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Invited Review

Interactions of Nitric Oxide-Derived Reactive Nitrogen Species with Peroxidases and Lipoxygenases

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Nitric oxide (NO) is a major free radical modulator of smooth muscle tone, which under basal conditions acts to preserve vascular homeostasis through its anti-inflammatory properties. The biochemistry of NO, in particular, its rapid conversion *in vivo* into secondary reactive nitrogen species (RNS), its chemical nature as a free radical and its high diffusibility and hydrophobicity dictate that this species will interact with numerous biomolecules and enzymes.

In this review, we consider the interactions of a number of enzymes found in the vasculature with NO and NO-derived RNS. All these enzymes are either homeostatic or promote the development of atherosclerosis and hypertension. Therefore their interactions with NO and NO-derived RNS will be of central importance in the initiation and progression of vascular disease. In some examples, (e.g. lipoxygenase, LOX), such interactions provide catalytic 'sinks' for NO, but for others, in particular peroxidases and prostaglandin H synthase (PGHS), reactions with NO may be detrimental. Nitric oxide and NO-derived RNS directly modulate the activity of vascular peroxidases and LOXs through a combination of effects, including transcriptional regulation, altering substrate availability, and direct reaction with enzyme turnover intermediates. Therefore, these interactions will have two major consequences: (i) depletion of NO levels available to cause vasorelaxation and prevent leukocyte/platelet adhesion and (ii) modulation of activity of the target enzymes, thereby altering the generation of bioactive signaling molecules involved in maintenance of vascular homeostasis, including prostaglandins and leukotrienes.

Keywords: Nitric oxide; Peroxidase; Lipoxygenase; Lipid; Prostaglandin; Myeloperoxidase

INTRODUCTION

Nitric oxide (NO) is a free radical signaling molecule that preserves vascular homeostasis through causing smooth muscle relaxation and preventing leukocyte and platelet adhesion to

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the endothelium. These classical signaling actions of NO are mediated exclusively through activation of soluble guanylate cyclase, and elevating intracellular concentrations of cyclic guanosine monophosphate (cGMP). As a free radical, the biochemistry of NO dictates that it interacts with numerous other signaling pathways, in particular with heme catalytic centers and free radical enzyme turnover intermediates.

Peroxidases and lipoxygenases (LOX) are two enzyme families that play central roles in vascular homeostasis and disease. Interactions of NO and NO-derived reactive nitrogen species (RNS), including nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) with catalytic centers of peroxidases and LOX are extensive and complicated, and have multiple consequences for maintenance of vascular homeostasis. These enzymes are ubiquitously expressed throughout the vasculature (Fig. 1), and their pattern of cytokine induction and activation indicate that generation of NO and its associated species will occur in tandem with enzyme turnover. The results of this are two fold. First, NO and NO-derived RNS will influence enzyme activity. This will result in modulation of eicosanoid product generation and peroxidase-dependent oxidative toxicity. Second, catalytic consumption of NO by peroxidase and LOX during turnover will alter levels of NO bioactivity, leading to loss of its vasoactive and anti-inflammatory properties. In this review, we summarize the known interactions of NO and NO-derived RNS with peroxidases (including heme peroxidases) and LOXs, and discuss what is known about the occurrence of these phenomena *in vivo*, and their contribution to vascular disease.

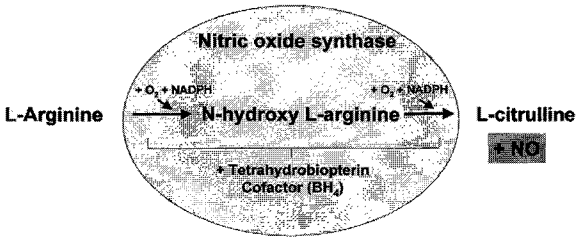

FIGURE 1 Generation of nitric oxide by nitric oxide synthases. Nitric oxide is generated by nitric oxide synthases thorough the oxidation of L-arginine and NADPH. During this process O_2 is reduced to H_2O. Tetrahydrobiopterin (BH_4) is an essential cofactor required by the enzyme.

THE BIOLOGICAL CHEMISTRY OF NO AND NO-DERIVED RNS

Nitric oxide is a free radical since it has an odd number of valence electrons. The unpaired 11th electron exhibited in the π^*2p anti-bonding orbital can be removed leading to generation of NO^+ (nitrosonium ion). Reduction of NO by addition of an extra electron to the second, unoccupied π^*2p orbital results in NO^- (nitroxyl ion) formation and a triplet ground state.[1]

The biological chemistry of NO predominantly occurs through reactions with other free radicals and transition metal ions leading to a complex mixture of products, several of which are potent oxidants and nitrating/nitrosating agents. Aerobically, NO reacts rapidly with O_2 forming the oxidant NO_2:

$$2NO + O_2 \rightarrow 2NO_2, \quad k = 2 \times 10^6\,M^{-2}\,s^{-1} \quad (1)$$

Biological exposure to NO_2 occurs through cigarette smoking, air pollution, reaction of endogenous NO with O_2,[2] and oxidation of NO_2^- by myeloperoxidase (MPO).[3,4] Reaction of NO_2 with another NO forms dinitrogen trioxide (N_2O_3), a potent 1- and 2-electron oxidant. In aqueous systems, N_2O_3 rapidly hydrolyzes to form nitrite (NO_2^-):

$$NO_2 + NO \rightarrow N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (2)$$

Formation of $ONOO^-$ occurs at near diffusion-controlled rates efficiently outcompeting superoxide dismutase (SOD) for superoxide ($O_2^{\cdot-}$).[5,6]

$$O_2^{\cdot-} + NO \rightarrow ONOO^-, \quad k = 6.7 \times 10^9\,M^{-1}\,s^{-1} \quad (3)$$

Peroxynitrite anion ($ONOO^-$) and its conjugate acid, peroxynitrous acid (ONOOH, $pK_a = 6.8$ at

37°C) can oxidize numerous biological targets including lipids, thiols, amino acid residues, DNA bases and low molecular weight antioxidants.[7–11] Also, $ONOO^-$ nitrates aromatic amino acids (e.g. tyrosine, tryptophan and phenylalanine) *in vitro* and possibly during inflammatory processes *in vivo*.[12–15] Reaction of $ONOO^-$ with CO_2 to form $ONOOCO_2^-$, redirects $ONOO^-$ reactivity towards nitration, and away from oxidation and hydroxylation.[10,16–20] Formation of $ONOO^-$ requires $O_2^{\cdot-}$, produced *in vivo* by several cell types following activation by pro-inflammatory stimuli.[21–24] Accessibility of $ONOO^-$ to biological targets is restricted by its fast decomposition rate ($t_{1/2} = 1$ s, 37°C, pH 7.4). Recent studies have shown that $ONOO^-$ can diffuse through biological membranes either through passive mechanisms, or through the erythrocyte anion channel.[25,26] This indicates that $ONOO^-$ will diffuse through biomembranes and into hydrophobic compartments.

Under acidic conditions, NO_2^- is protonated forming nitrous acid (HONO), a species which decomposes to yield both oxidants and nitrosating agents. Plasma nitrite concentrations can reach 0.5–13 μM,[27,28] 15 μM in respiratory tract lining fluid[29] and over 1 mM in saliva.[30,31] At least two biological compartments are acidic enough to allow HONO formation. In the stomach, pH varies from 2.5 to 4.5[32] while neutrophil phagolysosomes have a pH of 3.0–6.5.[33] This makes HONO formation highly likely, particularly during periods of excessive NO_2^- production (e.g. following induction of iNOS in sepsis or inflammation).

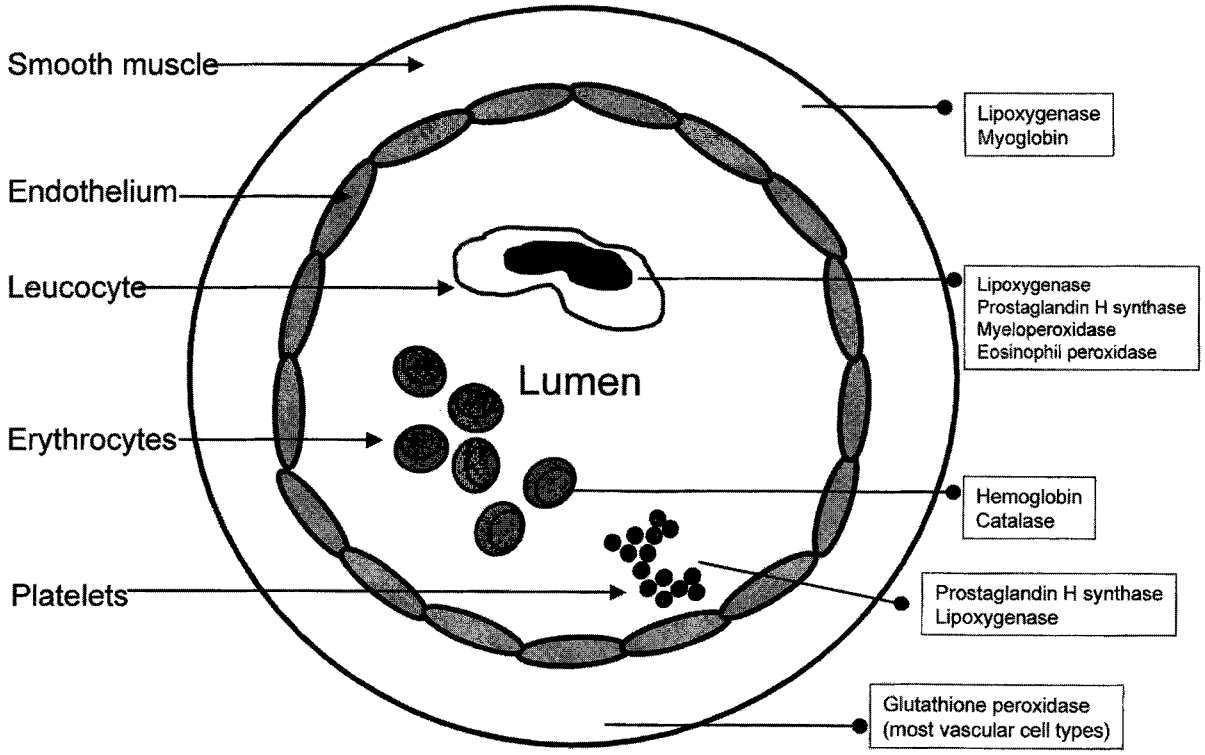

FIGURE 2 Cross section through a blood vessel showing locations of vascular peroxidases and lipoxygenases.

THE GENERATION OF NO BY NITRIC OXIDE SYNTHASES (NOS)

In biological systems, nitric oxide is generated through the conversion of L-arginine to L-citrulline by nitric oxide synthases (NOS) in the presence of molecular oxygen, NADPH and an essential cofactor, tetrahydrobiopterin (BH_4) (Fig. 2).[34] In the vasculature, NO is generated by endothelial NOS (eNOS). This constitutively-expressed enzyme produces NO in response to calcium influx (e.g. bradykinin, acetylcholine) for maintenance of vascular homeostasis.[35] The second NOS enzyme, neuronal NOS (nNOS) is expressed mainly in neuronal cells, but also certain other cell types, including kidney macula densa cells, β-pancreatic cells, skeletal muscle and epithelia of the lung, stomach and uterus.[36–40] Nitric oxide is produced by nNOS mainly as a neurotransmitter, but is also utilized for muscle tone regulation. The third NOS isoform, iNOS, is inducible in virtually all mammalian cells by a variety of pro-inflammatory stimuli, including tumor necrosis factor-α, interleukin-1β and lipopolysaccharide.[41] Nitric oxide produced by iNOS functions in pathogen killing, with its toxicity due to a combination of effects, including inhibition of respiration and cell division *via* NO reaction with catalytic iron centres, and direct cytotoxicity *via* formation of oxidants and nitrating/nitrosating species (incl. NO_2, N_2O_3, HONO and $ONOO^-$).[42–49]

THE ROLE OF NO IN THE VASCULATURE

Until the mid 1980s, NO was generally considered as an atmospheric pollutant with no significant biochemical activity *in vivo*. Its identification as the endothelial derived relaxation factor (EDRF) by Palmer *et al.*[50] rapidly changed the perception of its importance within the context of vascular biology.

Vascular tone is largely controlled *via* the action of NO on soluble guanylate cyclase (sGC) in vascular smooth muscle cells.[51] Nitric oxide generated by eNOS in response to agonists or shear, diffuses into the smooth muscle layer where it binds to the heme of sGC, causing a conformation change and activation. The subsequent rise in cyclic guanosine monophosphate (cGMP) activates protein kinase G,[52] resulting in a decrease in the levels of intracellular Ca^{2+} available to cause muscular contraction.[53,54] Also, eNOS-derived NO plays a central anti-inflammatory role in the vasculature *via* prevention of leukocyte and platelet adhesion to the endothelium.[55] Uncontrolled NO generation may have a detrimental effect on vascular homeostasis by causing a global drop in blood pressure (as can be associated with endotoxaemia or anaphylactic shock).[56] Conversely, chronic depletion of NO, or lack of its generation, can lead to hypertension and its associated downstream pathologies.[57,58] Controlling circulating NO concentrations within tight limits is an essential part of maintaining vascular homeostasis.

Nitric oxide treads a fine line between "hero and villain" within the vasculature: cultured monocyte-macrophages exert anti-oxidant effects through NO generation which attenuates lipid-peroxidising potential.[59] Paradoxically then, considering its role in relaxing vasomotor tone, NO can also be considered pro-atherogenic. The direct reaction of NO with other free radicals such as $O_2^{\cdot-}$ can generate pro-oxidants capable of modifying LDL and inactivating NOS.[60–63] Also, NO_2 formation that can occur through peroxidase oxidation or acidification of NO_2^- forms oxidizing and nitrating intermediates that can damage cell constituents, and may be centrally involved in the progression of vascular disease.[64]

NITRIC OXIDE REMOVAL IN THE VASCULATURE

Given the propensity of NO for liganding with heme iron (Fe), it was long assumed that the

principal mechanism of NO removal from the vasculature was through its reaction with oxyhemoglobin (oxyHb).[65] This reaction is fast ($k = 3.4 \times 10^7\ M^{-1}\ s^{-1}$)[66] and predicts that the half-life of NO in the vasculature would be only 2×10^{-6} s. This rate of reaction with oxyHb is too rapid to allow efficient diffusion of NO into the subendothelial layer.[67] Recent studies in microvessel preparations have shown that sequestration of hemoglobin into erythrocytes, combined with physiological flow, creates a "red cell depleted zone" between the inner face of the vascular endothelium and the lumen of the vessel containing the majority of the erythrocytes.[68,69] This zone effectively provides a diffusion barrier for NO removal by the red cells, and coupled with the rate at which the red cells flow through the vessel, led to the observation that, red cells do not scavenge EDRF activity, at least in this model system. Intriguingly, Pawloski *et al.*[70] have recently suggested that NO entering the erythrocyte is converted to S-nitrosothiol through its reaction with a conserved cysteine thiol group in the Hb molecule. The S-nitrosothiol can then be "released" from the cell in oxygen-poor tissues through an interaction with the anion exchanger AE1, suggesting targeted delivery of NO as opposed to simple random diffusion. Clearly then, scavenging of NO by oxyHb will be an important contributor to regulation of NO bioactivity *in vivo*, however the relative importance of this to other pathways (described below) has yet to be clarified. Similar to oxyHb, a central role for oxymyoglobin (oxyMb) present at high concentrations in cardiomyocytes, in regulating coronary blood flow and cardiac contractility through scavenging NO has been recently shown.[71]

The biological half life of NO under oxyHb-free conditions (0.1–3 s) is shorter than calculated rates of NO autoxidation,[72,73] suggesting that additional cell-dependent NO removal pathways exist. Also, in vascular diseases including hypertension and atherosclerosis, accelerated NO loss is often observed.[74–76] In hypertension, a role for superoxide ($O_2^{\cdot-}$) has been found, however this is only responsible for a proportion of the NO removed, since $O_2^{\cdot-}$ scavengers do not fully normalize blood pressure.[76] These observations suggest that cell-dependent catalytic scavenging of NO will regulate NO bioactivity under both physiological and pathophysiological conditions.

Recent work has examined whether NO removal may occur through its reaction with free radicals formed during turnover of enzymes that are expressed in vascular cells. For example, we have shown that 15 and 12/15-lipoxygenases (LOX) and prostaglandin H synthase (PGHS), all enzymes that play central roles in vascular homeostasis and disease can catalytically consume NO.[77,78] For some, activity was sufficient to remove μM concentrations of NO and efficiently block NO signaling (e.g. sGC activation, platelet aggregation).[77,78] Finally, the heme peroxidases, myeloperoxidase (MPO), lactoperoxidase (LPO), eosinophil peroxidase (EPO) and horseradish peroxidase (HRP) have been reported to scavenge NO catalytically as a reducing peroxidase substrate.[79,80] The localization of these and other potential NO scavenging enzymes within the vasculature is shown in Fig. 1. The subsequent sections of this review aim to summarise current thinking on the mechanistics and physiological importance of NO interactions with such enzymes.

INTERACTIONS OF NO AND NO-DERIVED RNS WITH PEROXIDASES AND LOX

The following sections focus on the interplay between the NO signaling pathway and LOX and peroxidase enzymes at a molecular and cell biology level. The interactions between these pathways are myriad and often complicated, including scavenging of NO by LOX/peroxidases, and modulation of LOX/peroxidases through the direct actions of NO or NO-derived

RNS. Since regulation of both the NO signaling pathway and LOX/peroxidase activities is central to the maintenance of vascular homeostasis, elucidating and understanding the interactions between these pathways is important.

Prostaglandin Endoperoxide-H Synthase

Prostaglandin H synthases (PGHS) are heme-containing enzymes also referred to as cyclooxygenases (COX). Two isoforms exist; PGHS-1 is constitutively expressed and found in platelets, endothelial cells, the kidney and gastro-intestinal tract while PGHS-2 is inducible and has been identified in macrophages and fibroblasts. Arachidonic acid oxygenation by PGHS generates prostaglandins and thromboxanes which play key roles in modulating vascular cell function (e.g. platelet aggregation and smooth muscle constriction are promoted by thromboxane A2, but inhibited by prostacyclin). Synthesis involves a two-step conversion. First, the enzyme oxidizes arachidonic acid to a cyclic endoperoxide, prostaglandin-G_2 (PGG_2) by a cyclooxygenase activity, then a peroxidase reduces the peroxide to a hydroxide, forming the endoperoxide, prostaglandin-H_2 (PGH_2).[81–84] The peroxidase and cyclooxygenase activities of PGHS separated by the heme prosthetic group, are located on opposite sides of the protein, and can function independently of each other although some crosstalk does occur.

It is now well established that there are numerous interactions between NOS and PGHS signaling pathways within the contexts of vascular biology and inflammation.[85–88] However, the precise interplay between NO and PGHS has yet to be fully clarified, with the literature citing a duplicitous role for NO, either enhancing, inhibiting or having no observable effects on PGHS activity or expression.[89–91] In several systems (including purified recombinant PGHS-2, intact platelets, endothelial cells and RAW-264.7 cells), NO highly stimulates prostaglandin generation.[89,92–95] However, others have found NO to be inhibitory towards PGHS,[91] or to have no effect on PGHS activity or expression.[90,96] In some cell types however (rat microglial cells and peritoneal macrophages), NO has been shown to suppress LPS-induced COX-2 expression, resulting in apparent enzyme inhibition.[97,98] Such differing effects may be attributable to the different NO exposure levels, and formation of secondary RNS, for example, $ONOO^-$ generated by NO reaction with activated macrophage-derived $O_2^{\cdot-}$, which can activate PGHS.[99]

Nitric oxide can interact with PGHS directly in several ways (Fig. 3). First, NO can form a ferrous-nitrosyl complex which is catalytically inactive. Tsai *et al.*[100] demonstrated the NO-heme interaction using stopped-flow spectrophotometry and described it as a favourable reaction of NO with the reduced ferrous heme of the enzyme with a K_d of 0.92 mM, which could displace the proximal histidine ligand to the prosthetic group. However, the concentrations of NO required to elicit even small inhibitory effects on PGHS activity are not found *in vivo* and the resting heme state of the enzyme is ferric, which has a low affinity for binding NO. Therefore, the generation of a ferrous-nitrosyl complex under physiological conditions is unlikely.

Activation of PGHS by hydroperoxide results in formation of a tyrosyl radical, proposed to be the oxidant responsible for hydrogen abstraction during cyclooxygenase turnover.[101,102] In this mechanism, ferric heme is oxidized by a hydroperoxide to an oxyferryl porphyrin π cation radical (compound I). Compound I then oxidizes a tyrosine, forming a tyrosyl radical, in turn being reduced to compound II ($Fe^{IV} = O$). Next, the tyrosyl radical oxidizes arachidonate, forming a carbon-centred radical.[103] Nitric oxide reacts with free tyrosyl radicals at nearly diffusion limited rates.[104] Recent work has shown that the PGHS-2 tyrosyl radical also reacts with NO, forming an iminoxyl radical that rearranges to 3-nitrotyrosine as the stable end-

product.[105] In contrast, addition of NO to PGHS-1 abolishes the active site tyrosyl radical and forms 3-nitrotyrosine, without detectable iminoxyl radical formation. Localization of 3-nitrotyrosine by tryptic digestion and peptide sequencing determined that the catalytic tyrosine residue of PGHS-1 was Tyrosine 385.[106]

A final way in which NO interacts directly with PGHS is as a reducing peroxidase substrate.[77,90] In this reaction, hydroperoxides stimulate catalytic NO consumption by the heme peroxidase activity of the enzyme, in a similar manner to other heme peroxidases (see below). At typical plasma concentrations of platelets, activation of PGHS-1 is sufficient to consume μM concentrations of NO and prevent its anti-aggregatory actions.[77] This demonstrates that catalytic scavenging of NO by PGHS-1 is an additional pro-aggregatory activity for this enzyme, in addition to generation of thromboxane A2.

While tyrosyl radical termination by NO proceeds at essentially diffusion-limited rates, it is intriguing that PGHS is not effectively inhibited *in vitro* by NO. Possible explanations are that NO-tyrosyl radical reactions are readily reversible and that by acting as a peroxidase reducing substrate, NO could contribute to enzyme activation.[77,90] Nitric oxide-derived $ONOO^-$ is an oxidizing peroxidase substrate for both PGHS-1 and PGHS-2, suggesting another mechanism by which NO could activate prostaglandin synthesis, especially under inflammatory conditions where $O_2^{\cdot-}$ production is elevated.[99,107]

In addition to direct effects on PGHS activity and induction, NO also influences substrate supply through modulation of phospholipase A2. However, studies are contradictory with NO blocking arachidonate release in thrombin-stimulated platelets or alternatively stimulating arachidonate release during interleukin-1 activation of pancreatic islet cells.[108,109]

The multiple and complicated interplay between NO-derived RNS and PGHS *in vitro* undoubtedly contribute to the complexity of effects observed in cellular systems and *in vivo*. It is likely that both inhibition and activation of

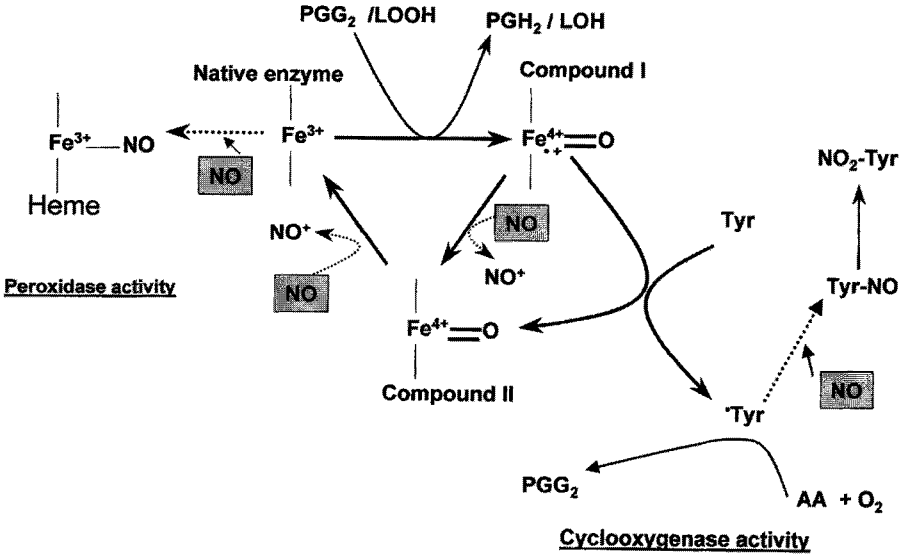

FIGURE 3 Catalytic cycle of prostaglandin H synthase showing sites of nitric oxide interaction. Prostaglandin H synthase exhibits two catalytic activities, cyclooxygenase and peroxidase. Nitric oxide can interact at multiple sites during turnover, as shown. AA, arachidonate; Tyr, tyrosine; $^{\cdot}$Tyr, tyrosyl radical; NO^+, nitrosonium ion; NO_2-Tyr, 3-nitrotyrosine; LOOH, lipid hydroperoxide; PGG_2, prostaglandin G2; PGH_2, prostaglandin H2; LOH, lipid hydroxide.

PGHS by NO combined with PGHS modulation of NO bioactivity simultaneously occur especially during conditions of upregulated prostaglandin and NO biosynthesis, such as inflammation.

Lipoxygenase

Lipoxygenases (LOX) are non-heme iron containing enzymes that oxidize unsaturated fatty acids to form hydroperoxides (LOOH). The unstable LOOH produced by LOXs are rapidly reduced to hydroxides (LOH), through the action of glutathione peroxidases (GPX). Isoforms of LOX are widely distributed throughout mammalian tissues with 5-LOX found chiefly in leucocytes, 12-LOX in platelets, 12/15-LOX in leukocytes and 15-LOX in reticulocytes and inducible in human monocytes by interleukins-4 and -13.[110–113] 15-LOX plays a central role atherogenesis, since (i) mRNA, protein and lipid products are found in human atheroma and (ii) inhibition prevents diet-induced atherosclerosis in rabbits.[114–118] Also, a central role for 12/15-LOX in vascular diseases was recently demonstrated by the observations that 12/15-LOX knockout mice do not develop either (i) atherosclerosis when crossed with Apo E-deficient mice or (ii) streptozotocin-induced diabetes.[119,120]

Lipoxygenases contain a catalytic non-heme iron that alternates between Fe^{2+} and Fe^{3+} forms. Resting enzyme is predominantly reduced and requires oxidation by LOOH before it can catalyze dioxygenation. Oxidation of arachidonate or linoleate by the ferric enzyme is shown (Fig. 4).

Nitric oxide has long been known as an inhibitor of soybean and mammalian LOXs, including platelet 12- and reticulocyte 15-LOX.[96,121–124] Initially, inhibition was suggested to result from reversible formation of a ferrous iron-nitrosyl complex, followed by oxidation to a ferric species, which is sensitive to peroxide activation.[123,124] However, formation of the nitrosyl complex requires high, non-physiological concentrations of NO.[121,125] Recent studies have shown that at lower biological NO concentrations, reversible transient inhibition of LOX occurs that coincides with catalytic NO consumption.[78] Kinetic analyses suggested that NO reacts with the enzyme-bound lipid peroxyl radical ($E_{red}LOO^{\cdot}$). Following this, dissociation and subsequent hydrolysis of the organic peroxynitrite species (LOONO) generates LOOH and NO_2^- as products. As NO addition occurs after dioxygenation of the fatty acid, the LOX product profile is unchanged, however turnover is transiently inhibited until the NO is fully consumed (Fig. 4).[78]

Catalytic consumption of NO by purified 15-LOX can suppress NO activation of purified sGC indicating that LOX-derived lipid peroxyl radical intermediates can effectively compete with the heme of sGC for NO.[79] Also, we have recently seen that linoleate-stimulated NO removal in porcine monocytes, which express leucocyte type 12/15-LOX, can completely block activation of monocyte sGC.[126] This indicates that cellular LOX expression is sufficient to catalytically consume biologically significant quantitites of NO and is likely to have a significant impact on NO signaling in the vasculature.

Heme Peroxidases

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are abundantly expressed in phagocytic white blood cells, with MPO constituting approximately 5% of total neutrophil protein.[127] Classically their role has been considered one of host defence, generating hypochlorite (HOCl) from hydrogen peroxide (H_2O_2) and chloride ions (Cl^-).[128,129] Heme peroxidases interact with NO and NO-derived RNS through multiple pathways that modulate their activities, and these interactions may play

a role in host defence responses when both high levels of neutrophil activation and NO generation co-exist.

A number of studies have demonstrated that heme peroxidases, including, MPO, horseradish peroxidase (HRP) and lactoperoxidase (LPO) can directly oxidize NO_2^-, following activation by H_2O_2. This occurs presumably *via* a one-electron pathway to form NO_2, and results in nitration of phenolic substrates and tyrosine residues in proteins.[4,130,131] Also, MPO can oxidize NO_2^- indirectly *via* HOCl presumably forming NO_2Cl.[132,133] Studies with purified peroxidases have shown that pathophysiological levels of NO_2^- (1–50 μM) compete with physiological substrates including chloride (Cl^-), bromide (Br^-) and thiocyanate (SCN^-) for oxidation. Second order rate constants for NO_2^- reaction with compound I to form compound II, and compound II to ferric MPO at pH 7 are $2\times10^6\,M^{-1}\,s^{-1}$ and $5.5\times10^2\,M^{-1}\,s^{-1}$ respectively.[134] This suggests that NO_2^- could be a biological substrate of heme peroxidases.[4] In addition, NO_2^- can enhance MPO-dependent Cl^- oxidation by recycling the inactive MPO Compound II.

Mammalian and plant peroxidases are well known to oxidize phenolic compounds such as tyrosine. This occurs through a one-electron mechanism forming intermediate tyrosyl radicals (Tyr), which combine with another Tyr to form dityrosine, or oxidize other biological substrates.[135] Simultaneous oxidation of NO_2^- and tyrosine produces NO_2 and Tyr in close proximity, and the subsequent reaction of these two species yields nitrotyrosine (NO_2Tyr), an established marker of inflammatory-mediated oxidative protein damage.[136,137] A role for MPO in generating NO_2Tyr *in vivo* has been proposed by several groups. In one study, human polymorphonuclear neutrophils (PMN) activated with 12-D-tetradecanoylphorbol-13-acetate (TPA) in the presence of physiologic levels of NO_2^- (1–50 μM) nitrated phenolic substrates and tyrosine residues in synthetic peptides.[132] Tyrosine nitration under these conditions was found to be dependent on MPO and H_2O_2. Exposure of activated PMN to pathophysiological fluxes of NO also led to tyrosine nitration that was almost exclusively inhibited by catalase and azide (an MPO inhibitor). This suggested that the mechanism

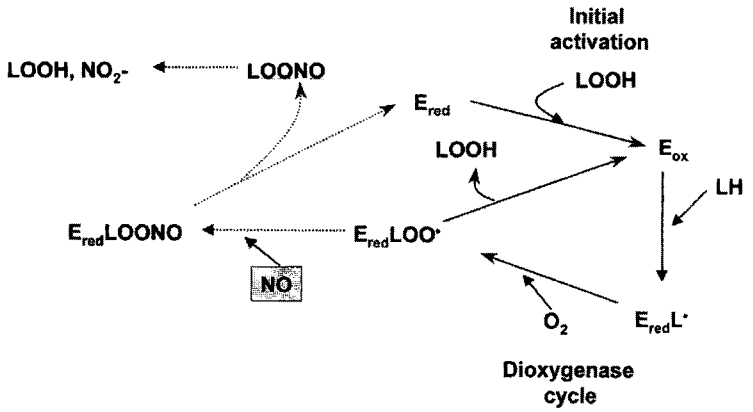

FIGURE 4 Catalytic cycle of lipoxygenase showing reaction of nitric oxide with lipid peroxyl radical ($LOO^·$) generated during enzyme turnover. Lipoxygenase oxidizes unsaturated fatty acids to lipid hydroperoxides as shown. Nitric oxide terminates $E_{red}LOO^·$ forming an organic peroxynitrite, $E_{red}LOONO$, that dissociates leaving the reduced enzyme (E_{red}), and finally hydrolyzes to LOOH and NO_2^-. E_{red}, inactive ferrous enzyme; E_{ox}, ferric enzyme; LH, unsaturated fatty acid; LOOH, lipid hydroperoxide; $E_{red}L^·$, reduced enzyme with bound lipid alkyl radical; $E_{red}LOO^·$, reduced enzyme with bound lipid peroxyl radical; LOONO, organic peroxynitrite, E_{red} LOONO, reduced enzyme with bound organic peroxynitrite; NO_2^-, nitrite; O_2, oxygen.

of NO_2-Tyr formation was $ONOO^-$-indepen--independent but required NO-derived NO_2^-. Finally, NO_2^- enhanced the levels of chlorinated products produced by activated PMN, confirming the role of NO_2^- in catalytically recycling MPO. Similar results have been found with human monocytes which stimulated in the presence of an exogenously added NO donor promoted LDL protein nitration and lipid oxidation through a number of pathways.[138] Here, low rates of NO flux caused nitration and oxidation that was inhibited by catalase and peroxidase inhibitors, but not by SOD, suggesting a role for MPO. Higher rates of NO caused nitration and oxidation that was sensitive to SOD, but not peroxidase inhibitors, indicating a role for $ONOO^-$. Collectively, these data reveal that NO_2^- oxidation by a broad range of heme peroxidases (i.e. MPO, LPO, EPO, and HRP) can lead to nitration *in vitro*, and suggest that this may be a plausible mechanism for achieving tyrosine nitration *in vivo*.

Heme peroxidases including MPO, LPO and HRP are oxidized by $ONOO^-$.[139,140] With HRP, formation of compound I followed by a slower conversion to compound II occurs, however, MPO and LPO appear to form compound II without intermediate formation of compound I. Second order rate constants for formation of MPO compound II, LPO compound II and HRP compound I at pH 7 are $6.2\times10^6\,M^{-1}s^{-1}$, $3.3\times10^5\,M^{-1}s^{-1}$ and $3.2\times10^6\,M^{-1}s^{-1}$ respectively.[139]

Nitric oxide can interact in several ways with heme peroxidases. With MPO, high levels of NO inhibit catalysis through formation of a stable six coordinate low spin nitrosyl complex, MPO-Fe(III)-NO.[141] A ferrous nitrosyl complex also forms with NO, however with an association constant that is over an order of magnitute slower.[141] In contrast, low levels of NO enhance MPO substrate peroxidation by promoting formation of compound II from compound I, and native enzyme from compound II (Fig. 5).[79,141] Similarly, NO also serves as a reducing peroxidase substrate for HRP, LPO and EPO.[79,80,140] Nitric oxide has been proposed as a physiological substrate for MPO and EPO,[79] however NO consumption by leukocyte peroxidases has not yet been reported in cells or *in vivo*.

Catalase is a heme-peroxidase ubiquitously expressed throughout mammalian tissues that is involved in protecting cells from reactive oxygen species.[142] Nitric oxide can inhibit catalase by forming a ferric heme complex. Paradoxically $O_2^{\cdot-}$ (also an inactivator of the enzyme) can reverse this inhibition, and H_2O_2 generated from $O_2^{\cdot-}$ dismutation can displace the heme-bound NO to gradually regenerate the active enzyme.[143] The significance of NO-mediated inhibition of catalase within the vasculature could be important within the context of vasorelaxation if H_2O_2, the principal substrate for catalase, is considered to be an endothelial derived hyperpolarizing factor (EDHF).[144] Inhibiting catalase could effectively prolong the time, H_2O_2 is present in the vasculature behaving as an EDHF and therefore may potentiate the vasorelaxing effects of NO. However, Li *et al.*[145] suggested that catalase inhibits NO generation from mouse peritoneal macrophages, challenged with interferon gamma and lipopolysaccharide, through a mechanism that involved decreasing levels of BH_4 (the NOS essential cofactor). As with the other heme-peroxidases, the homeostatic significance of NO interplay with catalase remains to be fully dissected.

Glutathione Peroxidase

Glutathione peroxidase (GPX) catalyses the reduction of lipid hydroperoxides (LOOH) generated by activated vascular cells and platelets. The enzyme is located ubiquitously throughout mammalian systems and is induced in response to oxidative stress.[146] A role for GPX in the metabolism of nitrosothiols was suggested

by the observation that the potency of S-nitrosoglutathione (GSNO) could be increased by addition of exogenous GPX.[147] Initially this was suggested to involve GPX catalysis of GSNO decomposition to NO.[147] In addition, a role for GPX in mediating transnitrosation between S-nitrosocysteine and glutathione was proposed. However, a later study showed that GSNO decomposition by GPX does not require co-substrates, and can be mediated directly by diselenides.[148]

Some studies have suggested reduced GPX can act catalytically as a $ONOO^-$ reductase, and thus protect cells from nitration.[149,150] However, others have shown that reaction of $ONOO^-$ with the ionized selenol of the GPX selenocysteine lead to enzyme inactivation.[151] Finally, inactivation of GPX by NO mediated S-nitrosation of a critical thiol group in the enzyme's active site has been shown.[152] Such mechanisms may have implications for cellular cytoxicity by effectively removing an important protective enzyme from a cell's available arsenal of countermeasures to oxidative stress.

IMPLICATIONS OF LOX AND PEROXIDASE INTERACTIONS WITH NO AND NO-DERIVED SPECIES*IN VIVO*

Nitric oxide and NO-derived RNS intereact with LOX and peroxidases *via* multiple mechanisms *in vitro* that are now being extensively studied by many groups. Since these pathways are centrally involved in the regulation of vascular homeostasis, understanding their interactions at a molecular level is critical to elucidating their involvement in the development of vascular disease.

Both PGHS and LOX isoforms are upregulated in human and animal vascular disease[114–116,153–159] and in some examples, their inhibition normalizes blood pressure.[158,160–162] Catalytic NO consumption by PGHS and LOX indicate additional mechanisms by which these enzymes might alter blood pressure, in addition to generation of vasoactive prostanoids, and are the first demonstrations of controlled NO removal by enzymatic catalysis in mammalian cells. Finally, studies using iNOS knockout mice

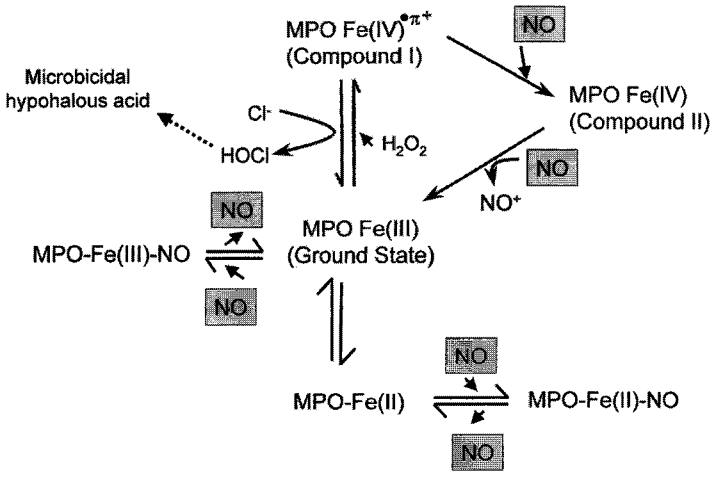

FIGURE 5 Catalytic cycle of myeloperoxidase showing sites of nitric oxide interaction. Myeloperoxidase oxidizes chloride forming hypochlorous acid. Sites of NO interaction with MPO are shown. MPO, myeloperoxidase; Cl^-, chloride; HOCl, hypochlorous acid; NO^+, nitrosonium ion.

have shown altered urinary prostaglandin E2 levels, indicating direct interactions between NO and PGHS *in vivo*.[163]

Human atherosclerotic lesions contain NO_2-Tyr at nearly 100-fold higher levels than those observed in low density lipoprotein from healthy volunteers,[164] and contain active MPO.[164–167] Intriguingly however, Brennan *et al.*[168] have recently demonstrated that MPO deficient mice are more prone to developing atherosclerosis hinting at perhaps an anti-atherogenic role for the enzyme in mice and revealing a potentially key difference between human and murine atherogenesis. Within an inflammatory context, both NO_2-Tyr and MPO are co-localized in rheumatoid joints,[169] and the lungs of patients with acute pulmonary inflammation.[170,171] Observations that microglial cells in human multiple sclerosis lesions are immunoreactive for iNOS, NO_2Tyr and MPO,[172–174] further suggest a peroxidase-dependent mechanism of tyrosine nitration may occur *in vivo*. However, since MPO is commonly an index of neutrophil infiltration, it is also likely to indicate sites of significant $O_2^{\cdot-}$ and hence, $ONOO^-$ generation. This makes elucidating the chemical nature of nitrating intermediates responsible for generation of NO_2Tyr *in vivo* difficult and at best speculative.

Currently, knowledge regarding interactions between LOX, peroxidases and NO is mainly from *in vitro* studies. While great progress has been made at that level, the challenge for the future is to more extensively determine which reactions are involved in the maintenance of vascular homeostasis and initiation/progression of disease. Clarification of the roles of vascular-located LOXs and peroxidases on the modulation of NO bioactivity and conversely, the roles of NO-derived RNS in regulating enzyme activity, particularly within the context of inflammatory cardiovascular pathologies, will aid development of effective therapies to halt or reverse the progression of vascular disease.

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