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EPISODE II:
Return of the Antioxidant: Nitric Oxide

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Abbreviations:

$AscH^-$	ascorbate
DHA	dehydroascorbic acid
DHA-22:6ω3	docoshexaenoic acid
$L^\bullet$	lipid radical
$LO^\bullet$	lipid alkoxyl radical
$LOO^\bullet$	lipid peroxyl radical
LOOH	lipid hyrdoperoxide
LPO	lipid peroxidation
NADPH	nicotinamide adenine dinucleotide phosphate
$NO^\bullet$	nitric oxide
NO_2^-	nitrite
$NO_2^\bullet$	nitrogen dioxide radical
NOS	nitric oxide synthase
$ONOO^-$	peroxynitrite
PUFA	polyunsaturated fatty acids
TOH	α-tocopherol
$TO^\bullet$	tocopherol radical

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Abstract

The biological function of nitric oxide ($NO^{\bullet}$) is topic of intense study and yet its role remains elusive. The evasive and dichotomous biological function of $NO^{\bullet}$ is due to its diverse chemistry. For example, $NO^{\bullet}$ behaves as both a pro-oxidant and antioxidant. This paper will briefly discuss how $NO^{\bullet}$ is produced, highlight $NO^{\bullet}$ pro-oxidant capabilities by reacting with superoxide to form peroxynitrite, and demonstrate its antioxidant capabilities. The main production of $NO^{\bullet}$ occurs enzymatically through three isoforms of nitric oxide synthase. Nitric oxide has a rate constant 10,000 times higher than the antioxidant, α-tocopherol, when reacting with lipid peroxyl radicals. Recent studies have also shown that $NO^{\bullet}$ can act as a chain-breaking antioxidant against lipid peroxidation.

Introduction

There is great interest in the role of nitric oxide (oxidonitrogen(•) or $NO^{\bullet}$) in biology because of its many diverse functions. It is involved in signaling, vasodilatation [1-4], neurotransmission [5], destruction of pathogens [4,6], a precursor to oxidizing and nitrating species [7,8], as well as an antioxidant [9-14]. However, its diverse chemistry and its biological activity sometimes are seemingly contradictory, behaving both as a pro-oxidant and as an antioxidant. The paper will briefly address the chemistry of producing $NO^{\bullet}$ in a biological system as well as demonstrate $NO^{\bullet}$ antioxidant abilities. Nitric oxide, the antioxidant, will also be compared kinetically to Vitamin E.

Biological Production of Nitric Oxide

The biological function of $NO^{\bullet}$ might be related to how it is produced as well as the amount produced. Nitric oxide can be produced endogenously *via* two pathways: non-enzymatic and enzymatic.

Non-enzymatic Pathway:

Under some physiological conditions (*i.e.*, low pH), nitrite (NO_2^-) can be reduced to $NO^{\bullet}$ in the presence of ascorbate ($AscH^-$), as seen in the following reaction [15]:

$$2NO_2^- + AscH^- + 3H^+ \rightarrow 2NO^{\bullet} + DHA + 2H_2O \quad (1)$$

The non-enzymatic production has been demonstrated to occur in the stomach, on the surface of the skin, in the ischemic heart, and infected nitrite-containing urine [15].

Enzymatic Pathway:

Nitric oxide synthase (NOS) mediates a 5-electron oxidation of L-arginine to L-citrulline. This process also uses nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. There are three different forms of NOS:

Neuronal (nNOS, NOS1 or Type I),

Inducible (iNOS, NOS2 or Type II),

Endothelial (eNOS, NOS3 or Type III)

Both the nNOS and eNOS isoforms are constitutively active and are regulated by the concentration of calcium *via* a calmodulin interaction. Inducible NOS on the other hand can also be activated by inducers that participate in inflammatory diseases (*i.e.*, atherosclerosis, diabetes, septic shock, and others) [17]. Endothelial NOS and nNOS, are believed to produce biological concentration in the nanomolar range, while the third isoform, iNOS, can produce micromolar concentrations. Nitric oxide synthase is a membrane bound protein, anchored by myristoylation and palmitoylation to the cytoplasmic side of the endoplasmic reticulum, golgi, or plasma membrane. The lipid anchors are preferentially found in cholesterol and glycolipid rich membrane domains. The compartmentalization of NOS appears to be important for providing local levels of $NO^{\bullet}$ [16,17].

Lipid Peroxidation

Oxidative stress is a disruption in the cellular pro-oxidant antioxidant balance [18]. As the balance shifts towards pro-oxidants, potential damage in the form of oxidized DNA, proteins, and lipids can occur. Lipid peroxidation (LPO) can be defined as the oxidative deterioration of lipids containing two or more carbon-carbon double bonds. The propensity of polyunsaturated fatty acids (PUFAs) to undergo LPO is due to the *bis*-allylic methylene hydrogens that are more susceptible to hydrogen abstraction by oxidants than fully saturated lipids [19]. Lipid peroxidation has three major components: initiation, propagation and termination [20].

$$\text{L-H} + \text{oxidant}^{\bullet} \longrightarrow \text{L}^{\bullet} + \text{oxidant-H} \qquad \textit{(initiation)} \qquad (2)$$

$$\text{L}^{\bullet} + \text{O}_2 \longrightarrow \text{LOO}^{\bullet} \qquad k_3 \approx 10^9\ \text{M}^{-1}\ \text{s}^{-1}\ [20] \quad \textit{(propagation cycle)} \qquad (3)$$

$$\text{LOO}^{\bullet} + \text{LH} \longrightarrow \text{L}^{\bullet} + \text{LOOH} \qquad k_4 \approx 50\ \text{M}^{-1}\ \text{s}^{-1}\ [20] \quad \textit{(propagation cycle)} \qquad (4)$$

$$\text{L}^{\bullet} + \text{L}^{\bullet} \longrightarrow \text{non-radical products} \qquad \textit{(termination)} \qquad (5)$$

$$\text{L}^{\bullet} + \text{LOO}^{\bullet} \longrightarrow \text{non-radical products} \qquad \textit{(termination)} \qquad (6)$$

LPO is an important research topic due to its involvement with various conditions including inflammation, ischemia/reperfusion, and vascular disease [21,22]. It also plays a crucial role in the mechanisms of various disease treatments such as photodynamic therapy of cancer, therapeutic hyperthermia, and chemotherapy of cancer.

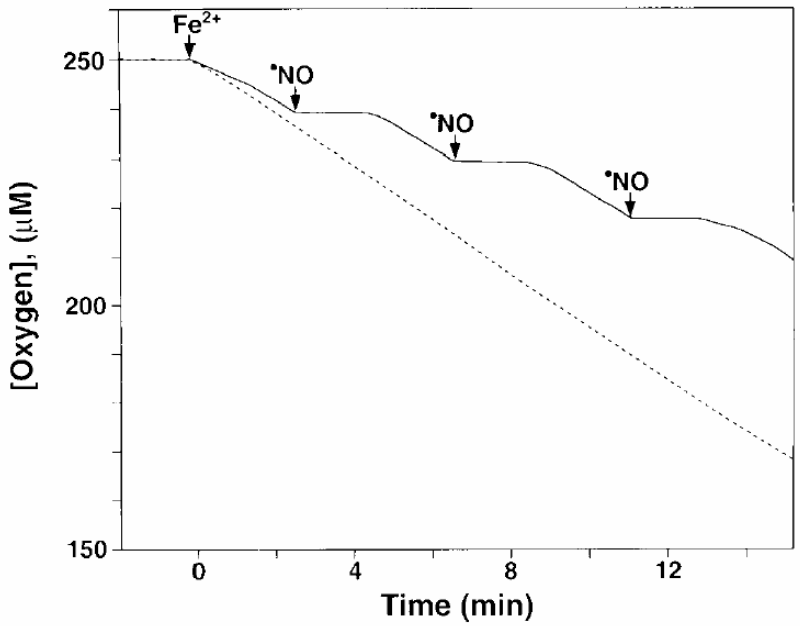

Figure 1. Inhibition of lipid peroxidation by bolus additions of $NO^{\bullet}$. Using DHA-22:6ω3-enhanced HL-60 cells, LPO was induced using 20 μM Fe^{2+} addition. Lipid peroxidation is demonstrated through the decrease in O_2 concentration in the system; showing that O_2 is being consumed. The additions of 1.8 μM $NO^{\bullet}$ inhibited further LPO as long as $NO^{\bullet}$ was present. From Kelley *et al.* (1999) [14].

Nitric Oxide as an Antioxidant

Antioxidants, independent of being preventive or chain-breaking, can be defined as "any substance that, when present at low concentrations compared to an oxidizable substrate, significantly delays or prevents the oxidation of the substrate" [23]. Chain-breaking antioxidants stop the propagation cycle of LPO. Nitric oxide has been shown to protect endothelial cells, fibroblasts, hepatocytes, intestinal epithelium, and cardiomyocytes against oxidative stress induced cytotoxicity [9-13].

Nitric oxide can serve as a chain-terminating antioxidant by reacting with peroxyl radicals ($LOO^{\bullet}$), as shown in **Reaction 7**.

$$LOO^{\bullet} + NO^{\bullet} \rightarrow LOONO \qquad k_7 = 2 \times 10^9\ M^{-1}\ s^{-1}\ [24] \qquad (7)$$

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \qquad k_8 = 8 \times 10^4\ M^{-1}\ s^{-1}\ [24] \qquad (8)$$

Comparatively between the two antioxidants of α-tocopherol, (**Reaction 8**), and $NO^{\bullet}$ there is significant advantage for $NO^{\bullet}$. The rate constants between both $NO^{\bullet}$ ($k_7 = 2 \times 10^9\ M^{-1}\ s^{-1}$) and α-tocopherol ($k_8 = 8 \times 10^4\ M^{-1}\ s^{-1}$) with $LOO^{\bullet}$ demonstrate a rate constant 10,000 times faster than the nitric oxide rate constant.

The LOONO can then undergo a hydrolysis reaction (**Reaction 9**) to form a lipid hyrdoperoxide (LOOH) and a nitrite.

$$LOONO + H_2O \rightarrow LOOH + NO_2^- \qquad [25] \qquad (9)$$

$$LOONO \rightarrow LO^{\bullet} + NO_2^{\bullet} \qquad [25] \qquad (10)$$

Another possible reaction is that the LOONO can become a lipid alkoxyl radical and nitrogen dioxide radical ($NO_2^{\bullet}$). The $LO^{\bullet}$ can continue the lipid peroxidation cycle while the $NO_2^{\bullet}$ could nitrite proteins, lipids, and DNA in the surrounding area.

Experimental data demonstrating nitric oxide's ability to inhibit LPO is shown in **Figure 1** by Kelley *et al.* (1999) [14]. In this experiment a human leukemia cell line, HL-60, was enriched with the polyunsaturated fatty acid, docoshexaenoic acid (DHA-22:6ω3), and then exposed to an oxidant inducing LPO. The initiation process produces a carbon-centered lipid radical ($L^{\bullet}$) that reacts with O_2 to form a lipid peroxyl radical ($LOO^{\bullet}$) (**Reaction 11**) consequently we can monitor the O_2 consumption in the system subsequently allowing us to monitor LPO of the HL-60 cells.

$$\mathbf{L^{\bullet} + O_2 \rightarrow LOO^{\bullet}} \qquad \mathbf{(}11\mathbf{)}$$

The addition of $NO^{\bullet}$ inhibited further oxygen consumption consequently Kelley *et al.* (1999) were able to deduce that $NO^{\bullet}$ was acting as a chain-breaking antioxidant. The inhibition of LPO however only occurs as long as $NO^{\bullet}$ is present in the system.

The work by Kelley *et al.* (1999) illustrated that 1.8 µM $NO^{\bullet}$ could inhibit LPO, but we must now examine the lower $NO^{\bullet}$ concentration threshold that is capable of inhibiting LPO. Using a similar experimental design, Tony Fischer examined the $NO^{\bullet}$ concentration when oxygen consumption was re-initiated. A 1.4 µM $NO^{\bullet}$ bolus was added to DHA-22:6ω3-enhanced HL-60 cells and at the peak of the $NO^{\bullet}$ bolus 20 µM final concentration of Fe^{2+} was added to the system. The decay of $NO^{\bullet}$ and oxygen were simultaneously monitored. **Figure 2** shows that when the $NO^{\bullet}$ level reached a range of 50-100 nM (marked by the red lines), oxygen consumption drastically increased; marking the induction of LPO. Inhibition of LPO occurs for approximately 120 seconds after the addition of Fe^{2+}. This unpublished data further demonstrates that $NO^{\bullet}$ is a chain-breaking antioxidant.

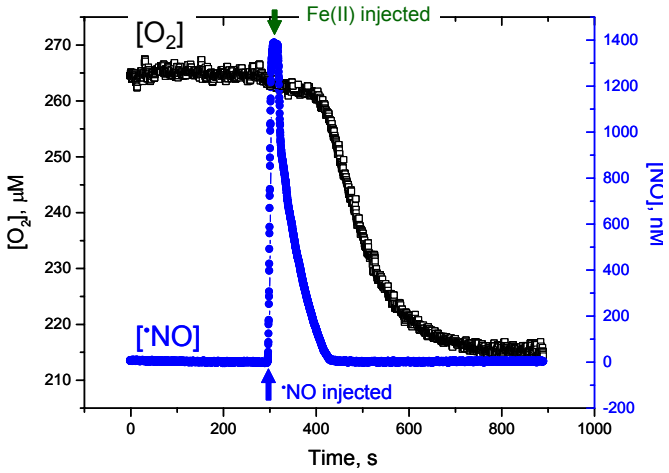

Figure 2. Induction of LPO when $NO^{\bullet}$ levels drop to 50-100 nM in the presence of an oxidant. Oxygen and $NO^{\bullet}$ concentrations were monitored in DHA-22:6ω3-enhanced HL-60 cells for the induction of LPO. The oxidant, 20 µM Fe^{2+}, was added at the peak of the 1.4 µM $NO^{\bullet}$ bolus.
From unpublished work by Tony Fischer.

Discussion and Conclusions

The diverse chemistry of $NO^{\bullet}$ portrays its various biological functions and is inversely proportional to its simplicity. On the one hand, $NO^{\bullet}$ can be a pro-oxidant, yet on the other $NO^{\bullet}$ is a potent antioxidant that has a rate constant 10,000 times faster than α-tocopherol. Nitric oxide has been shown to protect a variety of cells against cytotoxicity as well as be a chain-breaking anti-oxidant. Future studies of $NO^{\bullet}$ will hopefully elicit out more details about how the molecule functions under various biological conditions. These details will help researchers understand and exploit the full potential of $NO^{\bullet}$ as an antioxidant.

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