PEROXYNITRITE-MEDIATED \( \alpha \)-TOCOPHEROL OXIDATION IN LOW-DENSITY LIPOPROTEIN: A MECHANISTIC APPROACH

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Abstract—Previous reports proposed that peroxynitrite (ONOO\(^-\)) oxidizes \( \alpha \)-tocopherol (\( \alpha \)-TOH) through a two-electron concerted mechanism. In contrast, ONOO\(^-\) oxidizes phenols via free radicals arising from peroxo bond homolysis. To understand the kinetics and mechanism of \( \alpha \)-TOH and \( \gamma \)-tocopherol (\( \gamma \)-TOH) oxidation in low-density lipoprotein (LDL) (direct vs. radical), we exposed LDL to ONOO\(^-\) added as a bolus or an infusion. Nitric oxide (\( \cdot \)NO), ascorbate, and CO\(_2\) were used as key biologically relevant modulators of ONOO\(^-\) reactivity. Although \( \approx 80\% \) \( \alpha \)-TOH and \( \gamma \)-TOH depletion occurred within 5 min of incubation of 0.8 \( \mu \)M LDL with a 60 \( \mu \)M bolus of ONOO\(^-\), an equimolar infusion of ONOO\(^-\) over 60 min caused total consumption of both antioxidants. \( \gamma \)-Tocopherol was preserved relative to \( \alpha \)-TOH, probably due to \( \gamma \)-tocopheroxyl radical recycling by \( \alpha \)-TOH. \( \alpha \)-TOH oxidation in LDL was first order in ONOO\(^-\) with \( \approx 12\% \) of ONOO\(^-\) maximally available. Physiological concentrations of \( \cdot \)NO and ascorbate spared both \( \alpha \)-TOH and \( \gamma \)-TOH through independent and additive mechanisms. High concentrations of \( \cdot \)NO and ascorbate abolished \( \alpha \)-TOH and \( \gamma \)-TOH oxidation. Nitric oxide protection was more efficient for \( \alpha \)-TOH in LDL than for ascorbate in solution, evidencing the kinetically highly favored reaction of lipid peroxyl radicals with \( \cdot \)NO than with \( \alpha \)-TOH as assessed by computer-assisted simulations. In addition, CO\(_2\) (1.2 \( \mu \)M) inhibited both \( \alpha \)-TOH and lipid oxidation. These results demonstrate that ONOO\(^-\) induces \( \alpha \)-TOH oxidation in LDL through a one-electron free radical mechanism; thus the inhibitory actions of \( \cdot \)NO and ascorbate may determine low \( \alpha \)-tocopheryl quinone accumulation in tissues despite increased ONOO\(^-\) generation. © 2003 Elsevier Inc. All rights reserved.

Keywords—Nitric oxide, Peroxynitrite, Carbonate radical, \( \alpha \)-Tocopherol, \( \gamma \)-Tocopherol, Ascorbate, Free radical, Antioxidant, Low-density lipoprotein oxidation

INTRODUCTION

Peroxynitrite (ONOO\(^-\)), the product of the diffusion-limited reaction between nitric oxide (\( \cdot \)NO) and superoxide (\( \mathrm{O}_2^- \)), represents a key biological oxidizing and nitrating species [1,2]. Evidence for the in vivo formation of ONOO\(^-\) requires evaluation of several biochemical and pharmacological parameters [3]. The involvement of ONOO\(^-\) in atherosclerosis is supported by the observed simultaneous production of \( \cdot \)NO and \( \mathrm{O}_2^- \) by vascular cells, the \( \mathrm{O}_2^- \)-dependent modulation/impairment of \( \cdot \)NO signaling, and the demonstration of protein and lipid nitration in vivo [4–12]. Moreover, 3-nitrotyrosine is elevated in plasma of humans at increased risk of developing coronary artery disease [13], and protein nitration and vascular disease progression inhibition by metalloporphyrin ONOO\(^-\) decomposition catalysts has been reported [14]. However, other nitrating agents such as myeloperoxidase/H\(_2\)O\(_2\)/NO\(_2\) may also account for protein and lipid nitration observed during inflammation in the vasculature [15–19].

Oxidative modification of low-density lipoprotein (LDL) is widely considered to be critical for initiation
of atherogenesis [20], where conversion of native LDL into a more atherogenic form by ONOO⁻ as well as myeloperoxidase-generated reactive nitrogen species is likely involved [17,21–24]. Peroxynitrite-oxidized LDL can be recognized by scavenger receptors of vascular cells, upregulating reactive oxygen and nitrogen species production and enhancing inflammation and foam cell formation [9,25–28]. However, the mechanisms of ONOO⁻-mediated oxidation of LDL are only partially understood, probably due to its complex chemistry. In fact, ONOO⁻ and its conjugated acid ONOOH can oxidize organic molecules (1) directly; (2) through H⁺ or CO₂-catalyzed homolysis of its O–O bond yielding the highly oxidizing nitrogen dioxide (ONO₂⁻), hydroxyl (·OH), and carbonate anion free radical (CO₃⁻) (or CO₂); or (3) by both routes [1]. It has been reported that ONOO⁻ added as a bolus does not lead to important LDL lipid peroxidation [29–31]. Instead of lipid double bonds, it is proposed that apolipoprotein B-100 (apo B-100) and carotenoids are the preferred targets of ONOO⁻ in LDL [29–32]. In contrast, we have recently shown that low infusion rates of preformed ONOO⁻ can efficiently oxidize LDL lipids, causing extensive lipid–protein adduct formation [33].

α-Tocopherol (α-TOH) is probably the most important reductant in LDL. Previous work proposed that ONOO⁻ oxidizes LDL α-TOH via a direct reaction mechanism where ONOO⁻ acts as a two-electron (nonradical) oxidant [34]. Kinetic and mechanistic studies have revealed that various phenols (i.e., tyrosine, ubiquinol, and some polyphenols) are oxidized via one-electron free radical pathways [35–37]. In addition, the roles in ONOO⁻-mediated lipid and α-TOH oxidation of CO₂, which catalyzes ONOO⁻ homolysis into ·NO₂ and CO₃⁻, and ascorbate, which effectively reduces α-tocopheroxyl radical (α-TOX) back to α-TOH, have not been elucidated [30,38].

Although not frequently considered, evidence supports that ·NO constitutes an integral part of the antioxidant machinery present in LDL at the vascular wall and in cellular membranes [39,40]. Nitric oxide exerts its lipid oxidation protective actions through almost diffusion limited termination reactions with lipid peroxyl radicals (LOO·) yielding nitrogen-containing bioactive lipids [12,41]. Nitric oxide can also rapidly react with most initiating radicals, including ONOO⁻-derived radicals [42]. Interestingly, it has been shown that ·NO spares α-TOH in LDL and liposomes exposed to peroxyl radicals derived from azo-compounds [43,44].

Considering the above-mentioned aspects, we hypothesized that biologically relevant fluxes of ONOO⁻ induce α-TOH and lipid oxidation in LDL via one-electron radical pathways that would be potently inhibited by ·NO. We therefore investigated the mechanisms of ONOO⁻-mediated oxidation of α- and γ-TOH in LDL and their modulation by physiological levels of relevant intravascular modulators of ONOO⁻ reactivity: ·NO, ascorbate, and CO₂.

EXPERIMENTAL PROCEDURES

Materials

(Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]-diazen-1-ium-1,2-diolate (DETA NONOate) was from Cayman Chemical Company (Ann Arbor, MI, USA) and 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC-12) from Dojindo Laboratories, Japan. Ethanol, methanol, 2-propanol, acetonitrile, and hexane (all of HPLC grade) were supplied by Mallinckrodt UltimAR. All other reagents were from Sigma (St. Louis, MO, USA).

Preparation of ONOO⁻ and ·NO donors

Peroxynitrite was synthesized from acidified nitrite and hydrogen peroxide as previously described and its concentration determined by absorbance at 302 nm (ε = 1670 M⁻¹ cm⁻¹ [33,41]). Residual H₂O₂ was eliminated with MnO₂ and remaining nitrite was <30% of ONOO⁻ content. Peroxynitrite was added to LDL either as a bolus or as a continuous flux using a motor-driven syringe (SAGE Instruments, Boston, MA, USA) as before [33,41]. Stock solutions of DETA NONOate and NOC-12 were prepared in 50–150 mM potassium phosphate buffer, pH 9.5, and ·NO release rates were measured in the buffer used for LDL incubations (200 mM potassium phosphate, 100 μM DTPA, pH 7.4, 37°C), following absorbance changes at 250 nm [45] and by the oxyhemoglobin method [33,44].

Low-density lipoprotein oxidation

Human LDL was obtained from plasma of normolipidemic and apparently healthy donors and was purified by ultracentrifugation followed by HPLC gel filtration as before [46]. Alternatively, if higher-concentration preparations were needed, it was purified by double ultracentrifugation followed by filtration through a PD-10 column. Oxidation was initiated by either bolus addition or an infusion of ONOO⁻ in the presence or absence of ·NO donors and/or ascorbate. In some experiments, bicarbonate was added to LDL from a freshly prepared stock solution (500 mM, pH 7.4). As stock solutions of ONOO⁻ contained 0.7 N NaOH, a set of control reactions had equimolar NaOH infused into the LDL samples. Decomposed ONOO⁻ for reverse-order-of-addition controls was obtained by incubation of ONOO⁻ (3–30 mM) with phosphate buffer,
Lipid analysis

Conjugated dienes were determined as described in Ref. [50] with minor modifications. Samples prepared by cold methanol/sample (9/1 v/v) extraction followed by vortexing and centrifugation. The simultaneous RP-HPLC detection of a 10-mM SDS, for 120 min at 37°C with 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP). The extent of oxidation of α-TCH and γ-TCH in LDL was determined by RP-HPLC [52]. To avoid oxidation by nitrogen oxides derived from decomposition in methanol of the NO donors, samples (20 μl) were mixed with cold methanol (180 μl) adjusted at pH 10 ± 0.5 by preincubation with 10 μl of 500 mM phosphate buffer, pH 10.5, followed by vortexing and centrifugation. The simultaneous RP-HPLC detection of α-TCH, γ-TCH and α-tocopheryl quinone was performed after methanol elution at a flow rate of 1 ml min⁻¹. The detection system included a Gilson Model 122 fluorometric detector (λex = 295 nm, λem = 330 nm) and a Gilson Model 155 UV/ VIS detector (dual-wavelength mode, λ1 = 266 nm, λ2 = 234 nm). Concentrations of α-TCH and γ-TCH were calculated by interpolation in the corresponding standard curves. The α-tocopheryl quinone standard was synthesized and purified as in Ref. [53] and its concentration was determined by its absorbance at 266 nm (ε = 19,000 M⁻¹ cm⁻¹) [53].

RESULTS

Peroxy-nitrite-induced lipid oxidation in LDL: bolus versus infusion

Infusion of ONOO⁻ into LDL preparations caused CL-OOH formation in parallel with conjugated diene accumulation (Fig. 1A). In contrast, ONOO⁻ added as a bolus produced only a 10-fold lower yield of lipid oxidation than equimolar ONOO⁻ infused at a constant rate over 200 min (Fig. 1A). As previously reported [30], increasing concentrations of ONOO⁻ added as a bolus caused small amounts of CL-OOH and conjugated dienes (~5% yield based on added ONOO⁻, not shown). When LDL was exposed to successive bolus additions of ONOO⁻, oxygen consumption after addition of the first bolus was ~8% and increased up to ~15% for each of the following bolus additions (Fig. 1B). An ~10-s lag phase of oxygen consumption after bolus addition of ONOO⁻ was consistently observed, even if α-TCH and γ-TCH were already consumed. When ONOO⁻ was infused into LDL, a 24 ± 2 min lag time of oxygen consumption was observed (Fig. 1C) that correlated with lag phases of conjugated dienes and CL-OOH formation (Fig. 1A).

α-Tocopherol and γ-tocopherol oxidation: bolus versus infusion

Although a 60 μM bolus of ONOO⁻ caused ~80% α-TCH and γ-TCH depletion within 5 min of incubation (Fig. 2A), an equimolar infusion of ONOO⁻ over 60 min caused total consumption of both antioxidants (Fig. 2A). Reverse-order addition experiments did not produce any significant consumption of these antioxidants even when longer incubation times were allowed (not shown). Comparison of the time courses of α-TCH and γ-TCH depletion during ONOO⁻ infusion versus independent bolus of ONOO⁻ at different initial concentrations showed that both infusion and bolus at low initial concentration (~30 μM) were equally efficient (Figs. 2B and 2C). γ-TCH was less susceptible to ONOO⁻-mediated oxidation than α-TCH (Figs. 2B and 2C). In fact, the end of the lag phase of oxygen consumption during infusion (Fig. 1C) was accompanied by an acceleration of γ-TCH oxidation and an ~50% depletion of α-TCH in both bolus and infusion additions (Figs. 2B and 2C). In contrast, slow infusion of ONOO⁻ was more efficient than bolus only at high initial concentrations of ONOO⁻ bolus. α-Tocopheryl quinone formation paralleled α-TCH depletion, yielding 1 mol α-tocopheryl quinone/mol of oxidized α-TCH (Fig. 2B). The same stoichiometry was observed when ONOO⁻ was added as a bolus (Fig. 2C). In addition, α-TCH consumption linearly depended on ONOO⁻ infusion rate, with a yield of ~10% with respect to ONOO⁻.
To determine the amount of ONOO\(^-\) available to oxidize \(\alpha\)-TOH we studied the oxidation yield as a function of LDL concentration (Fig. 3B). \(\alpha\)-TOH oxidation and \(\alpha\)-tocopherol quinone formation increased up to 2 \(\mu\)M LDL (ONOO\(^-\)/LDL molar ratio of 25/1) and was accompanied by a stoichiometric depletion of \(\alpha\)-TOH. The maximum oxidation yield determined with respect to ONOO\(^-\) was \(\sim 12\%\), in a process that was not affected by the addition of nitrite (NO\(_2\), 0.05–1.0 mM, not shown).

Fig. 1. LDL lipid oxidation induced by ONOO\(^-\). LDL (0.4 mg protein ml\(^-1\)) in phosphate buffer 200 mM, pH 7.4, 37°C, was exposed to ONOO\(^-\) added as bolus or as a 1 \(\mu\)M min\(^{-1}\) infusion. (A) Lipid oxidation product accumulation: Open symbols, conjugated dienes (CD); solid symbols, CL-OOH; squares, infusion; circles, bolus additions. Oxygen uptake (B) after sequential bolus additions of ONOO\(^-\) (130 \(\mu\)M) or (C) during 1 \(\mu\)M min\(^{-1}\) infusion of ONOO\(^-\). Data shown are representative of three independent experiments.

Fig. 2. \(\alpha\)- and \(\gamma\)-tocopherol oxidation by ONOO\(^-\) bolus versus infusion additions. (A) RP-HPLC analysis of \(\alpha\)-TOH and \(\gamma\)-TOH during LDL oxidation by ONOO\(^-\) (60 \(\mu\)M): (a) native LDL; (b) bolus addition (\(t = 5\) min); (c) ONOO\(^-\) infusion (1 \(\mu\)M min\(^{-1}\) for 60 min). A representative chromatogram is shown. (B) Time course of \(\alpha\)-TOH and \(\gamma\)-TOH consumption and \(\alpha\)-TQ formation during ONOO\(^-\) infusion (1 \(\mu\)M min\(^{-1}\)) into LDL. (C) Final concentrations of \(\alpha\)-TOH, \(\gamma\)-TOH and \(\alpha\)-tocopherol quinone after bolus additions of ONOO\(^-\) (0, 10, 20, 50, and 100 \(\mu\)M). Data in (B) and (C) represent means \pm SD. \(\alpha\)-TOH and \(\alpha\)-TQ data are relative to the initial \(\alpha\)-TOH concentration. \(\gamma\)-TOH is expressed with respect to its initial concentration.
Nitric oxide and ascorbate inhibition of \( \text{ONOO}^- \)-induced \( \alpha \)-tocopherol oxidation

\( \cdot \text{NO} \) (300 nM min\(^{-1}\) from NOC-12) or ascorbate spared \( \alpha \)-TOH in LDL, displaying additive antioxidant actions toward \( \alpha \)-TOH and LDL lipids when added in concert (Fig. 4A and Table 1). Table 1 shows that ascorbate had a greater inhibitory effect on CL-OOH formation than on conjugated diene accumulation. This is not due to CL-OOH reduction directly ascribed to ascorbate, because ascorbate does not reduce emulsified CL-OOH (not shown). A potent dose-dependent inhibition of \( \alpha \)-TOH (Fig. 4B) and \( \gamma \)-TOH (not shown) consumption by \( \cdot \text{NO} \) fluxes released from NOC-18 \((t_{\frac{1}{2}} \sim 500 \text{ min at pH } 7.4, 37^\circ \text{C})\) was also observed (Fig. 4B), with 50% inhibition at \( \sim 300 \text{ nM min}^{-1} \). Additionally, ascorbate was able to dose-dependently spare \( \alpha \)-TOH in LDL exposed to \( \text{ONOO}^- \) infusion (not shown). Interestingly, \( \cdot \text{NO} \) fluxes decreased rates of ascorbate consumption by \( \text{ONOO}^- \) infusion in a dose-dependent manner, with a 50% inhibitory effect at \( \sim 1200 \text{ nM min}^{-1} \) (Fig. 4B). Results of computer-assisted simulations were similar to experimental results on \( \alpha \)-TOH depletion only if

![Graph A](image1.png)

**Fig. 3.** Dependence of \( \alpha \)-TOH oxidation on \( \text{ONOO}^- \) infusion rate and LDL concentration. (A) Initial rates of \( \alpha \)-TOH consumption in LDL exposed to increasing \( \text{ONOO}^- \) fluxes \((0–5.0 \text{ µM min}^{-1}\)). Representative data of two independent experiments are shown. (B) \( \alpha \)-Tocopheryl quinone \((\alpha \text{-TQ})\) formation in LDL \((0, 0.2, 0.4, 0.8, 1.2, 2.0, 4.0, \text{ and } 8.0 \text{ µM})\) after exposure to \( \text{ONOO}^- \) \((50 \text{ µM, bolus addition})\). Data are means ± SD.

![Graph B](image2.png)

**Fig. 4.** Nitric oxide and ascorbate inhibition of \( \text{ONOO}^- \)-dependent oxidation of \( \alpha \)-TOH in LDL and ascorbate. (A) LDL was exposed to \( \text{ONOO}^- \) \((0.8 \text{ µM min}^{-1}, \text{squares})\) in the presence of 20 µM NOC-12 \((\sim 300 \text{ nM min}^{-1}, \text{circles})\), 20 µM ascorbate (point-up triangles), and NOC-12 plus ascorbate (point-down triangles). Representative data from three experiments performed at different initial concentrations of NOC-12 and ascorbate are shown. (B) Nitric oxide-mediated inhibition of LDL \( \alpha \)-TOH (black solid squares) and ascorbate (black solid triangles) oxidation during independent exposure to \( \text{ONOO}^- \) infusions \((1 \text{ µM min}^{-1})\). Experimental data (black solid symbols) are compared with computer-assisted simulation data for \( \cdot \text{NO} \) inhibition of \( \alpha \)-TOH oxidation in the presence of oxidizable lipids (open diamonds) and in their absence (open circles). Nitric oxide was delivered from 0 to 2 mM NOC-18 \((0–2.5 \text{ µM min}^{-1})\). Data are percentages with respect to the initial rate of antioxidant consumption in the absence of \( \cdot \text{NO} \). Means ± SD were calculated after normalization of data from three independent experiments.
oxidizable lipids were included. In the absence of oxidizable lipids simulations indicate that α-TOH would be protected by ·NO similarly to ascorbate (Fig. 4B).

LDL was incubated in the presence of ONOO⁻ (1.0 μM min⁻¹) as in Fig. 4. Twenty micromolar NOC-12 (300 nM min⁻¹ ·NO) and/or 20 μM ascorbate were added before starting ONOO⁻ infusion. Control experiments were performed in the absence of antioxidants. Data represent means ± SD of three independent experiments. Statistical analysis was performed using ANOVA and Student–Newman–Keuls tests for all six groups. *p < .05 compared with NOC-12 and ascorbate. **p < 0.05 compared with NOC-12. ***p < 0.05 compared with ascorbate-conjugated diene group.

Table 1. Protective Effects of ·NO and Ascorbate on LDL Lipid Oxidation

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<tr>
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<th>Conjugated dienes (% inhibition)</th>
<th>CL-OOH (% inhibition)</th>
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<tbody>
<tr>
<td>NOC-12</td>
<td>49 ± 11</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>40 ± 9</td>
<td>75 ± 4***</td>
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<tr>
<td>NOC-12, ascorbate</td>
<td>77 ± 9*</td>
<td>92 ± 8**</td>
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Carbon dioxide inhibition of ONOO⁻-induced α-TOH and lipid oxidation

Bicarbonate in equilibrium with CO₂ at physiological concentrations inhibited lipid oxidation (Fig. 5A) in parallel with α-TOH consumption and α-tocopherol quinone formation (Fig. 5B). Moreover, CO₂ was able to inhibit tryptophanyl residue oxidation as evidenced by LDL fluorescence analysis (Fig. 5C). Finally, CO₂ inhibited ONOO⁻-induced apo B-100 fragmentation as evidenced by native apo B-100 band disappearance (Fig. 5D, upper panel) showing a less intense smear pattern in the regions of lower as well as higher molecular masses relative to that of native apo B-100 (Fig. 5D, bottom panel).

DISCUSSION

The mechanisms underlying ONOO⁻-induced α-TOH and lipid oxidation in LDL were explored, making
use of two approaches: (1) comparison of bolus versus fluxes of ONOO\textsuperscript{−} and (2) the actions of biologically relevant modulators of ONOO\textsuperscript{−} reactivity: \textbullet NO, ascorbate, and CO\textsubscript{2}. Herein, we show evidence supporting that: (1) ONOO\textsuperscript{−} induces \( \alpha \)-TOH oxidation in LDL through one-electron free radical-mediated oxidations; (2) \( \leq 50\% \) of ONOO\textsuperscript{−}-derived \textbullet OH and \( \text{NO}_2\) finally cause \( \alpha \)-TOH oxidation; (3) \textbullet NO spares \( \alpha \)-TOH by its fast reactions with lipid radicals as well as ONOO\textsuperscript{−}-derived radicals; (4) \textbullet NO and ascorbate additively protect \( \alpha \)-TOH from ONOO\textsuperscript{−}-mediated oxidation; and (5) CO\textsubscript{2} inhibits ONOO\textsuperscript{−}-mediated \( \alpha \)-TOH and lipid oxidation in LDL.

Although \( \alpha \)-TOH is not a major component of LDL it is consumed during the initial phases of LDL oxidation, thus constituting a preferential target of ONOO\textsuperscript{−} induced oxidants. Peroxynitrite-mediated LDL antioxidant depletion has been attributed to rapid direct oxidation reactions by ONOO\textsuperscript{−} or ONOOH [29,32, 34,54]. Nevertheless, our data support a radical-dependent mechanism in which \textbullet NO\textsubscript{2} and \textbullet OH initiate LDL oxidation. A concerted two-electron mechanism was discarded by analyzing the \( \alpha \)-TOH-sparing actions of ascorbate and \textbullet NO. Although ONOO\textsuperscript{−} directly reacts with ascorbate \textbullet AH\textsuperscript{−} [55,56],

\[
\text{ONOOH} + \text{AH}^- \rightarrow \text{NO}_2^- + \text{products} \quad k_1 = 236 \pm 1 \text{ M}^{-1} \text{s}^{-1},
\]

this reaction is kinetically unimportant at physiological concentrations of ascorbate [55]. The potent \( \alpha \)-TOH-sparing action of ascorbate can be explained on the basis of its ability to reduce the \( \alpha \)-tocopheryloxyl radical in LDL and bilayer membrane systems with the concomitant formation of ascorbyl radical anion \textbullet A\textsuperscript{−} [57,58]:

\[
\alpha\text{-TOH}^- + \text{AH}^- \rightarrow \alpha\text{-TOH} + \text{A}^- \quad k_2 = 3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}.
\]

An ascorbate-dependent two-electron concerted reduction of \( \alpha \)-tocopheryl quinone or any other two-electron \( \alpha \)-TOH oxidation of product back to \( \alpha \)-TOH can be discarded at physiological pH [59]. In addition, ascorbate rapidly reacts with aqueous \textbullet OH and \textbullet NO\textsubscript{2} [42]:

\[
\text{AH}^- + \text{\textbullet OH} \rightarrow \text{A}^- + \text{OH}^- \quad k_3 = 1.6 \times 10^8 \text{ M}^{-1} \text{s}^{-1},
\]

\[
\text{AH}^- + \text{\textbullet NO}_2 \rightarrow \text{A}^- + \text{NO}_2^- \quad k_4 = 1.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}.
\]

However, ascorbate oxidation by \textbullet OH and \textbullet NO\textsubscript{2} is likely outcompeted by their reactions with LDL targets. In summary, either by reacting with \( \alpha \)-TOH\textsuperscript{•} or by inhibiting the initiation of lipid oxidation, ascorbate is acting as a one-electron reductant.

The \( \alpha \)-TOH-sparing action of \textbullet NO can be explained by simple competition kinetics, as previously proposed [44]: \textbullet NO rapidly reacts with lipid peroxyl radicals (LOO\textsuperscript{•}, reaction 5 [60]), and this reaction is in competition with LOO\textsuperscript{•} reduction by \( \alpha \)-TOH (reaction 6 [61]) and unsaturated lipids (LH, reaction 7) [62]:

\[
\text{LOO}^- + \text{\textbullet NO} \rightarrow \text{LOONO} \quad k_5 = 2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}, \quad (5)
\]

\[
\text{LOO}^- + \alpha\text{-TOH} \rightarrow \text{LOOH} + \alpha\text{-TOH}^- \quad k_6 = 6 \times 10^5 \text{ M}^{-1} \text{s}^{-1}, \quad (6)
\]

\[
\text{LOO}^- + \text{LH} \rightarrow \text{LOOH} + \text{L}^- \quad k_7 = 30 \text{ M}^{-1} \text{s}^{-1}. \quad (7)
\]

Lipid double bonds are present at high concentrations in LDL (~0.9 M); nevertheless, the reaction of LOO\textsuperscript{•} with LH is kinetically unfavored if compared with reactions 5 and 6. Nitric oxide reactions with \textbullet NO\textsubscript{2} and \textbullet OH are also diffusion-limited [42]. Although these reactions are likely outcompeted by \textbullet NO\textsubscript{2} and \textbullet OH reactions with LDL targets, this may not be the case if \textbullet NO must diffuse long distances, before the facile diffusion into the LDL particle [39]. In fact, the one-compartment model simulation results (Fig. 4B) suggest that reaction–diffusion processes in the aqueous surroundings decrease \textbullet NO availability in LDL. As the \textbullet NO reaction with hindered phenoxy radicals is rapidly reversible [44,63], the potent \( \alpha \)-TOH-sparing action of \textbullet NO cannot be ascribed to \( \alpha \)-tocopheryloxyl radical trapping nor reduction. Finally, \textbullet NO is not expected to directly scavenge ONOO\textsuperscript{−} to any significant extent [64]:

\[
\text{\textbullet NO} + \text{ONOO}^- \rightarrow \text{\textbullet NO}_2 + \text{NO}_2^- \quad k_8 < 5 \times 10^{-2} \text{ M}^{-1} \text{s}^{-1}. \quad (8)
\]

Therefore, the potent inhibition by \textbullet NO and ascorbate on \( \alpha \)-TOH consumption in LDL exposed to ONOO\textsuperscript{−} provides strong evidence supporting a one-electron free radical mechanism of \( \alpha \)-TOH oxidation. Although ascorbate and \textbullet NO displayed an additive inhibitory action on \( \alpha \)-TOH consumption and conjugated diene accumulation, ascorbate-mediated inhibition of CL-OOH accumulation was greater (Table 1). This difference may be due to its participation as a cofactor in the already reported apo B-100 peroxidase activity [65]. Similarly to \( \alpha \)-TOH, we also observed that \( \gamma \)-TOH consumption by ONOO\textsuperscript{−} fluxes can be inhibited by
·NO and ascorbate (not shown). Therefore our results also support a one electron-mediated γ-TOH oxidation. A greater extent of α-TOH consumption relative to γ-TOH was observed, as previously reported [66]. Because reaction rate constants of LOO· reduction by α-TOH and γ-TOH are similar, this can be partially due to the ~15-fold higher α-TOH concentration in the particle with respect to γ-TOH. More important, α-TOH can repair an intermediate of γ-TOH oxidation yielding γ-TOH [66]. As LOO· reacts with γ-TOH yielding γ-tocopheroyl radical (γ-TO·), we propose that a recycling of γ-TO· by α-TOH is the most likely explanation for these results.

Although α-TOH oxidation in LDL is first order on ONOO− flux (Fig. 3A), kinetics favor a lipid radical-mediated mechanism of α-TOH oxidation. Peroxynitrite induces lipid oxidation in phospholipid vesicles free of α-TOH [67]. In fact, the LDL content of unsaturated lipids (LH) is almost three orders of magnitude greater than that of α-TOH while the rate constants of reaction of ·NO2 and ·OH with LH and trolox C, a water-soluble analogue of α-TOH, are similar [42]. Moreover, LDL lipid peroxidation increased after α-TOH depletion (Fig. 1). Therefore, in accordance with recent quantifications [61], we conclude that LOO· can preferentially react with α-TOH. This explains the low yields of lipid oxidation observed until α-TOH has been significantly consumed (~50%); thus, the rate of α-TOH consumption reflects the rate of lipid oxidation initiation (R1) in LDL. It is mostly accepted that indirect oxidations by ONOO− in the absence of CO2 and metals are ·OH and ·NO2 dependent, where free radical formation from ONOOH occurs with a yield near 30% [1]. Nevertheless, we demonstrate here that only ~12% of ONOO− is maximally available to induce α-TOH oxidation in LDL (Fig. 3B). This suggests competing reactions of ONOO−, ONOOH, and its derived radicals in LDL, which decrease R1 values. Peroxynitrite anion and ONOOH directly react with some amino acid residues [35] and possibly other LDL components.

To investigate the contribution of ·NO2 versus ·OH on lipid oxidation initiation processes we employed nitrite anion (NO2−), which at the higher concentrations tested is expected to rapidly react with ·OH, yielding ·NO2 and OH− (k = 1×10^10 M^−1 s^−1) [42]. The observed lack of effect of NO2− on α-TOH oxidation yields suggests that both ·NO2 and ·OH are involved in the initiation of lipid oxidation in LDL exposed to ONOO− [42]:

\[
\text{LH + ·NO}_2 \rightarrow \text{L}^* + \text{NO}_2^− \quad k_9 = 1 \times 10^9 \text{ M}^−1 \text{ s}^−1, \quad (9)
\]

\[
\text{LH + ·OH} \rightarrow \text{L}^* + \text{OH}^- \quad k_{10} = 1 \times 10^9 \text{ M}^−1 \text{ s}^−1. \quad (10)
\]

Although ONOO− induces an almost equal α-TOH oxidation yield added either as a bolus or as an infusion (Fig. 2), full propagation of lipid oxidation takes place only in the case of infusion, where the kinetic chain length of lipid oxidation is ~20 (Fig. 1). Thus, low rates of ONOO− infusion determine low concentrations of initiating and propagating (LOO·) radicals in LDL that favor propagation reactions as compared with the bolus addition condition. Computer-assisted simulations using a simple scheme of reactions were performed to assess the relative contribution of termination pathways [42,68,69]:

\[
\text{LOO· + LOO·} \rightarrow \text{LOOOOL} \quad k_{11} = 1 \times 10^5 - 1 \times 10^7 \text{ M}^−1 \text{ s}^−1, \quad (11)
\]

\[
\text{LOO· + ·NO}_2 \rightarrow \text{LOONO}_2 \quad k_{12} = 1 \times 10^9 \text{ M}^−1 \text{ s}^−1, \quad (12)
\]

\[
\text{L}^* + ·\text{NO}_2 \rightarrow \text{LNO}_2 \quad k_{13} = 1 \times 10^9 \text{ M}^−1 \text{ s}^−1. \quad (13)
\]

Simulations showed that the LOO· recombination reaction (reaction 11) largely predominates over the others. Nevertheless, propagatory fluxes could predominate over termination pathways during infusion if \(1 \times 10^5 \text{ M}^−1 \text{ s}^−1 \leq k_{11} \leq 1 \times 10^7 \text{ M}^−1 \text{ s}^−1\). Although ·NO2 can participate in termination processes (reactions 12 and 13), these are minor pathways compared with ·NO2-mediated LH oxidation (reaction 9).

The inhibition of ONOO−-induced LDL α-TOH and lipid oxidation by CO2 (Fig. 5) contrasts with previous reports of enhanced SIN-1-dependent lipid oxidation by CO2 [30,38], showing that in the presence of CO2 the reactivity of SIN-1 is different than that of authentic ONOO−. Although the inhibitory action of CO2 toward ONOO−-induced α-TOH oxidation could be explained by a hypothetical diminished direct reactivity of CO3− with α-TOH, it is well established that CO2 catalyzes and enhances ONOO−-dependent oxidation of other phenols [70]. Alternatively, we propose that the physicochemical properties of the CO3−/LDL system may explain these disparities. Carbonate radical is a strong acid [71] that is negatively charged under the physiological conditions employed here. Thus, the limited permeability of LDL to charged species and, to a lesser extent, the repulsive coulombic forces between CO3− and the predominantly negatively charged LDL surface will disfavor the diffusion of CO3− into the hydrophobic environments of the particle, thus decreasing the rate of initiation of lipid oxidation in LDL. Rather, recombination reactions in the aqueous phase and/or reaction with less lipophilic targets (i.e., surface exposed apo B-100 residues) may be
favored. Since apo B-100 fragmentation and tryptophan oxidation were also inhibited by CO₂ (Figs. 5C and 5D), we conclude that they may result, at least in part, from LOO- mediated reactions. In addition, these results may indicate that similar diffusional constraints are imposed by LDL to LH oxidation by CO₃⁻ as well as to oxidation of LDL-buried apo B-100 regions.

In summary, taken together our data support a free radical one-electron mechanism of ONOO⁻-induced oxidation of α-TOH in LDL. Thus, the antioxidant role of NO and its additive actions with ascorbate may be of great importance in vivo. We also evidenced the ability of CO₂ to inhibit lipophilic target oxidation in LDL, probably redirecting ONOO⁻ reactivity to hydrophilic moieties. Finally, our results are in agreement with almost independent and additive α-TOH-sparing actions of NO, ascorbate, and CO₂, thus expecting low yields of ONOO⁻-mediated vitamin E oxidation in vivo.

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REFERENCES


Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. Peroxynitrite...


### ABBREVIATIONS

apo B-100 — apolipoprotein B-100
CL-OOH — cholesteryl linoleate hydroperoxide
DETA NONOate — (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DTPA — diethylenetriaminepentaacetic acid
LDL — low-density lipoprotein
LOO· — lipid peroxyl radical
·NO — nitric oxide
·NO2 — nitrogen dioxide
NOC-12 — 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene
O2·− — superoxide anion
·OH — hydroxyl radical
ONOO− — peroxynitrite
ONOOH — peroxynitrous acid
SIN — 1,3-morpholinosydnonimine
α-TOH — α-tocopherol
γ-TOH — γ-tocopherol
α-TQ — α-tocopheryl quinone