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Nitric Oxide as a Cellular Antioxidant: A little goes a long way*

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Abstract

Nitric oxide (NO•) is an effective chain-breaking antioxidant in free radical-mediated lipid oxidation (LPO). It reacts rapidly with peroxy radicals as a sacrificial chain-terminating antioxidant. The goal of this work was to determine the minimum threshold concentration of NO• required to inhibit Fe²⁺-induced cellular lipid peroxidation. Using oxygen consumption as a measure of LPO, we simultaneously measured nitric oxide and oxygen concentrations with NO•- and O₂-electrodes. Ferrous iron and dioxygen were used to initiate LPO in docosahexaenoic acid-enriched HL-60 and U937 cells. Bolus addition of NO• (1.5 μM) inhibited LPO when the NO• concentration was greater than 50 nM. Similarly, using (Z)-1-[N-(3-ammonioethyl)-N-(n-propyl)amino]diazene-1-ium-1,2-diolate (PAPA/NO) as a NO• donor we found that an average steady-state NO• concentration of at least 72 ± 9 nM was required to blunt LPO. As long as the concentration of NO• was above 13 ± 8 nM the inhibition was sustained. Once the concentration of NO• fell below this value, the rate of lipid oxidation accelerated as measured by the rate of oxygen consumption. Our model suggests that the continuous production of NO• that would yield a steady-state concentration of only 10 – 20 nM is capable of inhibiting Fe²⁺-induced LPO.

Keywords

nitric oxide; free radical; lipid peroxidation; iron; HL-60; U937; oxygen consumption; antioxidant

Introduction

There is great interest in the role of nitric oxide (oxidonitrogen(•) or NO•) in biology because it can be a signalling molecule, a toxin, a pro-oxidant, and a potential antioxidant. It is involved in signalling in vasodilatation [1,2,3,4] and neurotransmission [5], a toxin in the destruction of pathogens [6,7], and a precursor to oxidizing and nitrating species [8,9]. However, its diverse chemistry and its biologic activity sometimes are seemingly contradictory. Nowhere is this contradiction more evident than in oxidative stress. Nitric oxide has been proposed to act as a pro-oxidant at high concentrations [10] or when it reacts with superoxide (O₂^{•-}) forming the highly reactive peroxynitrite (ONOO⁻) [11,12,13]. On the other hand, NO• can also inhibit oxidation; NO• can terminate chain reactions during lipid peroxidation, as observed in model lipid systems [14,15], low density lipoprotein oxidation [16,17,18,19] and in cells [20,21].

Oxidative stress is a disruption in the cellular pro-oxidant antioxidant balance [22]. As the balance shifts towards pro-oxidants, potential damage in the form of oxidized DNA, proteins, and lipids can occur. Lipid peroxidation can be defined as the oxidative deterioration of lipids containing two or more carbon-carbon double bonds. The propensity of polyunsaturated fatty

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acids (PUFAs) to undergo LPO is due to the *bis*-allylic methylene hydrogens, which are more susceptible to hydrogen abstraction by oxidants than fully saturated lipids [23].

Lipid peroxidation has three major components: initiation, propagation and termination [24],

where L-H represents a lipid, generally a polyunsaturated fatty acid moiety (PUFA). Nitric oxide can serve as a chain-terminating antioxidant by reacting with chain-carrying peroxy radicals

Nitric oxide can be produced both non-enzymatically and enzymatically. For example, under some physiological conditions (*e.g.*, low pH), NO_2^- can be reduced to $NO^{\bullet}$, non-enzymatically [26],

The family of nitric oxide synthase (NOS) enzyme mediates the 5-electron oxidation of L-arginine to L-citrulline producing $NO^{\bullet}$. There are three isoforms of NOS: neuronal (NOS1), inducible (NOS2), and endothelial (NOS3). Both NOS1 and NOS3 are constitutively expressed and are estimated to produce local $NO^{\bullet}$ concentrations in the nM range, while NOS2 activity is inducible and is estimated to produce concentrations of $NO^{\bullet}$ in the μM range [27].

Previous work by Kelley *et al.* has shown that $NO^{\bullet}$ can protect docosahexaenoic acid (DHA-22:6 ω 3)-enriched HL-60 cells against iron-induced oxidative stress [20], as determined by monitoring oxygen consumption as a measure of lipid peroxidation. These experiments demonstrated that $NO^{\bullet}$ is an effective antioxidant protecting against cellular LPO; a bolus addition of $NO^{\bullet}$ immediately slowed the rate of LPO; the inhibition time of LPO varied directly with the amount of $NO^{\bullet}$ introduced, and when $NO^{\bullet}$ reached low levels, rapid LPO resumed.

In the present work, our goal was to determine the minimum concentration of $NO^{\bullet}$ required to inhibit cellular LPO. Iron(II) and dioxygen were used as an oxidative stress to initiate LPO in DHA-22:6 ω 3-enriched HL-60 and U937 leukemia cells [28,29]. To determine the minimum levels of $NO^{\bullet}$ needed to blunt cellular LPO, we simultaneously measured the concentration of $NO^{\bullet}$ and the rate of oxygen consumption.

Materials and Methods

NO[•] Stock Solution

Nitric oxide gas was either obtained from a nitric oxide gas tank or prepared from an acidified sodium nitrite solution [30,31]. Because the nitric oxide from either source can be contaminated with other oxides of nitrogen, it was purified by passing it through NaOH (4 M) and then deionized (DI) water. The purified NO[•] gas was then bubbled through a gas sampling bottle containing degassed DI water and stored.

Donor

The NONOate donor, (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]diazene-1-ium-1,2-diolate (PAPA/NO), and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) were from Alexis (San Diego, CA).

NO[•] and O₂ Electrodes

For the bolus-addition experiments, simultaneous electrochemical measurements of nitric oxide and oxygen were made using a ISO-NO Mark II NO[•]-measurement system (WPI, Sarasota, FL) and ISO₂ oxygen system. Data from these instruments were imported to a PC using a Duo18 data recording system (WPI). The data recording system allowed for measurements at 0.2 s intervals. Both the NO[•] and O₂ probes were standardized daily according to the manufacturer's recommendations. The response time of the nitric oxide probe response was rapid (<10 s equilibration to 1.5 μM NO[•] by bolus injection). For the donor experiments, the steady-state NO[•] concentrations produced through the degradation of the NONOate donors were also determined using an Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota, FL). The NO[•] electrode was calibrated using two different techniques. The first method used bolus additions of increasing volumes of 50 nM NaNO₂ into 10 mL of reducing solution (0.1 M KI, 0.1 M H₂SO₄). The production of NO[•] *via* the reduction of NaNO₂ is illustrated by the following equation:

The second calibration method uses the copper-catalyzed [32] release of NO[•] from bolus additions of a 150 μM S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and 540 μM EDTA solution into a 0.1 M copper sulfate solution. SNAP decomposes to NO[•] and a disulfide byproduct according to the following equation:

Regardless of the calibration method used, an electrode response plot is generated. The NO[•] electrode generally has a linear response from 25–2000 nM where the slope of the line provides the current to concentration ratio for the electrode. At a range of 25–400 nM the linear response of the electrode normally provides an r²-value of 0.99. If the r-value begins to deviate, the electrodes are inspected for tears in the membrane, which affects the response. The electrochemical signal from the O₂ electrode was calibrated using the signal difference from aerated water (approximately 250 μM) and an argon-purged aqueous solution (0 μM).

Cell Culture

Human leukemia cells (HL-60, U937) were acquired from American Type Culture Collection (ATCC). Cells were grown in medium consisting of RPMI 1640 medium (Gibco) and 10% fetal bovine serum (10% FBS-RPMI) supplemented with L-glutamine (2 mM) at 37°C, 5% CO₂/95% air. Experiments were done in exponential growth phase. To alter the fatty acid

profile of cells they were grown in 10% FBS-RPMI medium supplemented with 32 μM docosahexaenoic acid, (DHA: 22:6 ω 3) for 48 h [23].

Lipid Peroxidation Measurements

HL-60

HL-60 cells were washed twice, first in RPMI-1640 medium then in PBS by centrifugation (300 g). Upon re-suspension, cell density was adjusted (3×10^6 cells/mL) for the LPO experiments [33,34]. Following baseline measurements of $[\text{O}_2]$ and $[\text{NO}^\bullet]$ in the ISO-NO chamber, FeSO_4 (10 μM final concentration; 2 μL of 10 mM stock at pH 2.0) was introduced to induce lipid peroxidation. The pH of the cell suspension, 6.5, was not significantly affected by the injection. The pH of 6.5 was chosen to avoid problems of iron solubility and consequently the reactivity of ferrous iron [35]. All reactions were performed at 25°C with magnetic stirring.

U937

Cellular LPO is measured *via* the rate of consumption of oxygen using an oxygen electrode system [20,21]; the World Precision Instruments (Sarasota, FL) system allowed simultaneous determination of $[\text{O}_2]$ and $[\text{NO}^\bullet]$. We suspended approximately 3.3×10^6 DHA-22:6 ω 3-enriched U937 cells per mL in chelated 10 mM sodium chloride solution (NaCl) containing 9000 mg L^{-1} of NaCl at pH $\cong$ 6.5 with a 1 mL $\text{NO}^\bullet$ chamber. The chamber was then sealed and the electrodes inserted. We used $\text{NO}^\bullet$ donors to measure the antioxidant effect of $\text{NO}^\bullet$ in iron-induced lipid peroxidation. The pH of both the Fe^{2+} solution and the donor was adjusted from their respective storage solutions to pH 6.5 prior to addition to the system.

Results

To determine the level of nitric oxide needed to blunt lipid peroxidation we used a leukemia cell culture model system in which the cells were enriched with the unsaturated fatty acid docosahexaenoic acid (DHA-22:6 ω 3) [23]. This enrichment results in the cells being more oxidizable; providing an ideal system to study cellular lipid oxidation. We used two complementary approaches to identify the minimum level of $[\text{NO}^\bullet]$ needed to blunt lipid peroxidation. In each approach cellular lipid peroxidation was initiated by the introduction of Fe^{2+} . In the first approach we introduced a bolus of $\text{NO}^\bullet$ (1.5 μM) followed immediately by 10 μM Fe^{2+} into suspensions of DHA-22:6 ω 3-enriched HL-60 cells. By simultaneously measuring $[\text{NO}^\bullet]$ and $[\text{O}_2]$, we could correlate changes in the rate of peroxidation, as measured by the rate of oxygen uptake, and the level of $\text{NO}^\bullet$. This allowed us to estimate the level of $\text{NO}^\bullet$ required to blunt cellular lipid peroxidation. When $\text{NO}^\bullet$ was introduced into PBS, PBS + Fe^{2+} , or PBS + cells it disappeared at a rate consistent with its known reaction with dioxygen,

The rate of this process is governed by a rare, third-order rate constant where $k = 2.4 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$ at 37°C [36]; oxygen concentration enters the rate equation as a first-order term, nitric oxide concentration as a second-order term. Analysis of the electrode data for the disappearance of $\text{NO}^\bullet$ vs time showed that neither the presence of cells nor ferrous iron produced a major change in the rate of disappearance of $\text{NO}^\bullet$, Table 1. Analysis of the data showed that under the conditions of our experiments, loss of nitric oxide was second-order in $[\text{NO}^\bullet]$ with a third order rate constant very similar to the published rate constant for the reaction of $\text{NO}^\bullet$ with O_2 in room temperature aqueous solution. However, upon the initiation of lipid peroxidation by the introduction of iron to the cellular peroxidation system, the rate of loss of $\text{NO}^\bullet$ accelerated. It no longer obeyed the kinetics of the reaction with dioxygen. Figure 1 depicts representative

experiments where $\text{NO}^\bullet$ and Fe^{2+} were injected in rapid succession. Consistent with previous observations [34,37], the introduction of ferrous iron results in a rapid initiation of cellular lipid oxidation as seen by uptake of oxygen. As observed in model systems and LDL oxidation [14,16] as well as cellular systems [20,21], when a bolus of $\text{NO}^\bullet$ (1.5 μM) was introduced, this rapid uptake of oxygen was delayed until the concentration of $\text{NO}^\bullet$ becomes very low, and then the rate of oxygen uptake accelerates.

To arrive at an estimate of the concentration of $\text{NO}^\bullet$ below which rapid oxygen uptake ensued, we plotted the data of Figure 1 as rate of peroxidation ($-\text{d}[\text{O}_2]/\text{dt}$) vs $[\text{NO}^\bullet]$ (rate-inhibitor plot) for relevant time points, Figure 2. Using this approach, we determined the concentration of $\text{NO}^\bullet$ at which the rate of cellular lipid peroxidation accelerates. At higher concentrations of $\text{NO}^\bullet$, the rate of peroxidation, as seen by the loss of O_2 , was generally less than $0.1 \mu\text{M s}^{-1}$, Figure 2A. However, when $[\text{NO}^\bullet]$ decreased to less than $\approx 250 \text{ nM}$, a notable increase in the reaction rate occurred and when $[\text{NO}^\bullet]$ decreased to $\approx 50 \text{ nM}$ another abrupt increase in the rate of lipid peroxidation occurred. If we define $[\text{NO}^\bullet]_{\text{Inh}(1/2)}$ as the concentration of nitric oxide required to limit the rate of lipid peroxidation to $1/2$ the maximal rate in any single experiment, we have estimated that $[\text{NO}^\bullet]_{\text{Inh}(1/2)} = 40 \pm 20 \text{ nM}$ ($n=3$), a value consistent with the proposed concentration of $\text{NO}^\bullet$ in many tissues [38,39,40,41]. As an additional way to view the results, we grouped sets of similar rates, as observed in rate-inhibitor plots from these experiments, into bins, Figure 2B. We found that the peroxidation rate is substantially higher when $[\text{NO}^\bullet] < 50 \text{ nM}$. These results suggest that only low nanomolar levels of nitric oxide are needed to serve as an effective lipid antioxidant.

The experiments above using bolus addition of stock solutions of $\text{NO}^\bullet$ produced quite high, short-lived initial levels of $\text{NO}^\bullet$. The experiments indicate that relatively low levels of $\text{NO}^\bullet$, $\approx 50 \text{ nM}$, are required to inhibit cellular LPO. High levels of $\text{NO}^\bullet$ associated with the bolus addition would not be common *in vivo*, rather a much lower steady-state level with minor modulation would normally be expected. To simulate this situation in our *in vitro* model we employed a nitric oxide donor (PAPA/NO). Our goal was to use a concentration of donor that would produce a quite low, quasi-steady-state level of $\text{NO}^\bullet$ and thereby avoid potential artifacts due to high transient levels of $\text{NO}^\bullet$. The half-life of nitric oxide donors varies considerably and is in general dependent on temperature and pH [42]. Under our experimental conditions the half-life of PAPA/NO is $\approx 20 \text{ min}$, data not shown. In the experiments using PAPA/NO as a continuous source of $\text{NO}^\bullet$, we used DHA-enriched U937 cells, a cell line very similar to HL-60 cells; we chose these cells because the myeloperoxidase of HL-60 could accelerate the oxidation of $\text{NO}^\bullet$ [43], although control experiments indicated this not to be a problem, data not shown.

Two representative experiments, using PAPA/NO as a $\text{NO}^\bullet$ donor, are presented in Figure 3 (data for all experiments are summarized in Table 2); panel **A** shows an experiment where the maximum level of $\text{NO}^\bullet$ reached only 20 nM , while in panel **B** the maximum level of $\text{NO}^\bullet$ was 65 nM . The 20 nM system failed to protect the U937 cells against the Fe^{2+} -induced lipid peroxidation while the 65 nM protected the cells for over 900 seconds. These lipid peroxidation experiments were repeated with various $\text{NO}^\bullet$ concentrations. Using the experimental data from the lowest $\text{NO}^\bullet$ concentration that provided protection, an average for the minimum level of $\text{NO}^\bullet$ required for protection was $72 \pm 9 \text{ nM}$; the average duration of protection was 11 ± 4 minutes; the $\text{NO}^\bullet$ -concentration when lipid peroxidation accelerates was $13 \pm 8 \text{ nM}$. In this model system, we observed two distinct LPO transitions as $[\text{NO}^\bullet]$ decreased. The introduction of Fe^{2+} initiated a burst of LPO and a high level ($\geq 75 \text{ nM}$) was required to contain this torrent of oxidation. However, once the $[\text{NO}^\bullet]$ fell below $\approx 15 \text{ nM}$ the antioxidant action of $\text{NO}^\bullet$ was not sufficient and LPO accelerated.

Discussion

Nitric oxide has a rich chemistry that includes its rapid reaction with peroxy radicals, rxn 6. This chain-termination reaction has a result similar to that of typical donor antioxidants such as vitamin E in that the chain carrying peroxy radical (LOO[•]) is removed. Thus, if the covalent product, LOON=O, is not very reactive, then NO[•] will serve as a chain-breaking antioxidant. The discovery that NO[•] can be an antioxidant in LDL oxidation [16] has generated interest in the importance and mechanism of its antioxidant action. In this work we have used *in vitro* cell models to estimate the minimum levels of NO[•] needed to inhibit Fe²⁺-O₂-mediated lipid oxidation.

We have observed that quite low levels of NO[•] are required to suppress LPO. When a bolus of NO[•] (≈1.5 μM) was introduced to rapidly peroxidizing cells, lipid peroxidation was blunted; at least 50 nM NO[•] was needed to maintain a low rate of LPO, preventing rapid LPO. Interestingly when a nitric oxide donor was used to generate low steady-state levels of NO[•], a lower level of NO[•] (> ≈15 nM) was required. However, the PAPA/NO-donor experiments reveal two phases for the antioxidant action of NO[•]. As observed here and in our previous work [20,21,23,28], the introduction of Fe²⁺ (20 μM) initiates a burst of cellular lipid peroxidation; to meet this burst [NO[•]] must be >75 nM. If the [NO[•]] is less than this, then cellular LPO cannot be contained. If the burst of peroxidation is quenched, then a much lower [NO[•]] is required to maintain a low rate of cellular lipid peroxidation, ≈15 nM in our experimental system. Once [NO[•]] falls below this level, lipid peroxidation accelerates. The experiments that used a bolus addition of NO[•], with initial concentrations of ≈1.5 μM also showed that only ≈50 nM of NO[•] was required to keep lipid oxidation processes in check. These somewhat different threshold levels of NO[•] can be understood by examining the rate of lipid peroxidation once it accelerates. In the bolus experiments, the rate of oxygen uptake is much greater than in the donor experiments, once acceleration occurs. This is consistent with a greater oxidative stress, *i.e.* greater rate of initiation in the bolus experiments, thus a higher level of NO[•] is required. These experiments demonstrate that only very low nanomolar levels of NO[•] are required to blunt cellular lipid oxidation. Ambient levels of NO[•] *in vivo* are estimated to be approximately these concentrations; *in vivo* concentrations would be higher during signalling events or during particular pathologies. These results suggest that NO[•] will be an effective antioxidant *in vivo* because only very low levels are required.

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Figure 1. Exogenous nitric oxide delays lipid peroxidation in HL-60 cells

Nitric oxide (◆ 1600 nM delivered as a single bolus addition) and iron(II) (10 μ M) were added sequentially. When NO• is present, the concentration of oxygen (■) remains near baseline levels for nearly 200 s after introduction of iron(II) (*). After NO• is sufficiently depleted, the rate of oxygen consumption increases dramatically. In the absence of NO•, oxygen (▲) consumption accelerated immediately upon introduction of iron(II).

Figure 2. The rate of oxygen consumption increases as $[NO^*]$ falls below a minimum level

A. The rate of oxygen consumption, $-d[O_2]/dt$, was calculated over 20-second intervals as NO^* is consumed. As the concentration of NO^* becomes limiting, the rate of lipid peroxidation increases. At approximately 50 – 100 nM NO^* , a sharp increase in the rate of oxygen consumption occurs. Shown are results from three independent experiments.

B. Lipid Peroxidation accelerates under limiting $[NO^*]$. The relative peroxidation rate was calculated by dividing the peroxidation rate at a given time by the maximal rate. Next, data points were divided into bins by $[NO^*]$. At $[NO^*] < 50$ nM, the rate of peroxidation is significantly increased, consistent with a role for nitric oxide in inhibiting lipid peroxidation kinetics. The results are from the analysis of three independent experiments.

Figure 3. A. $[\text{NO}^*]_{\text{ss}} < 50 \text{ nM}$ does not inhibit Fe^{2+} -induced LPO

A representative experiment using $0.38 \mu\text{M}$ PAPA/NO given initially (blue arrow) followed by $20 \mu\text{M}$ Fe^{2+} (red arrow) in 0.9% NaCl (pH 6.5) with DHA-22:6 ω 3-enriched U937 cells ($3.3 \times 10^6 \text{ mL}^{-1}$). The initial rate of oxygen consumption was $1.5 \mu\text{M}$ per minute and increased to $3.7 \mu\text{M}$ per minute.

B. $[\text{NO}^*]_{\text{ss}} > 50 \text{ nM}$ inhibited LPO. A representative experiment using $0.46 \mu\text{M}$ of PAPA/NO given initially (blue arrow) followed by $20 \mu\text{M}$ Fe^{2+} (red arrow) in 0.9% NaCl (pH 6.5) with DHA-22:6 ω 3-enriched U937 cells ($3.3 \pm 10^6 \text{ mL}^{-1}$). Inhibition of LPO was sustained for 10.3 min with a rate of oxygen consumption of $1.6 \mu\text{M}$ per minute, which increased to $5.3 \mu\text{M}$ per minute when $[\text{NO}^*]$ dropped to $< 5 \text{ nM}$.

Table 1

Nitric oxide decay in non-peroxidizing systems

Condition ^a	Rate Order for NO [•] ^b	$\frac{k^c}{10^6 \text{M}^{-2} \text{s}^{-1}}$
Aqueous	2	2.1 ^d
PBS	2	2.8 ± 0.6
PBS + Fe ²⁺ ^e	2	3.4 ± 0.3
HL-60 cells	2	3.4 ± 0.1

^aExperiments were done in air-saturated aqueous solutions with initial concentrations of NO[•] of approximately 1.6 μM.

^bThe rate order was calculated by plotting log(−d[NO[•]]/dt) vs. log[NO[•]]

^cThe rate constant was calculated from the slope of 1/[NO[•]] vs. t under the assumption −d[NO[•]]/dt = 4 k [O₂] [NO[•]]².

^dFrom [44].

^eFe²⁺ added was 10 μM, as in Figure 1.

Nitric oxide concentration and oxygen consumption during lipid peroxidation in DHA-22:6ω3-enriched U937 cells.

[NO] ^a nM	Initial ^b d[O ₂]/dt μM/min	Final ^c d[O ₂]/dt μM/min	Δ(O ₂)/dt ^d μM/min	Inhibition ^e min	[NO [•]] at Acceleration ^f nM
20	-1.5	-3.7	2.2	0	---
50	-2.3	-10.8	8.5	0	---
65	-0.05	-3.8	3.7	16.1	20
68	-1.6	-5.3	3.7	10.3	5
82	-0.8	-1.8	1.0	7.6	15
164	-0.2	-4.7	4.4	13.5	4
170	-0.3	-3.5	3.3	4.8	42
210	-0.4	-0.9	0.5	10.5	18
401	-2.2	-10.2	8.0	4.6	40

^a the quasi steady-state NO[•] concentration when Fe²⁺ was added; this table shows an example set of experiments;
^b the initial rate of O₂ consumption prior to the addition of 20 μM Fe²⁺;
^c the final rate of O₂ consumption after the addition of 20 μM Fe²⁺;
^d the change in the rate of O₂ consumption between the final and initial rates;
^e the time of inhibition from the point of the Fe²⁺ addition to the point of increased O₂ consumption;
^f the NO[•] concentration when the rate of O₂ consumption accelerates.