Free Rad. Res., Vol. 31, pp. 597–606
Reprints available directly from the publisher
Photocopying permitted by license only

Reactions of $^{\bullet}NO$, $^{\bullet}NO_2$ and Peroxynitrite in Membranes: Physiological Implications

STEVEN P.A. GOSS, RAVINDER J. SINGH, NEIL HOGG and B. KALYANARAMAN*

Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509, USA

Accepted by Prof. B. Halliwell

(Received 27 February 1999)

Nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$) are hydrophobic gases. Therefore, lipid membranes and hydrophobic regions of proteins are potential sinks for these species. In these hydrophobic environments, reactive nitrogen species will exhibit different chemistry than in aqueous environments due to higher local concentrations and the lack of hydrolysis reactions. The peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) can freely pass through lipid membranes, making peroxynitrite-mediated reactions in a hydrophobic environment also of extreme relevance. The reactions observed by these reactive nitrogen species in a hydrophobic milieu include oxidation, nitration and even potent chain-breaking antioxidant reactions. The physiological and toxicological relevance of these reactions is discussed.

Keywords: Nitric oxide, nitrogen dioxide, peroxynitrite, nitration, oxidation, membrane

INTRODUCTION

Nitric oxide ($^{\bullet}NO$) has many diverse biological activities, ranging from neuronal transmission to the regulation of vascular tone.[1] Similar to oxygen, $^{\bullet}NO$ is chemically inert with respect to most biological molecules and easily partitions into lipid environments. The relatively long half-life of $^{\bullet}NO$, approximately 1 s in biological systems,[2] allows $^{\bullet}NO$ to diffuse across several cell diameters.[3] These properties enable $^{\bullet}NO$ to act as a messenger molecule. An example of this is shown by the regulation of vascular tone; $^{\bullet}NO$ is generated by the vascular endothelium by a constitutive form of nitric oxide synthase (NOS), after which $^{\bullet}NO$ diffuses out of the cell and into the smooth musculature where it binds to guanylyl cyclase and initiates vascular relaxation.

Binding of $^{\bullet}NO$ to guanylyl cyclase illustrates one of the major biological targets of $^{\bullet}NO$, heme proteins. $^{\bullet}NO$ is also reactive to other free radicals, such as the superoxide anion ($O_2^{\bullet-}$) and the tyrosyl radical. Although $^{\bullet}NO$ is soluble in aqueous solutions, it has higher solubility in hydrophobic solvents.[4] This suggests that the concentration of $^{\bullet}NO$ may be higher in a lipid milieu and therefore highly relevant to reactions with lipid ($L^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) radicals.[5]

* Corresponding author. Tel.: 414-456-4035. Fax: 414-456-6512. E-mail: balarama@post.its.mcw.edu.

The steady state concentration range of •NO generated *in vivo* is about 10–100 nM.[6,7] •NO may increase to concentrations of 450 nM at the surface of endothelial cells following bradykinin stimulation[6] and up to 4 μM during cerebral artery occlusion.[8] Although much is known about the chemistry of •NO in aqueous solution, detailed investigations of the chemistry of •NO and other oxides of nitrogen in the hydrophobic interior of membranes or hydrophobic regions of proteins have only recently begun.

•NO AND MEMBRANES

The low-density lipoprotein (LDL) particle is a useful model for the investigation of both reactive oxygen and reactive nitrogen chemistry in a hydrophobic environment. The LDL particle consists of a single protein, apolipoprotein B-100 (Apo-B), which has a circumferential distribution around the particle.[9–14] The outer shell of the particle is composed of a monolayer of phospholipids and free cholesterol which surrounds a cholesterol-ester rich hydrophobic core[15] (Figure 1).

The generation of •NO by the endothelium has generated much interest in the role of •NO in LDL oxidation. •NO has been reported to have both antioxidant and pro-oxidant roles during its interaction with LDL.[5,16–21] Many of these contradictory investigations are the result of differences in choice of an appropriate •NO source. Although authentic •NO solutions are often used, care must be taken to reduce the contamination of these solutions with oxygen or higher oxides of •NO, such as $^{\bullet}NO_2$ or N_2O_3, which will exhibit entirely different chemistry. Other sources of •NO include a wide variety of compounds that decay to release •NO. The release of •NO from many of these donor compounds is usually dependent on light or catalysis by

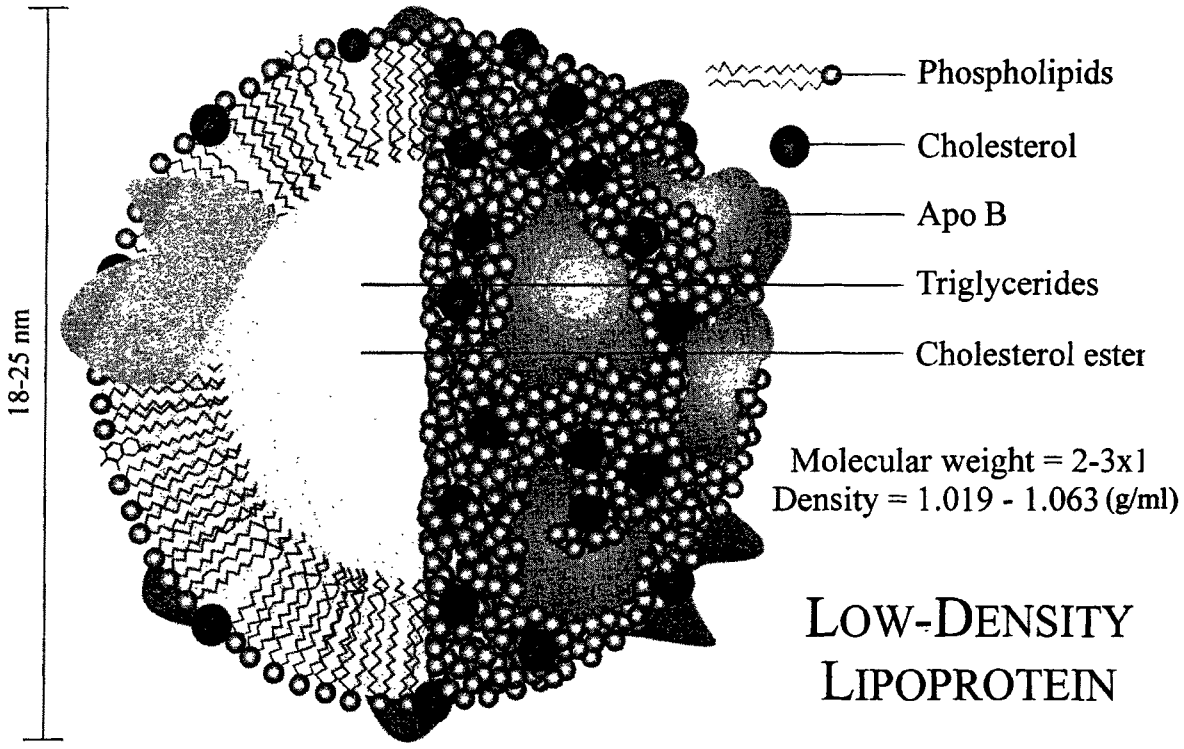

FIGURE 1 LDL structure.

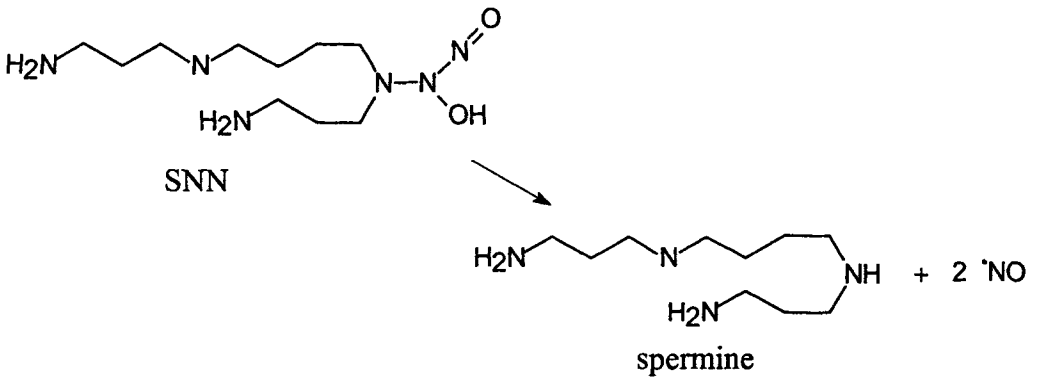

FIGURE 2 The thermolytic decomposition of SNN to •NO and spermine.

enzymes or metal ions,[22] making it difficult to determine the kinetics of •NO release. 1-Substituted diazen-1-ium-1,2-diolates, a group of •NO donor compounds commonly referred to as NONOates (Figure 2), thermolytically release two molecules of •NO from each molecule of donor compound. Since •NO release is independent of cofactors, these donor compounds have easily defined rates of decay, making them ideal tools for elucidating •NO mechanisms by kinetic modeling. In addition, both the decayed compound and secondary amine backbone are easily obtainable for use as controls. Rates of •NO release are affected by reaction conditions (e.g., pH, temperature, buffer), so it is important to verify and characterize •NO release in the experimental system under study, rather than rely on published half-lives. Verification of •NO release can be accomplished using an •NO electrode, a chemiluminescence detector or electron spin resonance with nitronyl nitroxide, a •NO scavenger.[23] The decay of the NONOate compound can be determined with a UV spectrometer at 250 nm.

It is believed that oxidation of the lipid component of LDL leads to the pro-atherogenic modification of the LDL particle.[24] Lipid peroxidation consists of three phases; initiation, propagation, and termination.[25] Initiation occurs upon the abstraction of a bis-allylic hydrogen from an unsaturated fatty acid which yields a lipid radical. Once initiation has occurred, peroxidation is propagated through a chain reaction mediated by lipid peroxyl radicals. Termination reactions remove these radicals by either radical–radical interactions or by reactions with chain-breaking antioxidants. Such antioxidants remove lipid peroxyl radicals through donation of a hydrogen atom to the radical, thus generating lipid hydroperoxide (LOOH). The resulting antioxidant radical is not sufficiently reactive to abstract a hydrogen atom and is therefore unable to participate in the propagation of oxidation. LDL is afforded some protection against oxidation in the form of a small contingent of endogenous antioxidants (consisting predominantly of vitamin E). However, once these antioxidants have been consumed, oxidation of the LDL particle results in a dramatic increase in the particle's LOOH content. Since antioxidants are the primary defense of the particle, monitoring endogenous antioxidant concentrations is a sensitive assay of LDL oxidation.

There is contradiction in the literature concerning whether •NO exhibits pro-oxidant[26–28] or antioxidant activity[29] with respect to the LDL particle. However when carefully handled, •NO does not affect the endogenous antioxidants α-tocopherol (α-TH), γ-tocopherol (γ-TH) or β-carotene, even when bolus addition of •NO solution is used.[30,31] Artifactual oxidation of α-TH in many studies most likely resulted from oxygen contamination or a failure to remove

contaminating nitrogen oxides from commercial •NO gas. Addition of •NO gas also exhibits an antioxidant activity towards lipid oxidation if bolus additions of low (sub-micromolar concentrations) are repeatedly made during the oxidation time-course[32] or by slow continuous infusion.[33]

(A) Transition Metal Ion-mediated LDL Oxidation

Copper-mediated LDL oxidation is dependent on the presence of pre-existing lipid hydroperoxides. When copper breaks down these lipid hydroperoxides, initiation occurs. Antioxidants such as α-TH donate a hydrogen atom to the lipid peroxyl radical to form a lipid hydroperoxide and break the propagatory chain reaction. Therefore, in the presence of α-TH, the kinetic chain length of the lipid peroxidation cycle is one. Figure 3 is a scheme of copper-mediated lipid peroxidation.

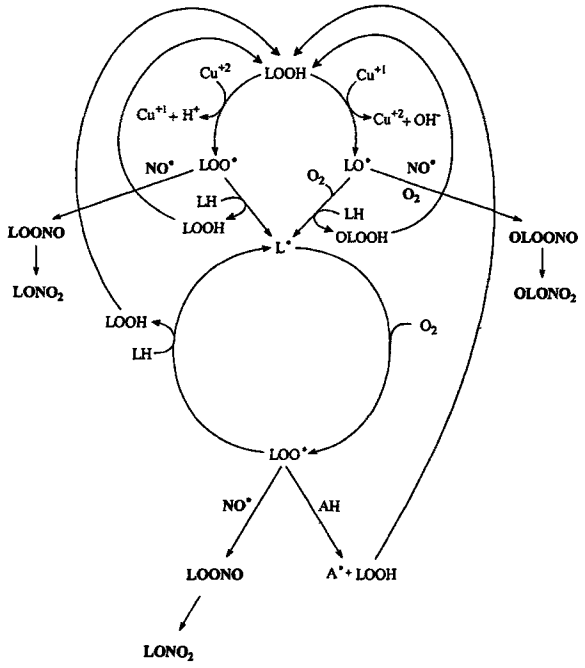

FIGURE 3 Copper-mediated lipid peroxidation in the presence of •NO. Bold symbols denote steps in which •NO scavenges lipid-derived radicals to inhibit oxidation.

As shown in Figure 3, each step of peroxidation will generate lipid hydroperoxides, making copper-mediated lipid peroxidation a complex autocatalytic reaction.

•NO is an extremely potent inhibitor of copper-mediated LDL oxidation as measured by TBARS formation, conjugated diene formation, changes in electrophoretic mobility, and α-TH depletion.[30,34] The kinetics of •NO inhibition of copper-dependent LDL oxidation suggest that •NO acts as a peroxyl radical scavenger, as previously reported.[5]

In contrast to phenolic antioxidants, the concentration dependence of the antioxidant effect of nitric oxide donor compounds is non-linear.[30] A possible mechanism for this is that •NO inhibits the propagation of lipid peroxidation by scavenging peroxyl radicals to form a lipid-nitroso adduct as shown in Figure 3. As a consequence, LOOH is not formed, resulting in a kinetic chain length of lipid peroxidation of zero in the presence of •NO. Since LOOH is not formed, further copper-dependent initiation is prevented. Therefore, when all of the endogenous LOOH has been converted to LOONO or OLOONO, the LDL will no longer be susceptible to copper-dependent oxidation. This will result in a non-linear concentration-dependent inhibition of oxidation by •NO. Support for this mechanism comes from the observation that when 13[S-(E,Z)]-hydroperoxy-9,11-octadecadienoic acid (HpODE, a lipid hydroperoxide) is added, a higher concentration of •NO donor compound is required to reach this non-linear region of the concentration-dependent inhibition than is needed in the absence of HpODE.[30]

Even in the presence of •NO donor compounds, LDL does eventually oxidize in the presence of copper. This may be due to the breakdown of LOONO to LOOH by hydrolysis or some other mechanism. If this occurs after the •NO donor compound has decayed, oxidation will occur. Recently it was demonstrated that two molecules of •NO are consumed for each chain reaction that is terminated.[32] This suggests that the products

formed from the breakdown of LOONO may also be scavenged in the presence of •NO. The stability of lipid-nitroso adducts in biological systems has not been fully determined.[33]

(B) Peroxyl Radical-Mediated LDL Oxidation

Azo compounds can initiate free radical chain reactions, including lipid peroxidation, by a metal ion-independent mechanism.[35] These molecules have the generic structure R–N=N–R and thermolytically decompose, by a double homolytic cleavage of the R–N bonds, to give nitrogen and the radical R•. This radical can either dimerize, forming R–R, or react with oxygen to give the peroxyl radical ROO•. It is this peroxyl radical that is thought to be the initiating molecule. Two azo initiators, 2,2′-azobis-2-amidinopropane HCl (ABAP), which is water-soluble, and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), which is lipid-soluble, have been extensively used to investigate lipid peroxidation. The advantage of using ABAP rather than copper to study the kinetics of LDL oxidation lies in the fact that ABAP decays very slowly. Consequently, if a high concentration of ABAP is used, radical production is effectively linear (because ABAP concentration decreases negligibly during the course of the experiment).

•NO is an extremely potent inhibitor of ABAP-mediated LDL oxidation as measured by TBARS formation, changes in electrophoretic mobility, and α-TH depletion. As with copper, a possible mechanism for the inhibitory effect of •NO upon lipid peroxidation is by scavenging peroxyl radicals to form a lipid-nitroso adduct.[35] Inhibition may also occur via a direct reaction between •NO and the initiating radical generated from the thermal breakdown of ABAP.[36] The effect of •NO, again, will differ from that of classical phenolic antioxidants such as α-TH in that •NO will act as an antioxidant by inhibiting lipid peroxidation with a kinetic chain length of zero. A scheme showing the various points at which •NO may have an effect on ABAP-mediated lipid peroxidation is shown in Figure 4.

•NO reacts with organic peroxyl radicals with a rate constant of $1–3 \times 10^9\,M^{-1}\,s^{-1}$.[37] The reaction between peroxyl radicals and α-TH has a rate constant of $5 \times 10^5\,M^{-1}s^{-1}$.[38] Therefore, •NO could be as effective an inhibitor of lipid peroxidation as α-TH at a much lower concentration (in the order of 10^4 times lower). Furthermore, •NO is a hydrophobic molecule and partitions favorably into LDL-lipid. •NO may then be available to react with all peroxyl radicals regardless of their orientation in respect to the antioxidant functional group or their location within the LDL particle. The generation of •NO at a slow and controlled rate may result in a steady state concentration of •NO sufficiently high to react with lipid peroxyl radicals as they are formed

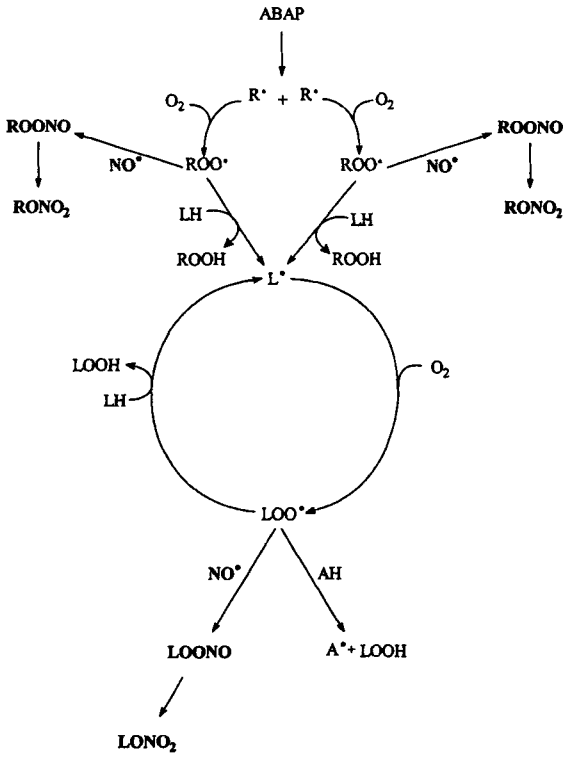

FIGURE 4 ABAP-mediated lipid peroxidation in the presence of •NO. Bold symbols denote steps in which •NO scavenges peroxyl radicals.

and thus inhibit both the initiation and propagation reactions of the lipid peroxidation chain reaction.

(C) Cell-mediated LDL Oxidation

The actual mechanism underlying biological oxidation of LDL is yet to be determined, however, many mechanisms have been suggested.[39] *In vitro* oxidative modification of LDL occurs when LDL is incubated in the presence of monocytes, macrophages, smooth muscle cells, endothelial cells, and neutrophils.[24,40–45] Macrophages stimulated with lipopolysaccharide and interferon-γ were shown to have a reduced ability to oxidize LDL.[19,21,46] This observation was attributed to the induction of iNOS and the formation of $^{\bullet}$NO because the NOS inhibitors MDL 100,248 and N^G-monomethyl-L-arginine were shown to reverse this effect. The addition of various $^{\bullet}$NO donors to unstimulated macrophages also prevented these cells from oxidizing LDL. This inhibition was observed with three different $^{\bullet}$NO donors that exhibited different mechanisms of $^{\bullet}$NO release.[47]

These studies clearly show that cell-dependent oxidation of LDL, which depends on the propagation of lipid peroxidation, is inhibited by the presence of $^{\bullet}$NO donors. As shown during both transition metal ion-mediated and peroxyl radical-mediated LDL oxidation, $^{\bullet}$NO may act as a peroxyl radical scavenger in this system.

$^{\bullet}NO_2$ AND MEMBRANES

$^{\bullet}$NO is not chemically inert in the presence of oxygen. The rate of the reaction between $^{\bullet}$NO and oxygen is proportional to the second power of the $^{\bullet}$NO concentration.[48,49] Consequently, this reaction will be greatly favored at the high, nonbiological concentrations of $^{\bullet}$NO generated by bubbling $^{\bullet}$NO gas through solutions.[16,20] In aqueous solution, the autoxidation of $^{\bullet}$NO to form reactive intermediates is thus too slow to be of any physiological significance.[50] However, both $^{\bullet}$NO and oxygen are considerably more lipophilic and, consequently, the autoxidation of $^{\bullet}$NO should be greatly accelerated in the hydrophobic milieu.[4] The oxidative reaction of $^{\bullet}$NO is mediated by $^{\bullet}NO_2$ or N_2O_3. These molecules are also hydrophobic. In the lipid phase, the hydrolysis of $^{\bullet}NO_2$ to nitrite and nitrate is minimal. As $^{\bullet}NO_2$ is a potent nitrating agent, it follows that membranes may represent an important site of biological nitration.

$^{\bullet}NO_2$ is also formed from autoxidation of $^{\bullet}$NO or from one-electron oxidation of the nitrite anion by peroxidases.[51,52] Several peroxidizing systems, including myeloperoxidase/H_2O_2 and copper, zinc superoxide dismutase/H_2O_2 have been shown to oxidize NO_2^- to $^{\bullet}NO_2$. $^{\bullet}NO_2$ will react with phenolic compounds to yield diagnostic products (see Scheme 1). $^{\bullet}NO_2$, a potent lipid-soluble oxidant, can abstract a hydrogen atom ($k \approx 10^5\,M^{-1}\,s^{-1}$ at pH 7.0) from the phenolic hydroxyl group of α-tocopherol (α-TH) and γ-tocopherol (γ-TH) to form the corresponding α-tocopheroxyl (α-T$^{\bullet}$) and γ-tocopheroxyl (γ-T$^{\bullet}$) radicals.[53] The reaction between α-T$^{\bullet}$ and $^{\bullet}NO_2$ is very rapid, forming a radical–radical recombination intermediate that rearranges to form the α-tocopheryl quinone (α-TQ). The reaction between γ-T$^{\bullet}$ and $^{\bullet}NO_2$ leads to a nitrated product, 5-nitro-γ-tocopherol (NGT). NCT and α-TQ can be separated and identified by HPLC.[54]

$$^{\bullet}NO_2 + \alpha\text{-TH} \rightarrow \alpha - T^{\bullet} + HNO_2$$

$$^{\bullet}NO_2 + \alpha\text{-T}^{\bullet} \rightarrow\rightarrow \alpha\text{-TQ}$$

$$^{\bullet}NO_2 + \gamma\text{-TH} \rightarrow \gamma\text{-T}^{\bullet} + HNO_2$$

$$^{\bullet}NO_2 + \gamma\text{-T}^{\bullet} \rightarrow \text{NGT}$$

$$^{\bullet}NO_2 + \text{NGT} \rightarrow {}^{\bullet}\text{NGT} + HNO_2$$

$$^{\bullet}\text{NGT} \rightarrow\rightarrow \text{products}$$

SCHEME 1

$ONOO^-$ AND MEMBRANES

As indicated earlier, $^{\bullet}NO$ reacts with $O_2^{\bullet-}$ at a nearly diffusion-controlled rate to form peroxynitrite.[55] This reaction appears to be ubiquitous in cellular systems.[56] Both peroxynitrite anion ($ONOO^-$) and its conjugate acid peroxynitrous acid (ONOOH) can cross lipid membranes at a rate comparable to that of water.[57] This rapid transmembrane diffusion of $ONOO^-$/ONOOH necessitates a better understanding of their oxidative and nitrosative reactions in membranes.

As with $^{\bullet}NO$ and $^{\bullet}NO_2$, the hydrophobic interior of biological membranes also influences nitration reactions of transmembrane targets by peroxynitrite (Figure 5). Recently, it has been reported that although the reaction between free tyrosine and peroxynitrite (added in bolus amounts) forms nitrotyrosine, neither SIN-1 (which decomposes to generate $^{\bullet}NO$ and $O_2^{\bullet-}$ at equal rates) nor the simultaneous addition of $^{\bullet}NO$ and $O_2^{\bullet-}$ results in tyrosine nitration.[58]

This paradox does not appear to exist with the nitration of γ-TH in membranes. Peroxynitrite preferentially nitrates membrane-bound γ-TH as compared to tyrosine in the aqueous phase and the addition of SIN-1 to liposomes containing γ-TH results in the formation of NGT.[59] This aspect is intriguing and raises interesting questions with respect to the mechanisms of phenolic nitration in the hydrophobic phase. Peroxynitrite-dependent nitration of tyrosine in the aqueous phase is likely to be very different from nitration of tyrosine residues in the membrane. In addition, the presence of either α-TH or γ-TH has an inhibitory effect on peroxynitrite-mediated tyrosine nitration.

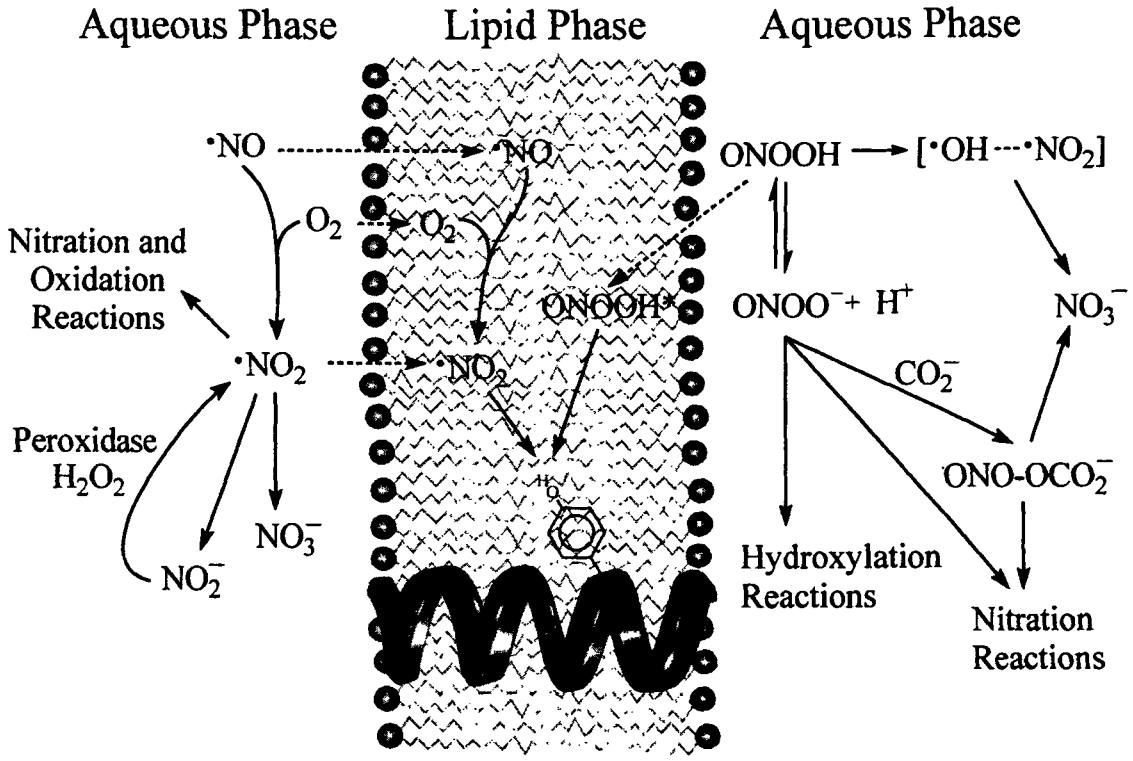

FIGURE 5 Nitration reactions in membranes. In the aqueous phase, reactive nitrogen species undergo hydrolysis and hydroxylation reactions. For example, $NO_2^{\bullet}$ hydrolyses to form NO_2^- and NO_3^- and peroxynitrite catalyzes hydroxylation reactions in the aqueous phase. In the lipid phase, the reaction mechanism appears to be dominated by nitration reactions.

CONCLUDING REMARKS

The reaction between •NO and lipid-derived radicals has major biological and biomedical ramifications.[60] As the rate constant between •NO and peroxyl radicals is nearly diffusion-controlled, this represents a potent chain-terminating reaction for lipid peroxidation. This property, coupled with the high solubility of •NO in hydrophobic membranes, makes •NO a unique gaseous antioxidant.

Although it is difficult to accurately measure the rate of biologically-mediated LDL oxidation in *in vivo*, it is likely to be fairly slow, if comparisons to cellular-mediated LDL oxidation can be made. The steady state concentration of •NO generated by the endothelium, and the likelihood of the presence of •NO in the sub-endothelial layer, suggest that the oxidative modification of LDL located in that area will be inhibited.

The slow generation of •NO by the vascular endothelium may represent a continuous source of antioxidant, playing an integral role in suppressing oxidative reactions within the vasculature. Impairment of •NO generation or acceleration of the rate of oxidation may be a critical component in both the early stages and the development of atherosclerosis.

Research focusing on the rapid transmembrane diffusion of $ONOO^-$/ONOOH may offer a better understanding of their oxidative and nitrosative reactions in membranes. Very little data concerning RNS-mediated nitration reactions in membranes exist in the literature.[4] Increased levels of nitrotyrosine and nitrated proteins have been detected in a variety of pulmonary and cardiovascular diseases, and neurodegenerative and chronic inflammatory disorders.[61,62] Clearly, a detailed understanding of the oxidative and nitrosative reactions of reactive nitrogen species in well-defined model membranes will provide new insight in to the development of therapeutic strategies to minimize oxidative and nitrosative processes in diseases.

Acknowle dgment s

This work was supported by a grant from the National Institutes of Health, HL47250. Material for this chapter was taken from the Ph.D. dissertation submitted by Steven Goss.[63]

References

[1] P.L. Feldman, O.W. Griffith and D.J. Stuehr (1993) The suprising life of nitric oxide. *Chemical Engineering News*, **71**, 26–38.
[2] M. Kelm and J. Schrader (1998) Nitric oxide release from the isolated guinea pig heart. *European Journal of Pharmacology*, **155**, 317–321.
[3] J. Lancaster (1998) Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 8137–8141.
[4] X. Liu, M.J.S. Miller, M.S. Joshi, D.D. Thomas and J.R. Lancaster Jr. (1998) Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 2175–2179.
[5] N. Hogg, B. Kalyanaraman, J. Joseph, A. Struck and S. Parthasarathy (1993) Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Letters*, **334**, 170–174.
[6] T. Malinski and Z. Taha (1992) Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor. *Nature*, **358**, 676–678.
[7] K. Shibuki and D. Okada (1991) Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature*, **349**, 326–328.
[8] T. Malinski, F. Bailey, Z.G. Zhang and M. Chopp (1993) Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *Journal of Cerebral Blood Flow and Metabolism*, **13**, 355–358.
[9] C. Yang, Z.W. Gu, M. Yang and A.M. Gotto Jr. (1994) Primary structure of apoB-100. *Chemistry and Physics of Lipids*, **67–68**, 99–104.
[10] S. Parthasarathy and J. Barnett (1990) Phospholipase A2 activity of low density lipoprotein: evidence for an intrinsic phospholipase A2 activity of apoprotein B-100. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 9741–9745.
[11] J.E. Chatterton, M.L. Phillips, L.K. Curtiss, R. Milne, J.C. Fruchart and V.N. Schumaker (1995) Immunoelectron microscopy of low density lipoproteins yields a ribbon and bow model for the conformation of apolipoprotein B on the lipoprotein surface. *Journal of Lipid Research*, **36**, 2027–2037.
[12] J.E. Chatterton, M.L. Phillips, L.K. Curtiss, R.W. Milne, Y.L. Marcel and V.N. Schumaker (1991) Mapping apolipoprotein B on the low density lipoprotein surface by immunoelectron microscopy. *Journal of Biological Chemistry*, **266**, 5955–5962.
[13] V.N. Schumaker, M.L. Phillips and J.E. Chatterton (1994) Apolipoprotein B and low-density lipoprotein structure:

implications for biosynthesis of triglyceride-rich lipoproteins. *Advances in Protein Chemistry*, **45**, 205–248.

[14] M.L. Phillips and V.N. Schumaker (1989) Conformation of apolipoprotein B after lipid extraction of low density lipoproteins attached to an electron microscope grid. *Journal of Lipid Research*, **30**, 415–422.

[15] H. Esterbauer, J. Gebicki, H. Puhl and G. Jurgens (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine*, **13**, 341–390.

[16] G.J. Chang, P. Woo, H.M. Honda, L.J. Ignarro, L. Young, J.A. Berliner and L.L. Demer (1994) Oxidation of LDL to a biologically active form by derivatives of nitric oxide and nitrite in the absence of superoxide. Dependence on pH and oxygen. *Arteriosclerosis and Thrombosis*, **14**, 1808–1814.

[17] V.M. Darley-Usmar, N. Hogg, V.J. O'Leary, M.T. Wilson and S. Moncada (1992) The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radical Research Communications*, **17**, 9–20.

[18] A. Graham, N. Hogg, B. Kalyanaraman, V. O'Leary, V. Darley-Usmar and S. Moncada (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Letters*, **330**, 181–185.

[19] W. Jessup, D. Mohr, S.P. Gieseg, R.T. Dean and R. Stocker (1992) The participation of nitric oxide in cell-free and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochimica et Biophysica Acta*, **1180**, 73–82.

[20] J.M. Wang, S.N. Chow and J.K. Lin (1994) Oxidation of LDL by nitric oxide and its modification by superoxides in macrophage and cell-free systems. *FEBS Letters*, **342**, 171–175.

[21] M.T. Yates, L.E. Lambert, J.P. Whitten, I. McDonald, M. Mano, G. Ku and S.J. Mao (1992) A protective role for nitric oxide in the oxidative modification of low density lipoproteins by mouse macrophages. *FEBS Letters*, **309**, 135–138.

[22] C.M. Maragos, D. Morley, D.A. Wink, T.M. Dunams, J.E. Saavedra, A. Hoffman, A.A. Bove, L. Isaac, J.A. Hrabie and L.K. Keefer (1991) Complexes of .NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *Journal of Medicinal Chemistry*, **34**, 3242–3247.

[23] J. Joseph, B. Kalyanaraman and J.S. Hyde (1993) Trapping of nitric oxide by nitronyl nitroxides: an electron spin resonance investigation. *Biochemical and Biophysical Research Communications*, **192**, 926–934.

[24] U.P. Steinbrecher, S. Parthasarathy, D.S. Leake, J.L. Witztum and D. Steinberg (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Sciences of the United States of America*, **81**, 3883–3887.

[25] A.W. Girotti (1985) Mechanisms of lipid peroxidation. *Free Radical Biology and Medicine*, **1**, 87–95.

[26] N.V. Gorbunov, A.N. Osipov, M.A. Sweetland, B.W. Day, N.M. Elsayed and V.E. Kagan (1996) NO-redox paradox: direct oxidation of alpha-tocopherol and alpha-tocopherol-mediated oxidation of ascorbate. *Biochemical and Biophysical Research Communications*, **219**, 835–841.

[27] E.G. Janzen, A.L. Wilcox and V. Manoharan (1993) *J. Org. Chem.*, **58**, 3597–3599.

[28] H. De Groot, U. Hegi and H. Sies (1993) Loss of alpha-tocopherol upon exposure to nitric oxide or the sydnonimine SIN-1. *FEBS Letters*, **315**, 139–142.

[29] N. Hogg, V.M. Darley-Usmar, M.T. Wilson and S. Moncada (1993) The oxidation of alpha-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Letters*, **326**, 199–203.

[30] S.P.A. Goss, N. Hogg and B. Kalyanaraman (1995) The antioxidant effect of spermine NONOate in human low-density lipoprotein. *Chemical Research in Toxicology*, **8**, 800–806.

[31] N. Hogg, R.J. Singh, S.P. Goss and B. Kalyanaraman (1996) The reaction between nitric oxide and alpha-tocopherol: a reappraisal. *Biochemical and Biophysical Research Communications*, **224**, 696–702.

[32] V.B. O'Donnell, P.H. Chumley, N. Hogg, A. Bloodsworth, V.M. Darley-Usmar and B.A. Freeman (1997) Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. *Biochemistry*, **36**, 15216–15223.

[33] H. Rubbo, R. Radi, M. Trujillo, R. Telleri, B. Kalyanaraman, S. Barnes, M. Kirk and B.A. Freeman (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *Journal of Biological Chemistry*, **269**, 26066–26075.

[34] S.P.A. Goss, N. Hogg and B. Kalyanaraman (1997) The effect of nitric oxide release rates on the oxidation of human low density lipoprotein. *Journal of Biological Chemistry*, **272**, 21647–21653.

[35] W.A. Pryor, T. Strickland and D.F. Church (1988) Comparison of the efficiencies of several natural synthetic antioxidants in aqueous sodium dodecyl sulfate micelle solutions. *J. Am. Chem. Soc.*, **110**, 2224–2229.

[36] C. Lagercrantz (1993) Spin trapping of nitric oxide (NO.) as aminoxyl radicals by its reaction with two species of short-lived radicals derived from azo compounds such as 2,2′-azobisisobutyronitrile and some aliphatic alcohols. *Free Radical Research Communications*, **19**, 387–395.

[37] S. Padmaja and R.E. Huie (1993) The reaction of nitric oxide with organic peroxyl radicals. *Biochemical and Biophysical Research Communications*, **195**, 539–544.

[38] E. Niki, T. Saito, A. Kawakami and Y. Kamiya (1984) Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *Journal of Biological Chemistry*, **259**, 4177–4182.

[39] U.P. Steinbrecher, H.F. Zhang and M. Lougheed (1990) Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biology and Medicine*, **9**, 155–168.

[40] S. Parthasarathy, D.J. Printz, D. Boyd, L. Joy and D. Steinberg (1986) Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis*, **6**, 505–510.

[41] M.K. Cathcart, D.W. Morel and G.M. Chisolm (1985) Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *Journal of Leukocyte Biology*, **38**, 341–350.

[42] T. Henriksen, E.M. Mahoney and D. Steinberg (1981) Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial

cells: recognition by receptors for acetylated low density lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, **78**, 6499–6503.
[43] T. Henriksen, E.M. Mahoney and D. Steinberg (1983) Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis*, **3**, 149–159.
[44] J.W. Heinecke, H. Rosen and A. Chait (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *Journal of Clinical Investigation*, **74**, 1890–1894.
[45] D.W. Morel, P.E. DiCorieto and G.M. Chisolm (1984) Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. *Arteriosclerosis*, **4**, 357–364.
[46] W. Jessup and R.T. Dean (1993) Autoinhibition of murine macrophage-mediated oxidation of low-density lipoprotein by nitric oxide synthesis. *Atherosclerosis*, **101**, 145–155.
[47] N. Hogg, A. Struck, S.P. Goss, N. Santanam, J. Joseph, S. Parthasarathy and B. Kalyanaraman (1995) Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric-oxide donors. *Journal of Lipid Research*, **36**, 1756–1762.
[48] D.A. Wink, J.F. Darbyshire, R.W. Nims, J.E. Saavedra and P.C. Ford (1993) Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. *Chemical Research in Toxicology*, **6**, 23–27.
[49] V.G. Kharitonov, A.R. Sundquist and V.S. Sharma (1994) Kinetics of nitric oxide autoxidation in aqueous solution. *Journal of Biological Chemistry*, **269**, 5881–5883.
[50] J.S. Beckman and W.H. Koppenol (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology*, **271**, C1424–C1437.
[51] J.B. Sampson, Y.Z. Ye, H. Rosen and J.S. Beckman (1998) Myeloperoxidase ane horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Archives of Biochemistry and Biophysics*, **356**, 207–213.
[52] A. van der Vliet, J.P. Eiserich, B. Halliwell and C.E. Cross (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *Journal of Biological Chemistry*, **272**, 7617–7625.
[53] R.J. Singh, S.P.A. Goss, J. Joseph and B. Kalyanaraman (1998) Nitration of gamma-tocopherol and oxidation of alpha-tocopherol by copper–zinc superoxide dismutase/H_2O_2/NO_2^-: Role of nitrogen dioxide free radical. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 12912–12917.
[54] R.V. Cooney, A.A. Franke, P.J. Harwood, V. Hatch-Pigott, L.J. Custer and L.J. Mordan (1993) Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 1771–1775.
[55] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall and B.A. Freeman (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 1620–1624.
[56] Y. Xia, V.L. Dawson, T.M. Dawson, S.H. Snyder and J.L. Zweier (1996) Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 6770–6774.
[57] S.S. Marla, J. Lee and J.T. Groves (1997) Peroxynitrite rapidly permeates phospholipid membranes. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 14243–14248.
[58] S. Pfeiffer and B. Mayer (1998) Lack of tyrosine nitration by peroxynitrite generated as physiological pH. *Journal of Biological Chemistry*, **273**, 27280–27285.
[59] S.P.A. Goss, N. Hogg and B. Kalyanaraman (1999) The effect of alpha-tocopherol on the nitration of gamma-tocopherol by peroxynitrite. *Archives of Biochemistry and Biophysics* (in press).
[60] H. Rubbo and B.A. Freeman (1996) Nitric oxide regulation of lipid oxidation reactions: formation and analysis of nitrogen-containing oxidized lipid derivatives. In *Methods in Enzymology*, pp. 385–394.
[61] J.B. Schulz, R.T. Matthews, M.M. Muqit, S.E. Browne and M.F. Beal (1995) Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *Journal of Neurochemistry*, **64**, 936–939.
[62] J.S. Beckman, Y.Z. Ye, P.G. Anderson, J. Chen, M.A. Accavitti, M.M. Tarpey and C.R. White (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biological Chemistry Hoppe-Seyler*, **375**, 81–88.
[63] S.P.A. Goss (1999) The antioxidant effect of nitric oxide on low-density lipoprotein. Ph.D. Dissertation, Medical College of Wisconsin, Milwaukee, WI.