Nitric oxide, peroxynitrite and lipoxygenase in atherogenesis: mechanistic insights

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Abstract

Nitric oxide (•NO) is a free radical species that diffuses and concentrates in the hydrophobic core of low-density lipoprotein (LDL) to serve as a potent antioxidant. Peroxynitrite, the product of the diffusion-limited reaction between •NO and superoxide anion, as well as lipoxygenase, represent relevant mediators of oxidative modifications in LDL. The focus of this review is the analysis of interactions between •NO, peroxynitrite and lipoxygenase during LDL oxidation, which are relevant in the development of the early steps as well as progression of atherosclerosis. The role of CO2 to redirect peroxynitrite reactivity in LDL, as well as the lipophilic antioxidant sparing actions of •NO, ascorbate and CO2 is also analyzed. In this context, the effects of novel potential pharmacological strategies against atherosclerosis such as MnIIIporphyrins will be discussed.

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1. Introduction

1.1. Lipid peroxidation in atherogenesis: mechanisms and consequences

Oxidation of lipids was first proposed as a key step in atherogenesis by Chisolm and colleagues who found that modification of low density lipoprotein (LDL) by cells or other mechanisms could cause it to become cytotoxic (Hessler et al., 1983; Morel et al., 1983, 1984). Following this, it was demonstrated that cell-modified LDL was converted by oxidative pathways to a form that is taken up by macrophage scavenger receptors (Henriksen et al., 1981). These key observations led to two decades of intense research on the role of lipid oxidation in atherogenesis, including a plethora of studies on the potential protective role(s) of antioxidant vitamins (tocopherol and ascorbate in particular) in preventing atherosclerotic vascular disease. While it is undisputed that lipid oxidation is a central feature of atherosclerosis, it is still unclear whether this process plays a causative role.
In particular, recent negative results from large scale supplementation studies using antioxidant vitamins have questioned this idea (Chisolm and Chai, 2000).

Lipid oxidation products identified in lesions and plasma of patients with vascular disease include cholesteryl hydroperoxides, hydroxides, epoxides, hydroxycholesterol derivatives, isoprostanes and reactive aldehydes. Some of these species are formed by enzyme-catalyzed reactions, for example in early lesions hydroxyeicosatetraenoic acids (HETEs) are predominantly 12/15-lipoxygenase-derived, whereas in late lesions, this enzyme stereospecificity is lost with non-enzymatic formation more predominant. For most lipid oxidation products found in vivo in atherosclerosis, the mechanisms of their generation is unknown, and likely to involve multiple pathways. These may include peroxidase-dependent oxidation, prostaglandin H synthases, lipoxygenases, and further unknown oxidative pathways. Many of these products are bioactive, for example aldehydes are cytotoxic and protein-reactive, and such biological effects may contribute to the pathogenesis of atherosclerosis through modulation of inflammatory responses (Uchida, 2000).

2. Nitric oxide

2.1. *NO diffusion into LDL

The oxidation of LDL components by oxygen-free radicals and related species is thought to be a key early event in the pathogenesis of atherosclerosis. 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 (Esterbauer et al., 1992; Kenar et al., 1996; Orlova et al., 1999). In addition, LDL contains lipophilic antioxidants, including α-tocopherol, carotenoids and ubiquinol-10 (Esterbauer et al., 1992).

In recent years a key player during this process has been identified, the nitrogen-centered free radical nitric oxide (*NO). Nitric oxide readily crosses biological membranes and can concentrate in hydrophobic compartments, being able to participate in chemical reactions both in aqueous and lipid phases (Denicola et al., 1996b). The diffusion of *NO into LDL has been studied taking advantage of the fact that *NO is a good collisional quencher of pyrene fluorescence. The idea is to select pyrene derivatives that could be incorporated into LDL at different depths i.e. the cationic 1-(pyrenyl)methyltrimethylammonium (PMTMA), located at the surface of LDL and the cholesteryl ester derivative 1-(pyrenyl)methyl 3-(9-octadecenoyloxy) 22,23-bisnor-5-cholenate (PMChO) which penetrates to the hydrophobic core of LDL. The pyrene moiety is responsible for the fluorescence (λ_{exc} = 337 nm, λ_{em} = 396 nm) and the substituent determines the location of the probe. Fluorescence quenching experiments were performed by measuring steady-state fluorescence intensities in the absence and presence of different *NO concentrations. Using the Einstein–Smoluchowsky equation, apparent diffusion coefficient of the quencher (D_{NO}) were estimated according to:

\[
D_{NO} = \frac{k_{NO} \times 10^{-10}}{\pi N R}
\]

where \( N \) is the Avogadro’s number and \( R \) is the sum of the molecular radii of the probe plus *NO. The determined apparent diffusion coefficient of *NO (D_{NO}) in native LDL was 2000 μm² s⁻¹, only half the value obtained for the probe in solution, which indicates that *NO can easy get access to the particle, even the hydrophobic core of LDL (Denicola et al., 2002). It is interesting to notice that the \( D_{NO} \) in erythrocyte plasma membrane is significantly lower, indicating that diffusivity of *NO in LDL exceeds that of biomembranes (Denicola et al., 1998). Nitric oxide diffusion to the hydrophilic surface as well as the hydrophobic core of LDL supports a beneficial role of *NO, protecting LDL from oxidation.

2.2. *NO inhibition of lipid oxidation in LDL: reaction with lipid radicals, inhibition of lipoxygenase and formation of nitrogen-containing lipids

Nitric oxide reacts at diffusion limited rates with superoxide anion (O_{2}^-) to form peroxynitrite (ONOO^−), \( k \sim 10^{10} \text{M}^{-1} \text{s}^{-1} \) (Kisnor et al., 1997; Radi et al., 2000), a reactive nitrogen species with strong oxidizing and nitrating properties. Nitric oxide can both stimulate and inhibit O_{2}^- induced lipid and lipoprotein oxidation (Rubbo et al., 1994, 1995, 1996). The prooxidant versus antioxidant outcome of these reactions are extremely dependent on relative concentra-
tions of individual reactive species (Goldstein et al., 2000; Jourd’heuil et al., 2001), where *NO only stimulates O$_2^•$-dependent lipid oxidation when production rates of *NO are less than or equivalent to rates of O$_2^•$ production (Rubbo et al., 1994). Thus, there is a dynamic competition between O$_2^•$ and lipid radicals for reaction with *NO. In fact, an optimal rate of *NO release is required for maximal suppression of lipid oxidation that depends upon the rate and the mechanisms of lipid oxidation. We demonstrated that during a-oxygen-initiated lipid oxidation, lag times correlated well with the presence of *NO levels above a critical level (Rubbo et al., 2000b). It is interesting to note that under physiological conditions in the vessel wall, the steady state concentration of *NOO$\cdot$ exceeds that of O$_2^•$, resulting in a high *NO/O$_2^•$ ratio where *NO could exert antioxidant actions (Wever et al., 1998).

Several pathways promote LDL oxidation in vitro but the physiologically relevant mechanisms for LDL oxidation in vivo are still to be defined, although it clearly involves free radical chain reactions (Heinecke, 1997). Peroxynitrite and cell lipoperoxidases can oxidatively modify LDL in vitro, in a process that can be inhibited by both chemically- and cell-derived *NO (Rubbo et al., 1995; Goss et al., 1997). Nitric oxide causes a prolongation of the lag time and inhibition of the propagation phase of LDL oxidation through its chain breaking activity (Goss et al., 1995; Rubbo et al., 1995; Sanguinetti et al., 2004). Moreover, fragmentation of apolipoprotein B-100 by oxidants, loss of amine groups and protein–lipid fluorescent adducts formation is prevented by *NO (Troitschansky et al., 2001). The formation of lipid peroxidation-dependent antigenic epitopes in oxidized LDL is also inhibited by *NO (Secchia et al., 1997). Nitric oxide has multiple physicochemical qualities that make it an effective lipid antioxidant, including (a) its ability to react with unsaturated lipid reactive species such as alkyl (L$^\bullet$), epoxylipid L(O)$^\bullet$, alkoxyl (LO)$^\bullet$ or peroxyl (LOO)$^\bullet$ radicals to yield potentially unstable radical-radical termination products (Padmaja and Huie, 1993; Rubbo et al., 1994, 1995), (b) its partition coefficient of 6.5 for n-octanol–H$_2$O permitting concentration in lipophilic milieu such as the hydrophobic core of LDL (Denicola et al., 2002) and (c) in the case of lipoxygenase, its capacity to inhibit lipoxygenase-dependent lipid oxidation via direct enzyme inactivation as a consequence of ferrous-nitrosyl complex formation (Kanner et al., 1992). However, this inhibition requires much greater concentrations of *NO than biologically achievable (Rubbo et al., 1995). This is in line with the fact that non-heme iron enzymes such lipoxygenase bind ligand at the iron site less tightly than heme proteins. At biologically relevant *NO concentrations, reaction of *NO with the reduced form of lipoxygenase leads to reversible enzyme inhibition via scavenging of LOO$^\bullet$, resulting in further inhibition of its redox/free radical chemistry (O’Donnell et al., 1999b; Coffey et al., 2001a, 2001b).

Nitric oxide scavenging of LO$^\bullet$ or LOO$^\bullet$ yields nitrogen-containing lipid derivatives that rearrange or further react to form non-radical end products of lipid peroxidation (Rubbo et al., 1994, 1995) as shown by the following equations:

$$\text{LO}^\bullet + \text{NO} \rightarrow \text{LOONO}$$  \hspace{1cm} (2)
$$\text{LOONO} \rightarrow \text{LONO}$$  \hspace{1cm} (3)
$$\text{LO}^\bullet + \text{NO} \rightarrow \text{LONO}$$  \hspace{1cm} (4)

The almost diffusion-limited reaction of *NO with LOO$^\bullet$ (3 × 10$^{3}$ M$^{-1}$ s$^{-1}$) (Padmaja and Huie, 1993) will be significantly more facile than the initiation of secondary peroxidation propagation reactions by LOO$^\bullet$ with vicinal unsaturated lipids (k = 30 M$^{-1}$ s$^{-1}$). In the case of linolenic acid, these products have been tentatively identified from their molecular weight as a nitrosoperoxolinolenate, hydroxyl-nitrosoperoxolinolenate and hydroperoxynitrosoperoxolinolenate (Rubbo et al., 1994). Some nitrogen-containing lipid intermediates appear to be highly unstable and may decompose to reinitiate radical processes. In fact, the product of the LOO$^\bullet$*NO combination reaction (LOONO) has at least two fates: (a) internal rearrangement to give the more stable LONO$^\bullet$ and (b) homolytic cleavage to LO$^\bullet$ and NO$^\bullet$ with rearrangement of LO$^\bullet$ to an epoxylipid radical L(O)$^\bullet$ followed by recombination of L(O)$^\bullet$ with NO$^\bullet$ (O’Donnell et al., 1999a). We have also demonstrated the formation of nitrogen-containing oxidized lipids during the oxidation of phosphocholine liposomes by lipoxygenase in the presence of *NO (Rubbo et al., 1995). Inhibition of lipoxygenase by *NO may contribute to the antiatherogenic properties of *NO that has been observed in both animal and human models.
Nitration of LDL is now shown to occur in vivo as a potential "footprint" of the critical role that \*NO and/or \*NO-derived reactive species play during lipid oxidation processes (Balazy et al., 2001; Lima et al., 2002; Baker et al., 2004). These observations taking together with the fact that \*NO can readily diffuse into the hydrophobic core of the LDL particle (Dencila et al., 2002) are in agreement with our hypothesis that \*NO represents a major endogenous lipophilic antioxidant.

2.3. \*NO–antioxidant interactions during ONOO\(^-\)–mediated LDL oxidation: \*NO and ascorbate sparing of LDL \(\alpha\)-tocopherol

\(\alpha\)-tocopherol, localized at the surface of the LDL particle, provides minimal protection to lipid components in the hydrophobic core of LDL. We have demonstrated that \*NO represents a key lipid-soluble chain-breaking antioxidant protecting lipophilic antioxidants such as \(\alpha\)-tocopherol from oxidation (O’Donnell et al., 1997; Rubbo et al., 2000b). Indeed, \*NO serves to spare \(\alpha\)-tocopherol, which is not consumed during lipid oxidation reactions until \*NO concentrations fall under a critical level. Since the reaction of LOO\(^*\) with \(\alpha\)-tocopherol (constant of \(k = 2.5 \times 10^6 \text{M}^{-1} \text{s}^{-1}\)) occurs with a rate constant three orders of magnitude less than for the reaction of LOO\(^*\) with \*NO and because \*NO may access to hydrophobic sites where \(\alpha\)-tocopherol is not present, \*NO could act more readily than or in concert with \(\alpha\)-tocopherol as an antioxidant defense against oxygen radical-derived oxidized lipid species. The \(\alpha\)-tocopherol sparing actions of \*NO can be explained by simple competition kinetics: \*NO rapidly reacts with LOO\(^*\) (reaction (2)) and this reaction is in competition with LOO\(^*\) reduction by \(\alpha\)-tocopherol (\(\alpha\)-TOH, reaction (5)) and by unsaturated lipids \(\rightarrow\) LOOH (reaction (6)):

\[
\text{LOO}^* + \alpha\text{-TOH} \rightarrow \text{LOOH} + \alpha\text{-TO}^* \quad (5)
\]

\[
\text{LOO}^* + \text{LOOH} + \alpha\text{-TO}\rightarrow \text{LOOH} + \alpha\text{-TOOH} \quad (6)
\]

Either \*NO or ascorbate at physiologically relevant low concentrations spare \(\alpha\)-tocopherol in LDL, displaying additive antioxidant actions toward \(\alpha\)-tocopherol and LDL lipids when added in concert (Botti et al., 2004). In addition, \(\alpha\)-tocopherol and \*NO can act cooperatively to inhibit lipid peroxidation processes, exhibiting greater antioxidant capacity than the pair \(\alpha\)-tocopherol/ascorbate (Rubbo et al., 2000b). This cooperative action of \(\alpha\)-tocopherol and \*NO results in increased lag times before the onset of autocatalytic peroxidative propagation reactions. Ascorbate can both directly reduce \(\alpha\)-tocopheroxyl radical to \(\alpha\)-tocopherol with a rate constant of \(1.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}\) and LOO\(^*\) to LOOH at the slower rate constant of \(k = 7.5 \times 10^4 \text{M}^{-1} \text{s}^{-1}\). When both antioxidants are present, ascorbate is consumed first and upon ascorbate depletion \(\alpha\)-tocopherol becomes oxidized, because LOO\(^*\) preferentially reacts with \(\alpha\)-tocopherol and ascorbate preferentially reduces \(\alpha\)-tocopheroxyl radical rather than LOO\(^*\). Because lipid radicals formed in lipophilic milieu do not readily partition into the bulk aqueous medium and \*NO has access to hydrophobic sites, it is conceivable that the pair \*NO/\(\alpha\)-tocopherol would be more efficient than ascorbate/\(\alpha\)-tocopherol in the inhibition of lipid oxidation processes.

2.4. Lipid peroxidation attenuates \*NO bioactivity via radical reactions with \*NO

The changes which occur during atherosclerosis include the loss of the control of vascular tone, an \*NO-dependent event (Rubbo et al., 2000a). The mechanisms accounting for endothelial dysfunction in hypercholesterolaemia include a decreased bioavailability of \*NO due to increased \*NO degradation by poorly characterized oxidative pathways which likely include both superoxide and additional radicals. Further in this review, the role of lipoxigenase-derived radicals in catalyzing \*NO decay and preventing \*NO signaling in vascular disease will be described.

3. Peroxynitrite

3.1. Peroxynitrite formation in the vascular wall

Endothelial and smooth muscle cells are thought to be sources of \(\text{O}_2^*\) in vascular disease (Griendling et al., 2000), in addition to the well-characterized production of \*NO by endothelial nitric oxide synthase (Schini-Kerth and Vanhoutte, 1995). These cells increase \(\text{O}_2^*\) production through NAD(P)/H oxidase activation in response to physiopathological

\[
\text{O}_2^* + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2
\]
stimuli, including angiotensin II and oxidized LDL (Griendling and Ushio-Fukai, 2000; Rueckerschloß et al., 2001). The simultaneous production of NO and O$_3^*$ by vascular cells may support ONOO$^-$ formation within the vascular wall (Pueyo et al., 1998; Warnholtz et al., 1999; Wattanapitayakul et al., 2000).

The involvement of ONOO$^-$ in atherosclerosis is also supported by the demonstration of O$_3^*$-dependent modulation/impairment of *NO signalling as well as the presence of protein and lipid nitration in vivo (Ignarro et al., 1987; Beckmann et al., 1994; Hobbs et al., 1994; Leuvenburgh et al., 1997b; Dijkhorst-Oei et al., 1999; Lima et al., 2003). It should be noted that other nitrating agents such as myeloperoxidase/H$_2$O$_2$/NO$_2^-$ may also account for protein and lipid nitration observed during inflammation in the vasculature (Eiserich et al., 1998; Podrez et al., 1999).

3.2. Peroxynitrite induces lipid and α-tocopherol oxidation in LDL by a radical mechanism: bolus versus infusion

Peroxynitrite can initiate lipid oxidation in membranes and lipoproteins (Rati et al., 1991; Darley-Utmar et al., 1992; Rubbo et al., 1994; Moore et al., 1995; Patel et al., 1996; Kaptiots et al., 1997; Thomas et al., 1998). Peroxynitrite-modified LDL binds with high affinity to scavenger receptors leading to the accumulation of cholesterol esters involved in the production of the fatty streak, characteristic of the atherosclerotic lesion (Darley-Utmar et al., 1992; Graham et al., 1993; Guy et al., 2001). However, the mechanisms of ONOO$^-$-mediated oxidation of LDL are only partially understood. For example, peroxynitrite anion (ONOO$^-$) protonates at physiological pH to peroxynitrous acid (ONOOH) that can oxidize organic molecules directly or through H$^*$ or CO$_2$-catalyzed homolysis yielding nitrogen dioxide (*NO$_2$), hydroxyl (*OH) or carbonate anion radical (CO$_3^{−}$) (Rati et al., 2000).

Bolus addition of ONOO$^-$ yields less than 10% of the lipid oxidation generated by ONOO$^-$ fluxes (Botti et al., 2004). This is in agreement with previous reports showing that ONOO$^-$ bolus addition does not lead to important LDL lipid peroxidation, where apolipoprotein B-100 and carotenoids are the proposed preferred targets of ONOO$^-$ in LDL (Pannala et al., 1998; Thomas et al., 1998; Panasenko et al., 2000). In contrast, low infusion rates of ONOO$^-$ can efficiently oxidize lipids in LDL causing cholesteryl linoleate hydroperoxide formation in parallel with conjugated diene accumulation and oxygen consumption (Troitschansky et al., 2001; Botti et al., 2004a,b). This is probably due to the fact that with bolus addition, ONOO$^-$ yields a burst of initiator radicals (i.e. *OH and *NO$_2$) from the homolysis of ONOOH that can also rapidly terminate lipid oxidation by radical-radical reactions. In contrast to the bolus addition, a flux of ONOO$^-$ generates a continuous source of lipid radicals, favouring propagation reactions.

Although α-tocopherol is not a major component of LDL it is consumed during the initial phases of LDL oxidation, thus constituting a preferential target of ONOO$^-$-induced oxidants. While ONOO$^-$-mediated LDL antioxidant depletion has been attributed to rapid direct oxidation reactions by ONOO$^-$ or ONOOH (Pannala et al., 1998; Kontush et al., 2000; Panasenko et al., 2000; Terentis et al., 2002), we postulate a radical-dependent mechanism in which *NO$_2$ and *OH initiate both lipid and α-tocopherol oxidation (Botti et al., 2004).

One of the major changes that occurs during LDL oxidation by ONOO$^-$ is the formation of lipid-protein adducts (Troitschansky et al., 2001) as a consequence of the extensive oxidative breakdown of polyunsaturated lipids. This yields hydroperoxides that decompose to aldehydes and other bioactive products that cross-link with free amino groups, making the LDL particle more electronceptive (Steinbrecher, 1987; Fruebis et al., 1992; Requesen et al., 1997; Tsai et al., 1998; Ikarka et al., 2000; Troitschansky et al., 2001). These adducts represent ligands for macrophage scavenger receptors that contribute to foam cell formation (Steinbrecher et al., 1989; Horkko et al., 1999; Boullier et al., 2000). Time courses of lipid-protein adduct formation in ONOO$^-$-treated LDL correlated well with the increase in lipid oxidation and amino group content depletion (Troitschansky et al., 2001). This strongly suggests that the decay in free amino groups that occurs during ONOO$^-$-mediated LDL oxidation mostly depends on the formation of lipid-protein adducts. These result from reactions between free amino groups of LDL with hydroperoxides (Fruebis et al., 1992) and aldehydes (e.g. malondialdehyde, hydroxynonenal), generated from hydroperoxide decomposition (Ikarka et al., 2000; Ushio, 2000). Therefore, the potent inhibition
by •NO and ascorbate on α-tocopherol consumption in LDL exposed to ONOO⁻ provide strong evidence supporting a one-electron free radical mechanism of α-TOH oxidation (Botti et al., 2004).

3.3. Redirection of ONOO⁻ reactivity in LDL by CO₂: apo B-100 nitration

Detection of nitrotyrosine in fatty streaks of coronary arteries in close association with foam cells, vascular endothelium and in the neointima of atherosclerotic lesions indicates •NO-derived oxidant-dependent reactions during both early and chronic stages of atherosclerosis, which result in the formation of highly immunogenic and potentially proinflammatory protein oxidation products (Beckmann et al., 1994). In addition, measurements of 3-nitrotyrosine in LDL isolated from human atherosclerotic lesions show that there is a striking 90-fold increase compared with circulating LDL (Leeuwenburgh et al., 1997a).

Human plasma contains 25 mM bicarbonate in equilibria with CO₂ which reacts rapidly with ONOO⁻ to form the reactive adduct 1-carboxylate-2-nitrosodioxidane (ONOOCO₂⁻) which rapidly decomposes to CO₂ and nitrate (Goldstein et al., 1998; Lymar and Hurst, 1998; Lyman and Hurst, 1998; Squatrito and Pryor, 1998; Bonini et al., 1999; Hodges and Ingold, 1999; Lehninger, 1999; Meli et al., 1999; Raddi et al., 2000). The second-order rate constant for the ONOO⁻–CO₂ reaction is 5.8 × 10⁴ M⁻¹ s⁻¹ at 37 °C (Denicola et al., 1996a). The ONOOCO₂⁻ adduct can perform one-electron oxidations as well as nitration reactions. Different pathways may account for the reactivity of ONOOCO₂⁻, i.e. rearrangement to nitrocarbonate anion, heterolytic cleavage to nitronium and carbonate anion or homolytic decomposition to yield CO₃•⁻ and •NO₂ (Raddi et al., 2000). We have recently observed that in LDL, bicarbonate in equilibrium with CO₂ at physiological concentrations inhibits lipid oxidation in parallel with α-tocopherol consumption (Botti et al., 2004). Moreover, CO₂ is able to inhibit ONOO⁻-induced apo B-100 fragmentation and enhances protein nitration (Botti et al., 2004). Thus, the limited permeability of LDL to charged species such as CO₃•⁻ and the repulsive forces between CO₃•⁻ and the predominately negatively-charged LDL surface will disfavour the diffusion of CO₃•⁻ into the hydrophobic environments of the particle, thus decreasing the rate of initiation of lipid oxidation in LDL. Rather, recombination reactions in the aqueous phase and/or reaction with less lipophilic targets (i.e. surface exposed apo B-100 residues) may be favoured (Fig. 1).

![Fig. 1. A radical-mediated mechanism of peroxynitrite-mediated lipid and α-tocopherol oxidation in LDL and the modulatory role of CO₂ and •NO (adapted from Botti et al., Free Rad. Biol. Med. 35, supplement 1, 283, 2003).](image-url)
3.4. A pharmacological approach: Mn(III)porphyrin plus uric acid inhibition of ONOO\(^-\)-mediated LDL oxidation

Manganese(III)porphyrins (Mn(III)porphyrins) possess several antioxidant properties, including their scavenging of \(\text{O}_2\)\(^{**}\), hydrogen peroxide, lipid radicals and ONOO\(^-\) (Batinic-Haberle et al., 1998; Lee et al., 1998; Day et al., 1999; Ferrer-Sueta et al., 1999). The scavenging properties of metalloporphyrins can be altered by modifying the porphyrin substituents (Batinic-Haberle et al., 1998; Day et al., 1999). One mechanism by which Mn(III)porphyrins scavenge ONOO\(^-\) involves the formation of an \(\text{O}_2\)Mn(IV) complex (\(k \sim 10^7 \text{M}^{-1} \text{s}^{-1}\)) and the subsequent reduction of \(\text{O}_2\)Mn(IV) by urate, ascorbate or glutathione (Ferrer-Sueta et al., 1999). This reduction step converts the simple scavenging of ONOO\(^-\) into a catalytic reduction cycle that is very efficient in the presence of excess of reductants (Lee et al., 1998; Ferrer-Sueta et al., 2002).

We recently demonstrated the protective role of three Mn(III)porphyrins differing in charge, alkyl substituent length and reactivity, on LDL exposed to low fluxes of ONOO\(^-\) in the presence of uric acid (Trostchansky et al., 2003). The Mn(III)porphyrin/urate system was able to inhibit cholesteryl ester hydroperoxide and lipid protein adduct formation, as well as \(\alpha\)-tocopherol oxidation. Interestingly, a shift from an anti- to a pro-oxidant action of the Mn(III)porphyrin was observed after uric acid was significantly consumed, supporting competition reactions between LDL targets and uric acid for the Mn(IV)=O complex. This likely results from reaction of Mn(IV) with lipid hydroperoxides, to form initiating and propagating lipid alkoxyl/peroxyl radicals, as previously shown (Bloodsworth et al., 2000).

Overall, the data is consistent with the catalytic reduction of ONOO\(^-\) in a cycle that involves a one-electron oxidation of Mn(III)–Mn(IV) by ONOO\(^-\) followed by the reduction back to Mn(III) by uric acid (Fig. 2). These antioxidant effects should predominate under in vivo conditions having plasma uric acid concentration range between 150–500 \(\mu\text{M}\) (Trostchansky et al., 2003). Other reductants including ascorbate at physiological concentrations could also support the antioxidant action of these compounds during LDL oxidation (Bloodsworth et al., 2000).

![Fig. 2. Protective mechanism of Mn(III)porphyrin/urate during peroxynitrite-mediated LDL oxidation (modified from Trostchansky et al., 2003).](image)
4. Lipoxygenase

Lipoxygenases (LOX) are non-heme iron-containing enzymes that catalyze arachidonate or linoleate oxidation to form linoleate or arachidonate hydroperoxides. Lipoxygenases contain a non-heme iron that cycles between Fe^{2+} and Fe^{3+} during turnover. Resting enzyme predominantly exists as Fe^{2+}, requiring oxidation before dioxygenation can occur. In mammalian cells, several LOX isoforms are known, named by their position of oxygen insertion into arachidonate. Their expression in vascular tissue is widespread, with sources including platelets (12-LOX), monocyte/macrophages (15-LOX, 12/15-LOX), neutrophils (5-LOX, 12/15-LOX), and smooth muscle cells (12/15-LOX). LOXs catalyze the oxidation of unsaturated fatty acids to hydroperoxides and other bioactive metabolites utilizing a non-heme iron active site (Kuhn and Thiele, 1999). 12/15-LOX, inserts molecular oxygen primarily at C12, but also at C15 forming mainly 12(S)-HPETE, and some 15(S)-HPETE by cytosolic glutathione peroxidases. Oxidation of arachidonate or linoleate by the ferric enzyme is shown (Fig. 3).

4.1. LDL oxidation by LOX and its potential role in atherogenesis

Most LOX isoforms, including 5-LOX (leukocytes) and 12-LOX (platelet-type), utilize free arachidonate substrate, however, the reticulocyte 15-LOX (human, rabbit), otherwise known as leukocyte 12/15-LOX (pig, rat, mouse) can oxidize complex lipids including membrane phospholipids and LDL, and also linoleate (Kuhn and Thiele, 1999). Its involvement in atherosclerosis was first proposed following observations of specific LOX products in early human and rabbit lesions, along with protein and mRNA expression, and that its inhibition prevents diet-induced atherosclerosis in rabbits (Yla-Herttuala et al., 1990; Yla-Herttuala et al., 1991; Kuhn et al., 1994, 1997; Folic et al., 1995; Hiltunen et al., 1995; Sendobry et al., 1997; Belkner et al., 1998; Bocan et al., 1998). Interestingly, established lesions contain oxidized lipids that have lost their stereospecificity, indicating non-enzymatic lipid peroxidation occurs later in atherosclerosis progression (Kuhn et al., 1994, 1997). Following from this work, several groups examined mechanisms and consequences of free and cell-expressed LOX oxidation of LDL, and found that this process formed a high-uptake LDL form, similar to that found in atherosclerotic lesions, and that this requires LDL receptor related protein, and can be enhanced by angiotensin II, lipoprotein lipase and secretory phospholipase A2 (Scheidegger et al., 1997; Sigari et al., 1997; Belkner et al., 1998; Xu et al., 2003; Zhu et al., 2003).

The generation of a 12/15-LOX knockout mouse enabled definitive experiments to be undertaken regarding the role of this enzyme in atherosclerosis. Backcrossing with Apo E or LDL receptor-deficient strains demonstrated that LOX plays a central role in atherosclerosis (Cyrus et al., 1999; George et al., 2001). Also, the extent of lipid oxidation in vivo in LOX−/−Apo E−/− versus LOX+/−Apo E−/− mice is significantly reduced, as measured by plasma isoprostane concentrations (Cyrus et al., 2001). Similarly, over expression of 15-LOX in vascular endothelium accelerates atherogenesis in Apo E−/− mice, although unexpectedly, selective macrophage over expression in rabbits is protective (Shen et al., 1996; Harats et al., 2000).

4.2. Lipoxygenase consumption of *NO and inhibition of *NO signalling in the vasculature

15- and 12/15-LOX isoforms play a central role in several vascular diseases associated with accelerated removal, including atherosclerosis, hypertension and diabetes (Jackson et al., 1981; Noszawa et al., 1990;
Yla-Herttuala et al., 1991; Stern et al., 1993; Kuhn et al., 1994, 1997; Lin et al., 1994; Folcik et al., 1995; Sendobry et al., 1997; Belkner et al., 1998; Bleich et al., 1999; Cyrus et al., 1999). Both 12/15- and 15-LOX consume \( \cdot \text{NO} \) during dioxygenase turnover (Fig. 4) (O’Donnell et al., 1999b; Coffey et al., 2001b). This results from the termination of an enzyme-bound lipid peroxyl radical with \( \cdot \text{NO} \), and results in reversible suppression of LOX turnover (O’Donnell et al., 1999b). Furthermore, using cells transfected with either 15- or 12/15-LOX, rapid removal of supraphysiological quantities of \( \cdot \text{NO} \) was observed. Finally, uptake of \( \cdot \text{NO} \) by 15- or 12/15-LOX prevents activation of sGC in vitro and in isolated porcine monocytes (O’Donnell et al., 1999b; Coffey et al., 2001b). Rates of linoleate-stimulated \( \cdot \text{NO} \) consumption by primary monocytes (\((1–2) \times 10^6 \text{nmol min}^{-1} \text{cells})\) are similar to rates of \( \text{O}_2^\cdot \) generation, and far exceed expected rates of \( \cdot \text{NO} \) generation from \( \text{nNOS} \). This indicates that 12/15-LOX has the potential to act as a significant \( \cdot \text{NO} \) sink in vivo.

Although the hydroxy lipid products of 12/15- and 15-LOX possess some bioactivity at a cellular level, they have not been considered major modulators of vascular function in vivo. These in vitro studies demonstrate a novel mechanism by which 12- and 12/15-LOX could contribute to the development of vascular disease, independent of lipid product bioactivity, namely by removing the vasodilatory and anti-inflammatory free radical, \( \cdot \text{NO} \).

5. Conclusions

The recent observations of the extremely fast and direct reactivity of \( \cdot \text{NO} \) with both free and LOX-bound lipid radicals as well as \( \text{O}_2^\cdot \) strongly support a central role for \( \cdot \text{NO} \) in regulating vascular atherogenic processes. Also, \( \cdot \text{NO} \) consumption through its reactions with lipid radical species will play an important role in inhibiting the vascular protective actions of \( \cdot \text{NO} \). Our data supports a free radical one-electron mechanism of \( \text{ONOO}^- \)-induced oxidation of \( \text{α- tocopherol in LDL} \). Thus, the antioxidant role of \( \cdot \text{NO} \) and its additive actions with ascorbate may be of great importance in vivo. We also evidenced the ability of \( \text{CO}_2 \) to inhibit lipophilic target oxidation in LDL, probably redirecting \( \text{ONOO}^- \) reactivity to hydrophilic moieties. Finally, the \( \text{α- tocopherol sparing actions of \( \cdot \text{NO} \), ascorbate and\( \text{CO}_2 \) will ensure low yields of \( \text{ONOO}^- \)-mediated \text{vitamin E} oxidation in vivo. Future pharmacological strategies against cardiovascular diseases should include \( \cdot \text{NO} \) precursors, LOX inhibitors and the development of novel compounds against \( \text{ONOO}^- \) toxicity, including \( \text{ONOO}^- \) scavengers such as \( \text{MnIII} \)-porphyrins.

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Fig. 4. Proposed mechanism of NO consumption by LOXs. \( \text{EOx} \): oxidized iron, \( \text{Ered} \): reduced iron, \( \text{LH} \): arachidonate, \( \cdot \text{L} \): lipid alkyl radical, \( \text{LOOO} \): lipid peroxyl radical, \( \text{LOOH} \): lipid hydroperoxide, \( \text{LOONO} \): organic peroxynitrite derivative, \( \text{NO}_2^- \): nitrite.

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