ISSN 0025-7680

SHOCK 1998: OXYGEN, NITRIC OXIDE
AND THERAPEUTIC PERSPECTIVES
International Symposium, Academia Nacional de Medicina
Buenos Aires, 30 April 1998

MEDICINA (Buenos Aires) 1998; 58: 361-366

NITRIC OXIDE AND PEROXYNITRITE IN LIPID PEROXIDATION

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Abstract Nitric oxide (·NO) can mediate tissue protective reactions during oxidant stress, as well as toxic and tissue prooxidant effects. Nitric oxide regulates critical lipid membrane and lipoprotein oxidation events, by 1) contributing to the formation of more potent secondary oxidants from superoxide (i.e. peroxynitrite) and 2) termination of lipid radicals to possibly less reactive secondary nitrogen-containing products (LONO, LOONO) which are in part organic peroxynitrites and are expected to be produced *in vivo*. Relative rates of production and steady state concentrations of superoxide and ·NO and cellular sites of production will profoundly influence expression of the differential oxidant injury-enhancing and protective effects of ·NO. Full understanding of the physiological roles of ·NO, coupled with detailed insight into ·NO regulation of oxygen radical-dependent reactions, will yield a more rational basis for the use of ·NO donors for therapeutic purposes.

Resumen ***Oxido nítrico y peroxinitrito en la peroxidación lipídica.*** El óxido nítrico (·NO) regula eventos críticos en procesos de lipoperoxidación de membranas o lipoproteinas mediante 1) su contribución a la formación de oxidantes secundarios más potentes como el peroxinitrito, 2) por su capacidad de terminación de reacciones de propagación lipídica con la concomitante formación de productos no radicalares del tipo nitrosolípidos (LONO, LOONO). Las velocidades relativas y concentraciones en el estado estacionario de ·NO y radicales libres del oxígeno, así como los sitios de producción celular de estas especies, determinan los efectos netos observados pro- o antioxidantes del ·NO. La mejor comprensión de los roles fisiológicos que el ·NO cumple en procesos oxidativos puede dar bases más racionales para su utilización con fines terapéuticos.

Key words: nitric oxide, peroxynitrite, superoxide, lipid oxidation, free radicals, antioxidants, lipids, low density lipoprotein

Nitric oxide (·NO, nitrogen monoxide) is an endogenously-synthesized free radical first characterized as a non-eicosanoid component of endothelial-derived relaxation factor, (EDRF)[1]. Nitric oxide is produced by a variety of mammalian cells including vascular endothelium, neurons, smooth muscle cells, macrophages, neutrophils, platelets and pulmonary epithelium[2]. The physiological actions of ·NO range from mediating vasodilation, neurotransmission, inhibition of platelet adherence/aggregation and the macrophage and neutrophil killing of pathogens. Many if not all of these effects are mediated by the activation of soluble guanylate cyclase, synthesis of cyclic guanosine 3',5'-monophosphate (cGMP) and the activation of a family of cGMP kinases[3].

Nitric oxide exerts potent actions in the regulation of cell function and tissue viability. Chemical reaction systems, cell and animal models and clinical studies have recently revealed an ability of ·NO to modulate reactions and pathologic processes long associated with the excess production and biological effects of reactive oxygen species. The focus of this review will be to discuss the observed pro-oxidant and antioxidant reactions of ·NO in the context of lipid oxidative processes based in our own recent observations that the protective effects of ·NO can often be ascribed to its antioxidant properties and its ability to redirect the reactivity of partially reduced oxygen species.

Lipid reactions of ·NO are an important area of focus for multiple reasons. First, this reactive species significantly concentrates in lipophilic cell compartments, with an n-octanol:water partition coefficient of 6-8:1. This solvation property will further enhance the ability of ·NO to regulate oxidant-induced membrane lipid oxidation. Second, ·NO reacts with lipid alkoxyl and peroxyl radicals (LO· and LOO·) at near diffusion-limited rates, inferring that both lipid peroxidation processes and reactions of lipophilic antioxidants will be influenced by local ·NO concentrations[4-6]. Third, the central role that ·NO plays in vascular diseases includes important reactivities of ·NO both as a signal transduction mediator and toward other

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free radical species i.e. superoxide ($O_2^{\cdot-}$) and LOO·. The issues to be addressed includes 1) the influences of ·NO and reactive species commonly associated with oxidant stress on lipid and lipoprotein systems, and 2) the mechanisms accounting for the protective effects of ·NO observed in pathological events associated with excess production of reactive oxygen species.

Nitric oxide reaction with superoxide

A critical reaction that ·NO undergoes in oxygenated biologic media is direct bimolecular reaction with $O_2^{\cdot-}$, yielding peroxynitrite ($ONOO^-$) at almost diffusion-limited rates ($6.7 \times 10^9\ M^{-1}\ s^{-1}$, ref. 7). This rate constant is ~3.5 times faster than the enzymatic disproportionation of $O_2^{\cdot-}$ catalyzed by superoxide dismutases (SOD) at neutral pH ($k_{SOD} = 2 \times 10^9\ M^{-1}\ s^{-1}$). Thus, $ONOO^-$ formation represents a major potential pathway of ·NO reactivity which depends on both rates of tissue ·NO and $O_2^{\cdot-}$ production and scavenging (e.g., local superoxide dismutase and oxyhemoglobin concentrations). Peroxynitrite has a half-life of <1 s under physiological conditions, due to proton-catalyzed decomposition of peroxynitrous acid (ONOOH) and competing target molecule reactions of ONOOH[8]. Nitric oxide will potentiate many aspects of $O_2^{\cdot-}$-mediated tissue damage via $ONOO^-$ formation. To date, it has been shown that $ONOO^-$ is a potent oxidant capable of a) directly oxidizing protein and non-protein sulfhydryls[9, 10], b) protonating to ONOOH, which exhibits both unique and hydroxyl radical (·OH)-like reactions via metal-independent mechanisms[11, 12] and c) reaction with metal centers to yield a species with the reactivity of nitronium cation (NO_2^+), an oxidizing and nitrating intermediate[13]. It is noteworthy that the mechanisms and extents of $ONOO^-$ reaction will be strongly influenced by CO_2/H_2CO_3, which is typically 25 mM in biological tissues and can significantly exceed this concentration during pathologic processes[14].

Nitric oxide can potentiate $O_2^{\cdot-}$-mediated tissue damage and leads to $ONOO^-$ formation, representing a major potential pathway of ·NO reactivity. Peroxynitrite is now being revealed as a key contributing reactive species in pathological events associated with stimulation of tissue production of ·NO, e.g., systemic hypotension, inhibition of intermediary metabolism, ischemia-reperfusion injury, immune complex-stimulated pulmonary edema, cytokine-induced oxidant lung injury, and inflammatory cell-mediated pathogen killing/host injury[15-17]. There is growing evidence that ·NO-mediated production of $ONOO^-$ readily occurs *in vivo*, underscoring the importance of understanding the target molecule reactions occurring during the coordinated production of oxygen and nitrogen-containing reactive species[18, 19].

Antioxidant reactions of nitric oxide

Since the reaction of ·NO with $O_2^{\cdot-}$ yields the potent oxidant $ONOO^-$, from a purely chemical point of view it would follow that a) an even broader array of target molecules would become susceptible to the toxic effects of reactive oxygen species when ·NO is present and b) ·NO will potentiate the toxicity of reactive oxygen species. While this is sometimes the case, it is evident that ·NO also exerts direct or indirect antioxidant actions in biological systems subjected to concomitant oxidant stress from excess production of reactive oxygen species. The following sections develop these concepts in more detail.

a) Nitric oxide reaction with lipid epoxyallylic and peroxyl radicals.

Nitric oxide has been observed to play a critical role in regulating lipid oxidation induced by reactive oxygen and nitrogen species and activated reticuloendothelial cells[5, 6, 20]. Nitric oxide (in some conditions) will stimulate $O_2^{\cdot-}$ induced lipid and lipoprotein oxidation and under other conditions mediate protective reactions in membranes by inhibiting $O_2^{\cdot-}$, copper and $ONOO^-$-induced lipid oxidation (Figure 1). The latter actions require higher (but still biologically relevant) rates of ·NO production. This revealed that oxygen radicals can serve critical roles as modulators of the biological reactions of ·NO. We now know that ·NO reacts with radical species including $O_2^{\cdot-}$ and lipid peroxyl radicals (LOO·) at almost diffusion-limited rate constants[4-6].

Nitric oxide has been reported to have contrasting effects on low density lipoprotein (LDL) oxidation. For

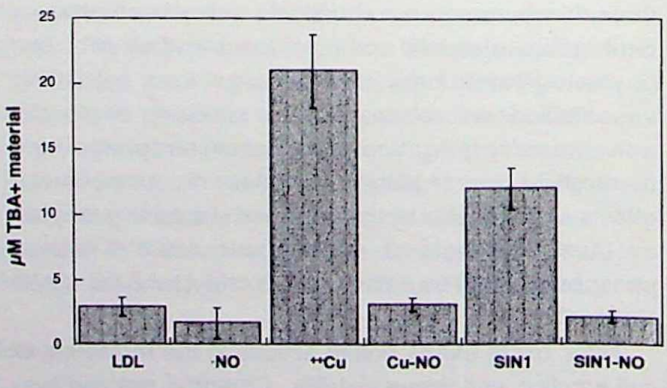

Fig. 1.- Nitric oxide inhibition of copper or SIN1-dependent low density lipoprotein oxidation. Human LDL was incubated for 3 hr at 37°C with 10 µM cupric sulfate or the peroxynitrite donnor SIN-1 (1 mM) in the absence and presence of 3 $\mu M\ min^{-1}$ ·NO production from 100 µM spermine NONOate. Lipid oxidation was assayed by thiobarbituric acid positive material formation at 532 nm.

both macrophage and endothelial cell model systems, increased rates of cell ·NO production via cytokine-mediated stimulation of inducible macrophage nitric oxide synthase gene expression and activity or exogenous addition of ·NO have been shown to inhibit cell and $O_2^{\cdot-}$-mediated lipoprotein oxidation[6, 21-23]. In contrast to these examples, the simultaneous production of ·NO and $O_2^{\cdot-}$ by 1,3-morpholino-sydnonimine-HCl (SIN-1) or the direct addition of $ONOO^-$ has been shown to oxidize lipoproteins to potentially atherogenic forms[24, 25]. Peroxynitrite-dependent tyrosine nitration reactions in areas of atherosclerotic vessel lipid deposition has also been shown to occur during both early and chronic stages of atherosclerotic disease[18].

Nitric oxide not only stimulates $O_2^{\cdot-}$ induced lipid and lipoprotein oxidation via $ONOO^-$ production, but will also inhibit $O_2^{\cdot-}$ and $ONOO^-$-induced lipid oxidation at slightly higher rates of ·NO production[5]. The prooxidant versus antioxidant outcome of these reactions which are sensitive to ·NO regulation are extremely dependent on relative concentrations of individual reactive species. For example, the continuous infusion of ·NO at various rates into LDL suspensions exposed to xanthine oxidase first stimulated and then inhibited formation of 2-thiobarbituric acid reactive products at rates of ·NO infusion greater than 3 $\mu M \cdot min^{-1}$ (Figure 2). Nitric oxide only stimulated $O_2^{\cdot-}$-dependent lipid peroxidation in LDL when production rates of ·NO were less than or equivalent to rates of $O_2^{\cdot-}$ production. Thus, there is a dynamic competition between $O_2^{\cdot-}$ and lipid radicals for reaction with ·NO. More investigation is required to understand the interaction of ·NO with lipid epoxyallylic radicals, the predominant species to which lipid alkoxyl radical (LO·) rearranges following cyclization[26].

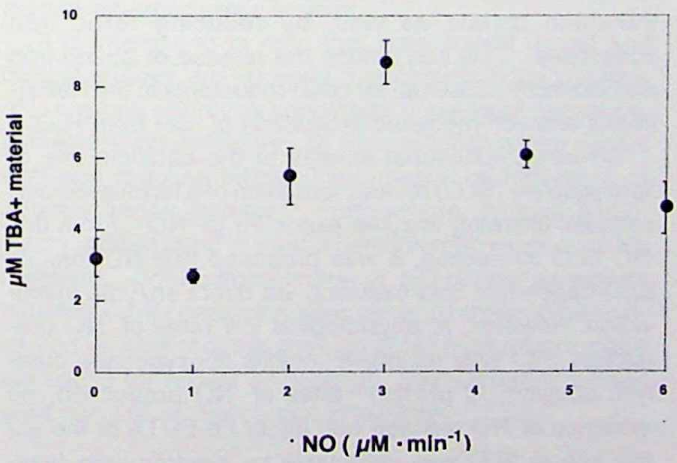

Fig. 2.- Pro- and antioxidant fates of nitric oxide on low density lipoprotein oxidation. Human LDL was incubated for 3 hr at 37°C with hypoxanthine/xanthine oxidase (3 $\mu M \cdot min^{-1}$ $O_2^{\cdot-}$ production) in the absence and presence of ·NO gas.
Low ·NO / $O_2^{\cdot-}$ ratios increase ·NO -mediated LDL oxidation via formation of peroxynitrite.
High ·NO / $O_2^{\cdot-}$ ratios decrease ·NO -mediated LDL oxidation by termination reaction of ·NO with peroxyl radicals.

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, with cholesteryl esters the most abundant lipid class found in LDL and cholesteryl linoleate the principal oxidizable lipid. Indeed, we observed that ·NO inhibited cholesteryl linoleate oxidation in LDL in a dose-dependent manner, with the concomitant formation of nitrogen-containing lipid adducts[27]. In addition, analysis of atherosclerotic human vessel lipid extracts by liquid chromatography-mass spectrometry analysis showed that cholesteryl linoleate oxidation products represented more than 85% of the total cholesteryl linoleate fraction of atherosclerotic vessels. At least 25% of the luminal cell and plaque cholesteryl linoleate fraction of atherosclerotic vessels consisted of nitrogen-containing oxidized lipid derivatives[27]. It is important to note that the products of ·NO termination of lipid radical species are unstable and may mediate a different spectrum of as yet undefined target molecule and pathologic reactions.

b) Nitric oxide-α-tocopherol interactions in lipid oxidation.

α-Tocopherol, a lipophilic chain-breaking antioxidant in biological membranes and lipoproteins acts by donating hydrogen atoms to chain-propagating peroxyl radical species (LOO·) to form the corresponding hydroperoxide[28]. Since the reaction of LOO· with α-tocopherol occurs at a rate three orders of magnitude less than for the reaction of LOO· with ·NO, ·NO could act more readily than α-tocopherol, as an antioxidant defense against oxygen radical derived oxidized lipid species. Based on comparison of relative rate constants, it is predicted that the termination of LOO· by ·NO will be significantly more facile than both the reaction of LOO· with α-tocopherol ($k=2.5 \times 10^6\ M^{-1} \cdot s^{-1}$) and the initiation of secondary peroxidation propagation reactions by LOO· with vicinal unsaturated lipids ($k= 30 - 200\ M^{-1} \cdot s^{-1}$).

In support of this argument, introduction of ·NO into lipid oxidation systems containing α-tocopherol results in preferential reaction of ·NO with lipid-derived radical species and prevents oxidation of α-tocopherol (Figure 3). One mechanism explaining the protection of α-tocopherol from oxidation by oxidizing lipids, can be the preferential reaction of ·NO with LO· and LOO· at significantly greater rates than a-tocopherol to yield nitrogen-containing radical-radical termination products. Another mechanism can be the direct reduction of α-tocopheroxyl radical (and possibly further oxidation states of α-tocopherol) by ·NO, thus regenerating reduced α-tocopherol and limiting the net extent of apparent

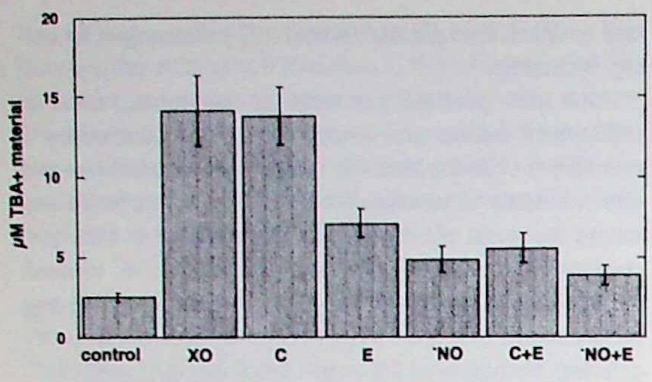

Fig. 3.- Inhibition of xanthine oxidase-induced linolenic acid oxidation by ·NO, α-tocopherol and ascorbate. Linolenic acid was incubated for 3 hr at 37°C (control), with 50 μM hypoxanthine/ 5 mU-ml xanthine oxidase in the presence of ascorbate (50 μM), α-tocopherol (50 μM), S-NONOate (100 μM) or a combination of both α-tocopherol plus S-NONOate or α-tocopherol plus ascorbate.
Nitric oxide and α-tocopherol exert similar cooperative lipid antioxidant activities than ascorbate plus α-tocopherol.

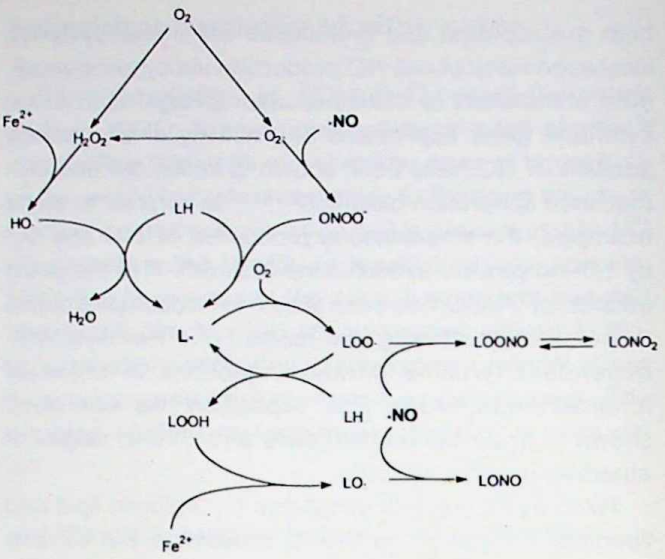

Fig. 4.- The double-edged action of nitric oxide on superoxide-mediated lipid oxidation.

α-tocopherol oxidation[29]. Nitric oxide is thermodynamically capable of inhibiting accumulation of α-tocopherol oxidation products via one electron reduction of α-tocopheroxyl radical, with $\Delta G^{o'}$= -5 Kcal/mol. Because lipid radicals in the lipophilic milieu do not readily partition into the bulk aqueous medium, we postulate that ·NO can act as a reductant of α-tocopherol in membrane and hydrophobic lipoprotein compartments, where reducing equivalents are not readily transferred from water-soluble reductants (eg. ascorbate, thiols, ref. 29). This mechanism could explain the observed additive antioxidant effects of ·NO and α-tocopherol in comparison with the pair ascorbate plus α-tocopherol (Figure 3). The mobility of α-tocopherol in the lateral plane of the membrane and its exact positioning in the membrane may restrict its antioxidant actions, in part explaining why ·NO can be much more facile at terminating lipid peroxyl radical species. Thus, because of a high reactivity with other radical species, a relatively lower reactivity of lipid radical-·NO termination products and an ability of ·NO to readily traverse membranes and lipoproteins, ·NO can effectively terminate radical species throughout all aspects of membrane and lipoprotein microenvironments. This can help maintain other tissue antioxidant defenses as well, during periods of oxidant stress.

c) Nitric oxide reactions with metals

Nitric oxide can react with metal centers in proteins including heme iron, iron-sulfur clusters and copper. Examples are the activation of soluble guanylate cyclase, a heme-containing enzyme, via the formation of an iron-nitrosyl complex[30]. It has been postulated that ·NO can exert a protective role towards metal complex and metalloprotein-catalyzed lipid oxidation, via formation of catalytically inactive metal iron-nitrosyl complexes, thereby modulating the pro-oxidant effects of iron and other transition metals[31]. Iron-nitrosyl complexes were detected in several proteins, including mammalian ferritin, transferrin, myoglobin and hemoglobin, albeit in the presence of high concentrations of ·NO[32]. It is important to note that the rate of ·NO reaction with most metal centers is significantly slower than for the almost diffusion-limited reaction of ·NO with either $O_2^{\cdot-}$ or LO· and LOO· species, critical for propagation of radical chain reactions. It should also be noted that ·NO can exert prooxidant effects with transition metals as well, by reducing ferric iron complexes. This can induce the release of bound iron and indirectly substitute for other reductants in the Haber-Weiss reaction-mediated production of ·OH from H_2O_2.

Structural-functional studies of the catalytic site of lipoxygenase (SLO) reveals formation of a ferrous-nitrosyl complex following enzyme exposure to ·NO. From this ·NO-SLO interaction, it was proposed that ·NO inhibits SLO-dependent lipid oxidation via direct enzyme inactivation. However, at physiological low rates of ·NO production, ·NO only minimally inhibits lipoxygenase catalytic activity[6]. At μM·min^{-1} rates of ·NO production, no evidence of ·NO reaction with either Fe-EDTA or the active site of SLO was detectable by electron spin resonance analysis[6]. From all of the above, it is concluded that the inhibitory effect of ·NO towards oxygen radical or SLO-dependent oxidation of multiple lipid and lipoprotein targets, as determined by multiple criteria, was due to termination of lipid radical chain propagation reactions rather than ·NO reaction with transition metals.

Inflammation and NO

A number of model systems for inflammation, vascular disease (atherogenesis, restenosis following angioplasty) and surgical problems (ischemia-reperfusion injury, graft reanastomosis) that include a pathogenic role for oxidant injury indicate that either endogenous NO biosynthesis or exogenous supplementation with sources of NO inhibit oxidant-dependent damage at both molecular and tissue functional levels. Many if not all of these studies have inflammatory injury as a common denominator.

Atherosclerosis is one example where this phenemenon occurs. The changes which occur during atherosclerosis includes loss of the control of vascular tone, an NO-dependent event. Increasing the availability of the substrate L-arginine for NO synthesis will restore vascular function, while inhibiting NO synthesis is pro-atherogenic[33-35].

Another early event in the atherosclerotic process is the chemical transformation of LDL through the initiation of oxidation. Probably in an attempt at host protection, oxidized LDL is taken up by macrophages, resulting in lipid-laden foam cells. These cells then become part of the problem, because of the effect of their secretory products on other cells in the lesion and the release of pro-oxidative enzymes such as 15-lipoxygenase. The balance between NO and $O_2^{\cdot-}$ production in the artery wall may also play a role in the oxidation of LDL (Figure 4). Peroxynitrite oxidizes LDL, causes a rapid depletion of several antioxidants (ascorbate, urate, protein thiols and ubiquinol) and releases copper ions from the plasma protein caeruloplasmin. Copper ions are powerful catalysts of LDL oxidation which have been detected in advanced human atherosclerotic lesions.

It is interesting to note that both animal model and clinical studies are showing that chronic administration of L-arginine improves endothelial dependent relaxation, decreases inflammatory cell accumulation at the vessel wall and reduces intimal hyperplasia, all hallmarks of atherosclerotic disease[36]. Furthermore, balloon angioplasty is often used to treat atherosclerotic vasoocclusive problems. Both administration of NO donors as well as transfection of constitutive nitric oxide synthase to balloon-injured vessels reduces the intimal cell hyperplasia, often the cause for repeat angioplasty, aortocoronary bypass graft surgery or myocardial infarction[37].

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Focusing on technology also highlights a powerful historical lesson: new technology enables frontier research. Many of the most extraordinary tools for doing science were invented by scientists themselves, out of frustration with the meager analytical means available to answer questions. But whatever the background of the instrument pioneers, their work open vistas in basic science. You can't understand the machinery inside a cell without an electron microscope. You can't see the most distant galaxies without a space telescope.

Hacer resaltar la tecnología también pone de relieve una poderosa lección histórica: una tecnología nueva permite una investigación de avanzada. Muchas de las herramientas para la investigación fueron inventadas por científicos con los precarios recursos a su alcance para resolver sus problemas. Pero cualquiera sea el origen de los pioneros en tecnología, su trabajo abre horizontes en ciencia básica. No se puede entender la maquinaria incluida en la célula sin un microscopio electrónico. No se pueden ver las galaxias distantes sin un telescopio.

Rodney W. Nichols

President, New York Academy of Sciences
Angles on Technology and Science, *The Sciences* March/April 1998