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Nitric Oxide Reaction with Lipid Peroxyl Radicals Spares α-Tocopherol during Lipid Peroxidation

GREATER OXIDANT PROTECTION FROM THE PAIR NITRIC OXIDE/α-TOCOPHEROL THAN α-TOCOPHEROL/ASCORBATE*

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Homero Rubbo¶‡§‖, Rafael Radi¶, Daniel Anselmi¶, Marion Kirk, Stephen Barnes§**, John Butler‡‡, Jason P. Eiserich‡§, and Bruce A. Freeman‡§ §§**

*From the ¶Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay, Departments of ‡Anesthesiology, §§Biochemistry and Molecular Genetics, **Pharmacology and Toxicology, and §Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama 35233, and the ‡‡Department of Biological Sciences, Salford University, Salford, United Kingdom*

The reactions of nitric oxide (•NO) and α-tocopherol (α-TH) during membrane lipid oxidation were examined and compared with the pair α-TH/ascorbate. Nitric oxide serves as a more potent inhibitor of lipid peroxidation propagation reactions than α-TH and protects α-TH from oxidation. Mass spectrometry, oxygen and •NO consumption, conjugated diene analyses, and α-TH fluorescence determinations all demonstrated that •NO preferentially reacts with lipid radical species, with α-TH consumption not occurring until •NO concentrations fell below a critical level. In addition, α-TH and •NO cooperatively inhibit lipid peroxidation, exhibiting greater antioxidant capacity than the pair α-TH/ascorbate. Pulse radiolysis analysis showed no direct reaction between •NO and α-tocopheroxyl radical (α-T•), inferring that peroxyl radical termination reactions are the principal lipid-protective mechanism mediated by •NO. These observations support the concept that •NO is a potent chain breaking antioxidant toward peroxidizing lipids, due to facile radical-radical termination reactions with lipid radical species, thus preventing α-TH loss. The reduction of α-T• by ascorbate was a comparatively less efficient mechanism for preserving α-TH than •NO-mediated termination of peroxyl radicals, due to slower reaction kinetics and limited transfer of reducing equivalents from the aqueous phase. Thus, the high lipid/water partition coefficient of •NO, its capacity to diffuse and concentrate in lipophilic milieu, and a potent reactivity toward lipid radical species reveal how •NO can play a critical role in regulating membrane and lipoprotein lipid oxidation reactions.

α-Tocopherol (α-TH),[1] a lipid-soluble membrane constituent, is a critical chain-breaking antioxidant in biological membranes and lipoproteins that acts by reducing chain-propagating peroxyl radical species (LOO•) to the corresponding hydroperoxide (LOOH, Refs. 1–3). The mechanism of reaction of α-TH with LOO• involves the loss of an H atom during reduction of LOO• to LOOH, yielding the α-tocopheroxyl radical (α-T•) at a rate constant of approximately 5×10^5 $M^{-1} \cdot s^{-1}$, depending on the alkyl chain length, degree of unsaturation, and esterified nature of the fatty acid undergoing peroxidation (4–6). α-Tocopheroxyl radical has only limited capability to propagate further radical chain reactions in most biological conditions because of resonance stabilization of the phenoxyl radical and reduction by ascorbate, thiols, or enzyme-dependent mechanisms (7).

The diffusible signaling molecule and inflammatory mediator nitric oxide (•NO) readily concentrates up to 20–25-fold in membrane and lipoprotein compartments, by virtue of a high lipid partition coefficient, charge neutrality, and small molecular radius (8, 9). There, •NO can avidly react with lipid epoxyallylic and alkoxyl radical (LO•) and LOO• species with rate constants of $\sim 2 \times 10^9$ $M^{-1} \cdot s^{-1}$, yielding both nitrogen oxide- and hydroperoxide-containing products (10–14). Based on relative rate constants, it is predicted that the reaction of •NO with LOO• will predominate over both the reduction of LOO• by α-TH and the initiation of secondary peroxidation propagation reactions by LOO• with vicinal unsaturated lipids ($k = 1.3 \times 10^3$ $M^{-1} \cdot s^{-1}$).

In the present study, we demonstrate that biologically relevant concentrations of α-TH and •NO can interact cooperatively to inhibit membrane lipid peroxidation, exhibiting greater antioxidant capacity than the pair α-TH/ascorbate. These inhibitory actions of •NO and α-TH toward lipid oxidation are a consequence of •NO preferentially terminating lipid radical-mediated chain propagation reactions, rather than prevention of α-TH loss by direct reduction of α-T•.

EXPERIMENTAL PROCEDURES

Materials—Egg phosphatidylcholine, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, and di-palmitoyl-glycero-3-phosphocholine were from Avanti Polar Lipids (Pelham, AL). Linoleic acid (18:2) was from Nu-Chek-Prep (Elysian, MI). Hexane and 1,1,3,3-tetramethoxypropane were from Aldrich. Sodium azide was obtained from

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‖ To whom correspondence should be addressed: Dept. de Bioquímica, Facultad de Medicina, General Flores 2125, 11800 Montevideo, Uruguay. Tel.: 5982-924-9561; Fax: 5982-924-9563; E-mail: hrubbo@fmed.edu.uy.

[1] The abbreviations used are: α-TH, α-tocopherol; •NO, nitric oxide; N_2O, nitrous oxide; NO^-, nitroxyl anion; $\cdot NO_2$, nitrogen dioxide; LOO•, peroxyl radical; LO•, alkoxyl radical; LOOH, hydroperoxide; •OH, hydroxyl radical; O_2^-, superoxide; $ONOO^-$, peroxynitrite; PC, phosphatidylcholine; DPPC, 1,2-palmitoyl-glycero-3-phosphocholine; 18:2, linoleic acid; α-T•, α-tocopheroxyl radical; AO•; phenoxyl radical TBA, 2-thiobarbituric acid; LC-MS, liquid chromatography-mass spectrometry; ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; SNN, spermine NONOate; GSNO, *S*-nitrosoglutathione.

Merck. α-Tocopherol (>99%) was from Alexis (San Diego, CA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP) was from Wako Chemicals (Richmond, VA). Nitrous oxide (N_2O, 99.999%) was from BOC Gases, and 1,3-propanediamine *N*-(4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl] (SNN) and spermine were from Cayman Chemical Co. (Ann Arbor, MI). All other reagents were from Sigma.

Preparation of ·NO and ·NO Donors—Solutions of ·NO were prepared by bubbling ·NO gas (Matheson, Madison, WI) for 30 min into argon-saturated deionized H_2O. Any nitrogen dioxide ($\cdot NO_2$) present was eliminated by first bubbling ·NO through 5 M NaOH. Before ·NO solutions were made, 100 ml of deionized/doubly-distilled water was purged with N_2 for 30 min to remove oxygen. The N_2-saturated solution was placed in series with the purified ·NO stream and continuously bubbled for 20 min to produce a saturated solution having a final ·NO concentration of approximately 1.8 mM. ·NO production rates and solution concentrations were measured by electrochemical detection using a ·NO-selective electrode (WPI Instruments, Ann Arbor, MI) calibrated by measuring ·NO liberated from KNO_2 in 0.1 M KI 0.1 M 0.1, and 0.1 M H_2SO_4, using the following reaction performed under anaerobic conditions: $2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$. *S*-Nitrosoglutathione (GSNO) was synthesized by combining equimolar (200 mM) concentrations of reduced glutathione and sodium nitrite in 0.5 M HCl followed by recrystallization to eliminate contaminating nitrite (15). Stock solutions of SNN were prepared in degassed 50 mM potassium phosphate buffer, pH 8.5. Either GSNO or SNN was added to buffer systems used for lipid oxidation reactions (100 mM potassium phosphate, pH 7.4, at 20 °C) to measure rates of ·NO release.

Lipid Oxidation—Phosphatidylcholine liposomes (PC) were prepared from 6 ml of a 25.4 mM (20 mg·ml^{-1}) lipid stock solution in chloroform as described previously (11, 12, 16). Briefly, solvent was removed *in vacuo* at 45–55 °C, and 6 ml of 10 mM potassium phosphate, pH 7.4, was added. The suspension was placed in a 4 °C water bath and sonicated 3 times for 30 s at 65 watts using a Branson sonifier. Liposomes were stored in the dark under argon and used within 24 h of preparation. Lipid peroxidation was performed with either 1 mg·ml^{-1} (2.5 mM) linoleic acid emulsified in 10 mM SDS, 50 mM sodium phosphate, pH 7.4, or 6 mg of phospholipid·ml^{-1} PC liposomes. Oxidation was initiated by the addition of the organic peroxyl radical initiator ABAP in the presence and absence of α-TH and ·NO. Nitric oxide was generated by the addition of GSNO, SNN, or by controlled infusion of anaerobically dissolved ·NO. ABAP-dependent oxygen consumption was measured polarographically using a water-jacketed cell fitted with a Clark-type oxygen electrode, model YSI 4004 (Yellow Springs Instrument Co., Yellow Springs, OH). Reactions were performed at 37 °C in a 1.8-ml water-jacketed chamber. All incubations were continually stirred at 37 °C, and aliquots were removed at indicated time points for measurement of tribarbituric acid-reactive substances (ϵ = 150 mM^{-1}·cm^{-1} at 532 nm) or diene conjugation (ϵ = 26 mM^{-1}·cm^{-1} at 234 nm) (11, 12, 17).

Lipid Analysis—Stock solutions of α-TH were freshly prepared in 100% ethanol. In some cases α-TH was quantitated fluorometrically after addition of 1 ml of ethanol and 5 ml of hexane to 1-ml reaction systems (18), with the fluorescence intensity of the organic phase measured at λ_{ex} = 295 nm and λ_{em} = 320 nm and calibrated against standard preparations of α-TH. Linoleic acid, α-TH, and α-TH oxidation products were also assessed by liquid chromatography-mass spectrometry (LC-MS). After oxidation reactions, methanol was added for a final concentration of 60% (v/v). Linoleic acid and its oxidation products were separated by reversed-phase high pressure liquid chromatography on a 10 cm × 2.1 mm inner diameter. Aquapore C_8 column at a flow rate of 0.2 ml/min using a linear 50–100% methanol gradient in 1% aqueous acetic acid (11). Negative ions were generated by passing eluates through an IonSpray interface, with an orifice potential of −60 V. Negative mass spectra were recorded over the m/z range from 200 to 600. The linoleic acid oxidation products included $[M - H]^-$ ions for 9- and/or 12-hydroxylinolenate (m/z = 295) and 9 and/or 12-hydroperoxolinolenate (m/z = 311). The α-TH parent molecule and its oxidation products were detected as the acetate adducts at m/z = 489 (α-TH), 505 (α-tocopherylquinone or 8-α-hydroxytocopherone), and 521 (8-α-hydroperoxytocophenone). Analyses were performed on an API III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) equipped with two MacIntosh Quadra 950 computers for data analysis.

Pulse Radiolysis—Radical reactions of α-TH or Trolox C were initiated by a short (20 ns), high energy electron pulse from a linear accelerator as described previously (19). Reactions were initiated in N_2O-saturated phosphate-buffered solutions (10 mM, pH 7.4) containing either 100 μM Trolox C or 50 μM α-TH and 100 mM sodium azide. In the case of α-TH, either 2 mM SDS was added to aid solubilization or α-TH was incorporated into 1,2-palmitoyl-glycero-3-phosphocholine (DPPC) liposomes. Liposomes were prepared by combining α-TH and DPPC (100 μM and 10 mM, respectively) in chloroform, brought to dryness in a rotary evaporator, reconstituted in 100 mM potassium phosphate, pH 7.4, and subjected to vortexing followed by sonication. In some reactions, an aliquot (0.1–0.8 ml) of the ·NO stock solution was added with a gas-tight syringe through a rubber septum into the sealed experimental apparatus containing the reaction mixture. Pulse radiolysis experiments were conducted within 5 min of ·NO addition. Azide radicals ($N_3^{\cdot}$) were formed in aqueous solutions of sodium azide (N_3^-) within about 0.1 μs after the pulse through the following Reactions 1–3.

$$H_2O \rightarrow {}^{\cdot}OH, H^{\cdot}, e_{aq}^-, H_2, H_2O_2, H_3O^+ \qquad \text{(Reaction 1)}$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \qquad \text{(Reaction 2)}$$

$$^{\cdot}OH + N_3^- \rightarrow N_3^{\cdot} + OH^- \qquad \text{(Reaction 3)}$$

Since N_3^- is present at high concentrations (100 mM), all the hydroxyl radical (·OH) formed is converted to $N_3^{\cdot}$. The generated $N_3^{\cdot}$ rapidly oxidizes phenolic substances (AOH) such as tyrosine, Trolox C, and α-TH to the corresponding phenoxyl radical (AO·, Refs. 19–21). Dosimetry was performed by the thiocyanate method (22). Initial radical yields were taken to be $G(N_3^{\cdot}) = G(^{\cdot}OH) + G(e_{aq}^-) = 5.6$ (100 eV)$^{-1}$, corresponding to a molar concentration of $G(N_3^{\cdot}) \times D \times 1.035 \times 10^{-7}$ M and were kept between 1 and 2 μM to ensure pseudo-first-order kinetics. The optical path length of the cell was 2.5 cm. Transient absorbance changes in Trolox C and α-TH were detected by kinetic absorbance spectroscopy at 430 nm (or over the range 380–500 nm when absorbance spectra were obtained). The transient signals were recorded by a Tektronix digitizer.

RESULTS

Inhibition of ABAP-dependent Linoleic Acid Oxidation by ·NO and α-TH—Addition of ABAP generates peroxyl radicals by spontaneous thermal decomposition at a constant rate and initiates lipid oxidation in phospholipid liposome membranes, a property essential for kinetic studies (6). An oxygen uptake rate of 0.3 ± 0.1 μM·min^{-1} at 37 °C was produced by 5 mM ABAP, corresponding to 300 nM·min^{-1} ABAP-derived peroxyl radicals (not shown). In the presence of linoleic acid (2.5 mM) the rate of oxygen uptake increased to 0.9 ± 0.1 μM·min^{-1} due to lipid peroxidation propagation reactions. Addition of ·NO from SNN prior to ABAP inhibited oxygen consumption during ABAP-initiated linoleic acid peroxidation in a dose-dependent manner (Fig. 1*A*). Under these conditions, SNN yielded 0–95 nM·min^{-1} ·NO. Similar results were obtained when ·NO was replaced with α-TH at higher concentrations (Fig. 1*B*). When partially inhibitory concentrations of both ·NO and α-TH were incubated with linoleic acid, the extent of inhibition was almost complete (Fig. 1*C*). In order to compare the response of the length of the lag phase before onset of autocatalytic lipid oxidation upon addition of both α-TH and ·NO, the concentration of antioxidant required for inhibition of linoleic acid oxidation was determined. The extent of conjugated diene formation in linoleic acid incubated with ABAP was inhibited by both ·NO and α-TH in a dose-dependent manner (Fig. 2, *A* and *B*) and occurred in concert with a reduction of net yields of TBA-reactive oxidation products (not shown). Whereas no lag times were observed in the absence of antioxidants, induction periods obtained in the presence of α-TH and/or ·NO were calculated from the intersect of the slope of the linear portion of the induction phase with the slope of the linear portion of the propagation phase. A 50% inhibition of ABAP-catalyzed linoleic acid oxidation was induced by the addition of 1 μM SNN (but not spermine alone), corresponding to a ·NO flux of 7 nM·min^{-1} for 1 h. By these same respective indices, 50 μM α-TH was required for 50% inhibition of linoleic acid oxidation. When α-TH and ·NO were added in combination, the increase in lag time was more than additive, inferring cooperative actions of α-TH and ·NO (Fig. 2*C*). More detailed dose-response studies showed that this interaction of ·NO and α-TH occurred at rates

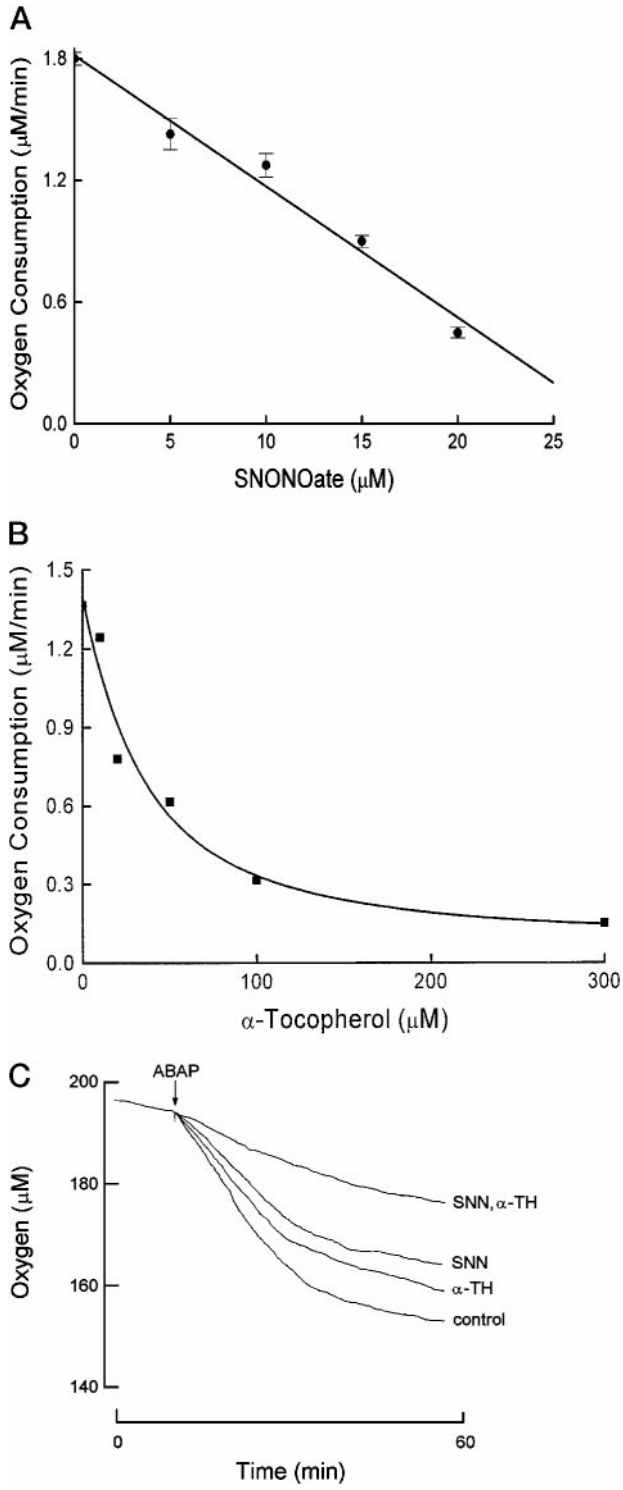

FIG. 1. **Nitric oxide and α-TH inhibition of oxygen consumption during ABAP-mediated oxidation of linoleic acid.** Oxidation of 1 mg·ml^{-1} linoleic acid emulsified in 10 mM SDS, 50 mM potassium phosphate buffer, pH 7.4, by 5 mM ABAP was monitored using a Clark-type oxygen electrode at 37 °C. Additions of SNN (5, 10, 15, and 20 μM), corresponding to ·NO fluxes of 20, 48, 69, and 95 nM·min^{-1}, respectively) (*A*), α-TH (10, 25, 50, 100, and 300 μM) (*B*), or 50 μM α-TH, 10 μM SNN, and 50 μM α-TH plus 10 μM SNN were made prior to ABAP addition (*C*).

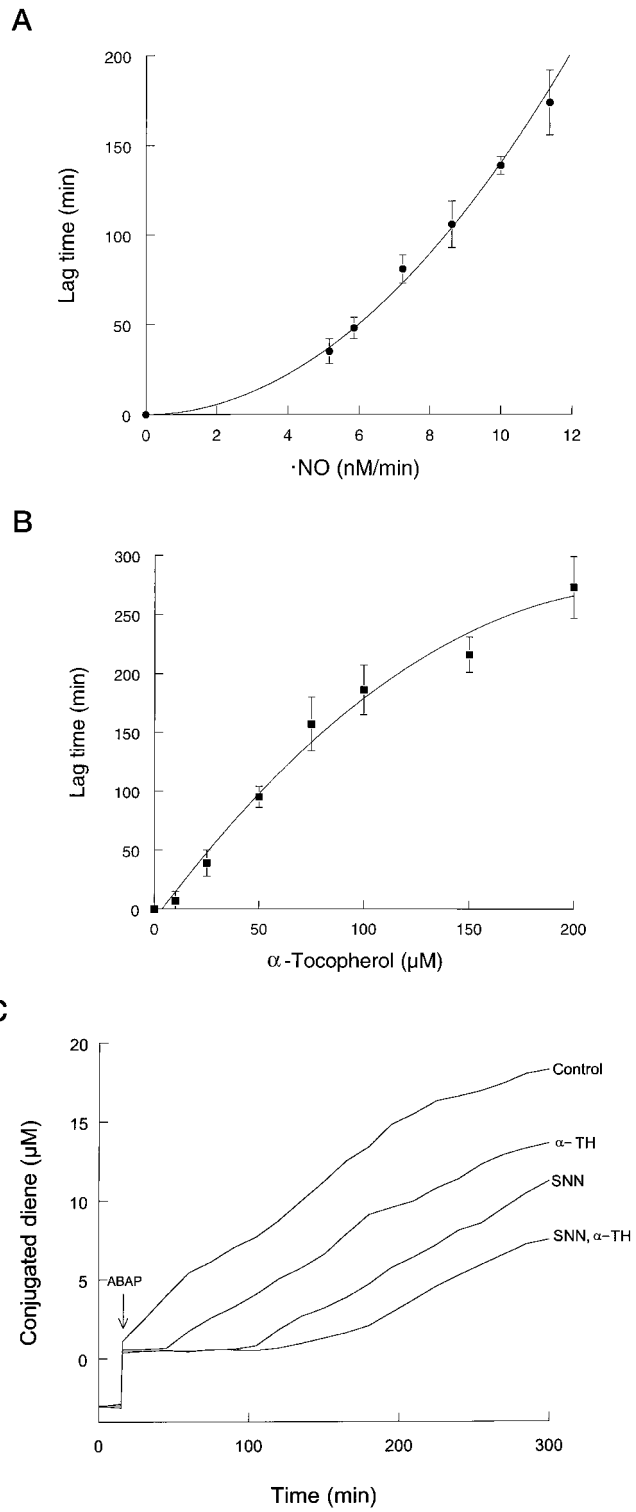

FIG. 2. **Dose-dependent inhibition of ABAP-induced linoleic acid oxidation by ·NO and α-TH.** ABAP (5 mM) was added to linoleic acid as before and incubated with stirring at 37 °C. Conjugated diene formation by linoleic acid was assessed spectrophotometrically at 234 where lag times were calculated for SNN (0, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 μM), corresponding to ·NO fluxes of 0, 5.2, 5.9, 7.0, 8.6, 10.0, 11.4, and 12.8 nM·min^{-1}, respectively) (*A*), α-TH (0, 10, 25, 50, 75, 100, 150, and 200 μM) (*B*), or 50 μM α-TH, 7.0 nM·min^{-1} ·NO from 1 μM SNN, and a combination of α-TH plus ·NO (*C*). Data represent mean ± S.D., $n = 3$.

of ·NO production greater than 10 nM·min^{-1} (Table I). It is important to note that lag times correlated with ·NO steady state concentrations, with lipid oxidation chain propagation reactions not occurring until ·NO concentrations fell below a critical level of ~10 nM (Fig. 3).

When comparing lipid oxidation inhibitory actions of the α-TH/ascorbate pair with α-TH/·NO, ·NO plus α-TH displayed greater antioxidant capacities toward ABAP-dependent liposome oxidation (Table II). Comparative extents of inhibition of

TABLE I
Experimental cooperative and theoretical additive effects of α-TH and ·NO toward inhibiting ABAP-dependent 18:2 oxidation

18:2 was oxidized as before in the presence of 50 μM α and varying concentrations of SNN (0, 0.5, 1, 2, 2.5, 3, and 4 μM). Data represent mean ± S.D., $n = 3$.

Lag time		Nitric oxide
Additive	Cooperative	
min		*nM/min*
0	0	0
120 ± 9	126 ± 8	5.9
148 ± 8	157 ± 7	7.2
181 ± 12	218 ± 14	10
210 ± 14	259 ± 14	11.4
256 ± 14	345 ± 19	12.8
293 ± 21	396 ± 22	15.5

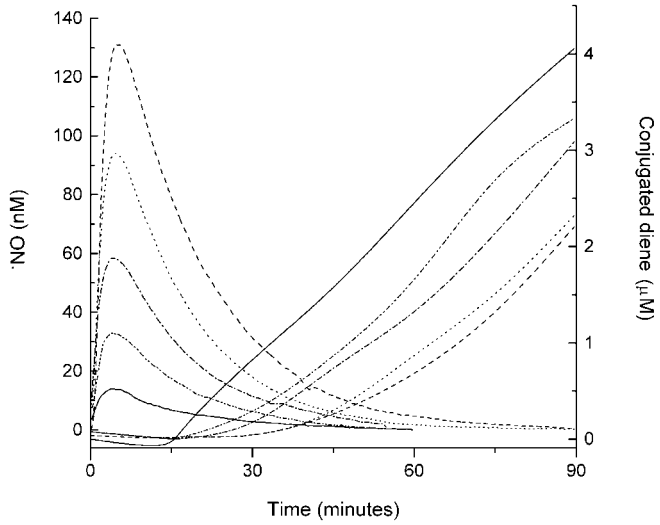

FIG. 3. **Nitric oxide consumption parallels inhibition of diene-conjugated formation during ABAP oxidation of linoleic acid.** Conjugated dienes and ·NO concentrations were monitored during oxidation of linoleic acid by ABAP as described in Fig. 2. Nitric oxide concentrations were monitored using a ·NO sensor after addition of SNN (0.5, 1, 1.5, 2, and 3 μM).

TABLE II
Inhibition of ABAP-mediated liposome oxidation by ·NO, α-TH, and ascorbate

PC liposomes (6 mg · ml^{-1}) were oxidized for 120 min as in Fig. 1 and monitored by thiobarbituric acid reactivity at 532 nm. Reactions were conducted in the presence of 50 μM α-TH, 1 μM SNN, 300 μM ascorbate or a combination of SNN plus α-TH or ascorbate plus α-TH. Data represent mean ± S.D., $n = 3$.

Condition	TBA reactive material
	μM
PC liposomes	3.8 ± 0.4
PC liposomes, ABAP	14.6 ± 1.4
+ Ascorbate	13.7 ± 1.5
+ α-TH	7.8 ± 0.8
+ SNN	6.5 ± 0.5
+ SNN, ascorbate	6.4 ± 0.4
+ SNN, α-TH	4.2 ± 0.3

lipid oxidation induced by 7 nM·min^{-1} ·NO plus 50 μM α-TH were only obtained when adding supra-physiological concentrations of ascorbate (300 μM) with α-TH.

Product Analysis of ·NO and α-TH Reaction with Oxidizing Lipids—Liquid chromatography-mass spectrometry (LC-MS) analysis of oxidation products of linoleic acid ($[M - H]^-$ ion = 279) induced by ABAP also revealed that ·NO and α-TH exert both independent and cooperative lipid antioxidant activities when present in concert (Table III). The $[M - H]^-$ ions of principal oxidation products were $m/z = 295$ (LOH) and 311 (LOOH), representing 8 and 21% of the total linoleic acid present, respectively. In the presence of α-TH, 7 and 18% of the same respective oxidation products were observed. Similar inhibition of linoleic acid oxidation was also afforded by SNN at 2 μM, yielding 3 and 12% of LOH and LOOH, respectively. When both α-TH and SNN were added to ABAP-initiated linoleic acid oxidation reactions, synergistic inhibition of linoleic acid oxidation occurred, with only 0.5% of linoleic acid being oxidized to LOH and LOOH. Similar product distribution was observed when SNN was substituted for GSNO (100 μM, data not shown).

In separate studies, the extent of α-TH oxidation to other species was proportional to the extent of ABAP-induced lipid hydroperoxide formation. Quantitative yields from a typical experiment are also summarized in Table III. Oxidation of α-TH was determined by LC-MS quantitation of α-TH loss and an accompanying increase in the major oxidation products having $m/z = 446$ (α-tocopherylquinone or 8-α-hydroxytocopherone) and $m/z = 462$ (8-α-hydroperoxytocophenone). Under conditions where ·NO produced by SNN inhibited ABAP-initiated linoleic acid oxidation, LC-MS analysis showed that α-TH oxidation was prevented by ·NO, as indicated by decreased yields in both the $[M - H]^-$ ions of α-TH oxidation products and the net extent of α-TH oxidation (from 75 to 31%) in the absence of ·NO.

Prevention of α-TH Loss by ·NO—In addition to the LC-MS studies, the extent of oxidation of α-TH was also quantitated by fluorescence spectroscopy (Fig. 4). α-TH oxidation, as indicated by loss of characteristic fluorescence emission, did not occur until later stages of SNN decomposition, where steady state concentrations of ·NO fell below 10 nM. Either ·NO or ascorbate protected α-T· from ABAP-mediated oxidation in a dose-dependent manner, with at least 1 order of magnitude lower concentrations of ·NO being required for comparable effects.

To explore the potential reaction of ·NO with α-T· and the reduction of α-T· in an ascorbate-like reaction, as predicted from thermodynamic calculations, pulse radiolysis studies were performed to reveal whether ·NO directly reacts with the phenoxyl radical of α-TH or Trolox C (not shown), the later being a water-soluble analog of α-TH. As shown in Fig. 5*A*, the reaction of $N_3^·$ with α-TH gave rise to characteristic phenoxyl radical absorbance spectra (λ_{max} ~430 nm) through the following Reaction 4.

$$AOH + N_3^· \rightarrow N_3^- + AO^· \qquad \text{(Reaction 4)}$$

This reaction is fast at pH 7.4 ($k = 6–8 \times 10^8$ $M^{-1}·s^{-1}$) and gives approximately 100% phenoxyl radical yield, consistent with previous studies (21). Monitoring α-TH phenoxyl radicals at 430 nm revealed that these species are extremely stable, as no evidence of radical decay could be observed over a 10-ms time scale (Fig. 5*B*). When ·NO (1–100 μM) was added prior to pulse radiolysis, no observable changes in the absorbance spectra or rates of decay of the α-TH and Trolox C (not shown) phenoxyl radicals were observed (Fig. 5*C*). These phenoxyl radicals were, however, reduced by ascorbate consistent with previous observations (22).

DISCUSSION

Nitric oxide inhibits superoxide ($O_2^{·-}$), peroxynitrite ($ONOO^-$), lipoxygenase, copper, and macrophage-dependent lipid and lipoprotein oxidation (11, 12, 14, 23–28). The principal mechanism underlying ·NO inhibition of lipid oxidation is a facile reaction with lipid alkoxyl or epoxyallylic and peroxyl radical intermediates, thus terminating lipid radical chain propagation reactions. Herein, we demonstrate the following: (*a*) ·NO represents a key lipid-soluble chain-breaking antioxi-

TABLE III

The influence of ·NO on ABAP-initiated linoleic acid oxidation and α-tocopherol oxidation product yields

Linoleic acid (18:2, 3.6 mM) was emulsified in 10 mM SDS, 50 mM potassium phosphate, pH 7.4, and oxidized by addition of 5 mM ABAP at 37 °C with constant stirring. Concomitant addition of 1 μM spermine NONOate and/or 50 μM α-TH was made at the same time, and 2 h later 18:2 and α-TH oxidation products were determined by LC-MS. 18:2 products (m/z = 295 and 311) were expressed as percent of unoxidized 18:2 (m/z = 279) with values normalized by an internal standard (heptadecanoic acid, m/z = 269). α-TH parent molecule (M_r = 430) and its oxidation products (M_r = α-TOH, 446 and α-TOOH, 462) were detected as the acetate adducts at m/z = 489, 505, and 521, respectively.

	Percent distribution							
	18:2 (L)	LOH	LOOH	α-TH	α-TOH	α-TOOH	% 18:2	% α-TH
M/z								
Condition	279	295	311	430	446	462	Oxidized	Oxidized
18:2	100	0	0				0	
18:2, ABAP	39	8	21				29	
+ SNN	61	3	12				15	
+ α-TH	44	7	18	25	21	36	25	75
+ SNN, α-TH	92	0.2	0.3	69	0	23	0.5	31

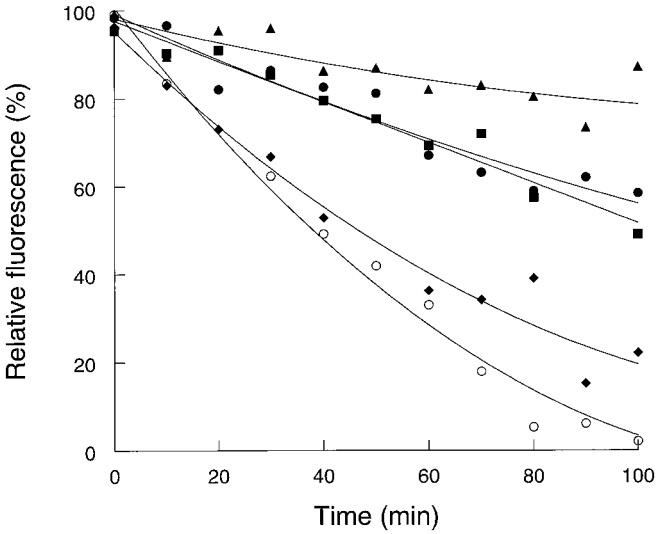

FIG. 4. **α-TH consumption in the presence of ascorbate or SNN.** PC liposomes (6 mg·ml^{-1}) were oxidized as before in the absence (○) and presence of SNN: 5 μM (■) and 10 μM (▲) or ascorbate: 100 μM (◆) and 300 μM (●). α-TH concentration was determined fluorometrically in separate aliquots from the same reaction (λ_{ex} = 295 nm, λ_{em} = 320 nm).

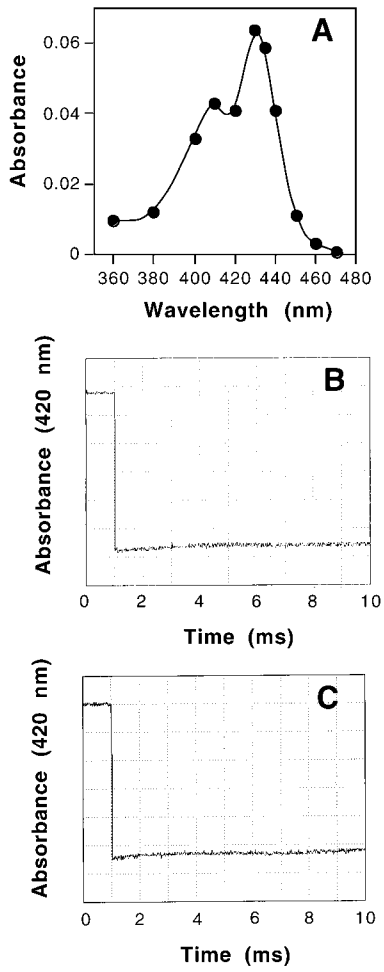

FIG. 5. **Pulse radiolysis analysis of ·NO reaction with α-TH radicals (α-T·).** N_2O-saturated solutions of α-TH (50 μM) were subjected to a single 20-ns pulse of high energy electrons in the presence of N_3^- as described under "Experimental Procedures" to produce (α-T·). Spectrum of the α-T· is shown in *A*. Transient kinetic profiles of α-T· were obtained from 1- to 10-ms time scale (*B*), and in the presence of ·NO (20 μM) prior to pulse radiolysis (*C*).

dant protecting α-TH from oxidation; (*b*) α-TH and ·NO can act cooperatively to inhibit lipid peroxidation processes, exhibiting greater antioxidant capacity than the pair α-TH/ascorbate; and (*c*) contrary to the thermodynamically predicted outcome, ·NO does not directly react with and reduce α-T·.

Linoleic acid oxidation by ABAP resulted in the formation of two principal hydroxy- and hydroperoxy- oxidation products, the formation of which was inhibited by the independent addition of either α-TH or ·NO (Figs. 1 and 2 and Table III). When both α-TH and ·NO were added, inhibition of lipid oxidation was almost complete. This cooperative action of α-TH and ·NO was confirmed by measurement of lag times before onset of autocatalytic peroxidative propagation reactions (Fig. 2 and Table I) and LC-MS analysis, where α-TH was not consumed in lipid oxidation reactions until decreasing concentrations of ·NO no longer preferentially inhibited lipid oxidation (Table III). A reaction between ·NO and either the carbon or peroxyl radical species formed during ABAP decomposition could potentially account for the inhibition of ABAP-dependent initiation of linoleic acid oxidation. It has been previously observed that at an oxygen consumption rate of 0.78 μM·min^{-1}, the rate of ·NO consumption was 1.5 μM·min^{-1}, indicating that for each ABAP peroxyl radical generated, approximately two molecules of ·NO were consumed in the absence of lipids (27). The potent lipid oxidation inhibitory effects that were observed at physiologically relevant (nM) concentrations of ·NO indicated that ·NO reaction with peroxyl radicals represents a rate-defining step in both the propagation and initiation phases of lipid oxidation.

Two mechanisms can explain the ability of ·NO to protect α-TH from oxidation, with the first and most critical being the reaction of ·NO with both LO· (this term is also used synonymously with epoxyallylic radical (L(O)·, see Ref. 29) and LOO· at significantly greater rates than α-TH (6, 10, 30), yielding nitrogen-containing radical-radical termination products (11, 12). The second potential mechanism is the reduction of α-T· by

·NO, thus regenerating α-TH consumed during reduction of lipid peroxyl radicals. Thermodynamic predictions suggest ·NO to be capable of limiting α-TH oxidation, via the one-electron reduction of α-T· to α-TH according to Reaction 5.

$$\alpha\text{-T}^{\cdot} + {}^{\cdot}\text{NO} + \text{OH}^{-} \rightarrow \alpha\text{-TH} + \text{NO}_2^{-} \qquad \text{(Reaction 5)}$$

This reaction has $\Delta G^{0\prime} = -5$ Kcal·mol^{-1} and $\Delta E^{0\prime} = +240$ mV, with the $E^{0\prime}$ for (α-T·/α-TH) = +480 mV and the $E^{0\prime}$ for (·NO/NO_2^-) = -240 mV (31, 32). Conversely, it has been reported that ·NO oxidizes α-TH to α-T· (33). Similarly, ·NO can be reduced to nitroxyl anion (NO^-) by 6-hydroxychromanes (34). In contrast to these latter suppositions, exposure of α-TH to physiological concentrations of ·NO under either aerobic or anaerobic conditions yielded no oxidation of α-TH (35, 36), inferring that significantly elevated levels of contaminating nitrogen dioxide (·NO_2) was responsible for the previously observed oxidation of α-TH by ·NO.

Pulse radiolysis analysis revealed that ·NO does not undergo direct reaction with the phenoxyl radical of α-TH (Fig.5) or its water-soluble analog Trolox C (not shown). This is in sharp contrast to the rapid reaction ($k = 2 \times 10^9$ M^{-1}·s^{-1}) that has been observed between ·NO and tyrosyl radicals (Tyr·, see Ref. 20). This marked difference in the reactivity of the phenoxyl radicals of tyrosine and that of α-TH with ·NO is likely a consequence of structural differences, which in turn impact on respective reactivities. Whereas α-TH and Trolox C contain methyl substituents attached *ortho-* to both carbons adjacent to the phenolic hydroxyl group, these sites in tyrosine are unsubstituted, being occupied by hydrogen atoms. Therefore, the phenoxyl radical of tyrosine is representative of a number of resonance forms where the radical may lie on the phenolic oxygen and the ring carbon atoms. However, with hindered phenolic radicals such as α-TH and Trolox C, the radical is predominantly associated with the phenolic oxygen. In contrast to the highly stable nature of α-TH radicals, free tyrosyl radicals rapidly decay over μs time scales (20) to produce dityrosine cross-links (*i.e.* formation of a carbon-carbon bond by combination of ring-carbon radicals to yield 3,3′-dityrosine, see Refs. 37 and 38). This position has also been determined to be the site of ·NO-Tyr· and ·NO_2-Tyr· reaction. Since α-TH and Trolox C radicals are not readily delocalized to ring carbons, the lack of reactivity between ·NO and phenoxyl radicals of α-TH and Trolox C would be expected. Indeed, previous studies have demonstrated that *O*-nitroso derivatives of α-TH rapidly undergo dissociation to form phenoxyl radicals and subsequent release of ·NO (34), implying that the equilibrium lies far to the right (see Reaction 6),

$$\alpha\text{-tocopherol-O-NO} \rightarrow \alpha\text{-T}^{\cdot} + {}^{\cdot}\text{NO} \qquad \text{(Reaction 6)}$$

Under conditions where linoleic acid emulsions were oxidized by ABAP-derived peroxyl radicals in the presence of α-TH, LC-MS analysis of α-TH oxidation products showed formation of the major species α-tocopherylquinone or 8-α-hydroxytocopherone and 8-α-hydroperoxytocophenone (Table III). The 8-α-hydroperoxytocopherone is formed by oxygen addition to α-T·, followed by hydrogen abstraction and hydrolysis to 8-α-hydroxytocopherone, which in turn rearranges to α-tocopherylquinone. Oxidation of α-T· by LOO· also yields 8α-(alkyldioxy)tocopherones, which may either hydrolyze to α-tocopherylquinone or become reduced, thus regenerating α-TH (5). Lipid peroxyl radicals can also add to the 8α position of α-T· in a radical-radical termination reaction, yielding 8α-(alkyldioxy)-tocopherones that account for approximately 50% of the α-TH consumed by LOO· in homogeneous solutions (5, 39, 40).

The mobility of α-TH in the lateral plane of the membrane and its positioning critically influence its antioxidant actions. The polar phenolic hydroxyl group of α-TH preferentially reacts with free radical species accessible at aqueous-lipophilic interfaces and, in the process, becomes oxidized to α-T· (4). α-Tocopheroxyl radicals in turn are less reactive than other lipid radicals (LO· or LOO·) but are susceptible to further oxidation. The reduction of α-T· to α-TH by biochemical reductants completes a one-electron redox cycle, an event proposed to account for the apparent antioxidant synergy between α-TH and ascorbic acid (7). The α-T· species that do not complete redox cycles can then react with a second LOO·, further contributing to the antioxidant actions of α-TH (5). Both ascorbate and α-TH can act in concert to inhibit lipid and lipoprotein oxidation via ascorbate-dependent reduction of α-TH (41, 42). Ascorbate can both directly reduce α-T· to α-TH with a rate constant of 1.5×10^6 M^{-1}·s^{-1} (6) and LOO· to LOOH at the slower rate constant of $k = 7.5 \times 10^4$ M^{-1}·s^{-1} (6). When both antioxidants are present, ascorbate is consumed first and upon ascorbate depletion α-TH becomes oxidized, suggesting that LOO· preferentially reacts with α-TH and that ascorbate preferentially reduces α-T· rather than LOO· (6).

Interestingly, α-TH but not ascorbate inhibits oxidation of lipids treated with azoperoxyl radical initiators (43, 44). Moreover, *in vivo* study of guinea pigs failed to provide evidence for the ability of ascorbate to decrease the turnover of α-TH in various tissue compartments (45). Because lipid radicals formed in lipophilic milieu do not readily partition into the bulk aqueous medium (where ascorbate is already present), it motivated the present comparison of ·NO/α-TH and ascorbate/α-TH inhibition of lipid peroxidation (Table II). The greater efficacy of ·NO/α-TH in protecting α-TH from ABAP-dependent liposome oxidation (Fig. 4) is likely due to the fact that, unlike other key lipid antioxidants (especially α-TH), ·NO can terminate lipid and possibly protein radical species with little or no regard to the spatial orientation of the radical intermediate within membrane or lipoprotein microenvironments. Also, by virtue of its high rate constant for reaction with lipid radical species, ·NO will protect other lipophilic antioxidants from oxidation to maintain tissue antioxidant defenses during periods of oxidant stress. These observations strengthen the concept that ·NO plays a central role in maintaining tissue homeostasis, not only by serving as an endothelial derived mediator of vascular relaxation but also by virtue of its potent lipid antioxidant actions in the absence of O_2^- (11). Lipoprotein oxidation, a critical component of atherogenic processes and defective vascular relaxation, is inhibited by not only α-TH but also chemically derived and cell-derived ·NO as well (12, 23–26). Thus, the recent observations that increased rates of vascular ·NO production are anti-atherogenic and can limit intimal thickening of injured vessels (46, 47) implies contributory antioxidant actions of ·NO via mechanisms reported herein. It still remains important to define the conditions of oxidant stress to membranes and lipoproteins that favor the pro-oxidant actions of ·NO, via O_2^--dependent formation of $ONOO^-$ (48–50), *versus* the presently reported oxidant-protective reactivities of ·NO.

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