover. E red indicates inactive native ferrous enzyme; E ox, active ferric enzyme; LH, unsaturated lipid substrate; and LOOH, lipid hydroperoxide product. Reaction of NO with enzyme-bound lipid peroxyl radicals suppresses lipid oxidation by forcing the enzyme to undergo an activation step during each catalytic cycle.
Studies of soybean LOX-1, purified rabbit reticulocyte 15-LOX, human 15-LOX in murine fibroblast PA317 cells, and porcine leukocyte 12-LOX in monocytes have shown that the reaction of NO with E saturated LOO results in turnover- dependent NO consumption (also M.J. Coffey and V.B. O’Donnell, unpublished data, 2001). This scavenging of NO effectively prevents activation of purified or monocyte soluble guanylyl cyclase (sGC), indicating that enzyme-bound lipid radicals can compete with the heme of sGC for NO binding and thus attenuate the bioactivity of NO in mammalian cells (Figure 8A).43

Through acting as a peroxidase-reducing substrate, NO is also consumed rapidly by both purified PGHS-1 plus arachidionate and by the A23187 or thrombin-activated PGHS-1 activity of human platelets.103 Rates of NO removal by platelets are fast enough to deplete micromolar NO levels and potentely prevent NO-dependent activation of platelet sGC, thus causing platelets to overcome the antiaggregatory effect of NO (Figure 8B). This reveals a second novel proaggregatory function for PGHS-1 in addition to its generation of proaggregatory eicosanoids—specifically, catalytic consumption of the antiaggregatory species NO.

Lipid Oxidation Products Regulate NO Bioactivity

Although NO production regulates the induction and activity of lipid oxidation enzymes, their eicosanoid products can conversely modulate rates of cellular NO production. In platelets, activation of nitric oxide synthase is inhibited by aspirin or indomethacin, an effect that is overcome by addition of thromboxane A2.128 In the murine macrophage cell line J774, induction of NOS2 by LPS is inhibited by indomethacin,129 inferring involvement of PGHS products. Activation of LOX also leads to increases in NOS2 expression. For example, the nonspecific LOX inhibitor nordihydroguaiaretic acid prevents induction of NOS2 in myocytes or smooth muscle by IL-1 or LPS, respectively.130,131 In addition, isolated peritoneal macrophages from 12-LOX knockout mice display 50% less NO2−/NO3− generation after interferon-γ/LPS challenge.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 NOS2.133,134

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.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.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.139–144 Several isoforms of PGHS and LOX are upregulated in both clinical and experimental cases of vascular disease,21,34,145–151 with inhibition of these enzymes normalizing blood pressure in some cases.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 prostanoids. 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 E2 levels, confirming direct interactions between NO signaling and PGHS pathways in vivo.99 Finally, the reactions of NO and NO-derived species (eg, NO2− or ONOO−) 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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