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# Reduction of a Nitroxide Spin Label by Native and Partially Oxidized Human Low-Density Lipoprotein

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**The reduction of nitroxide compounds *in vivo* has in most part been assigned to the reaction with reductants such as ascorbic acid, reduced thiols and quinols such as ubiquinol or by reaction with simple carbon-centred radicals. In this study a water-soluble nitroxide, 2,2,5,5-tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl (TPI), was exposed to both native and partially oxidized human low-density lipoprotein ($LDL_n$ and $LDL_{pox}$, respectively) and it was found that TPI decayed in each case to the corresponding hydroxylamine, 2,2,5,5-tetramethyl-4-phenyl-3-oxide-1-hydroxylimidazoline (TPHI). In particular, the reduction of TPI in the presence of $LDL_{pox}$ occurred via a complex mechanism involving the consumption of both α-tocopherol (α-TOH) and cholesteryl linoleate hydroperoxides (Ch18:2-OOH). The EPR signal of the nitroxide also diminished when TPI was exposed to the water-soluble vitamin E analogue, Trolox C, but neither Ch18:2-OOH nor linoleate hydroperoxides alone caused significant decay of the nitroxide signal. Together these results indicate that water-soluble nitroxides may be reduced in circulation by reaction with α-TOH in LDL, thereby adding to the complexity of the reduction of nitroxide spin labels *in vivo*.** © **1997 by John Wiley & Sons, Ltd.**

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## INTRODUCTION

Antioxidant effects of nitroxide spin labels are well established in the literature. For example, nitroxides have been used as antioxidants to trap carbon-centred radicals in polymerization processes,[1] water-soluble nitroxides and their corresponding hydroxylamine derivatives have also been shown to act as potent bioantioxidants[2–5] and nitroxides have been implicated in the inhibition of ischaemia reperfusion damage.[6–8] In addition, the reduction of nitroxides *in vivo* has been used to determine several biologically relevant parameters such as clearance rates in the liver[9,10] and the location of reductive sites of membranes[11] and cells.[12,13] The reduction of spin labels to their corresponding EPR-silent hydroxylamine products *in vivo* (for a comprehensive review, see Ref. 14) together with the reported antioxidant activity of nitroxides has led us to investigate the interaction of the water-soluble nitroxide 2,2,5,5-tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl (TPI) with isolated native and partially oxidized human low-density lipoprotein ($LDL_n$ and $LDL_{pox}$, respectively) to determine whether TPI can interact directly with components of the lipoprotein particle.

*Abbreviations*: ascorbate ($AH^-$); 2,2′-azobis(2-amidopropane) dihydrochloride (AAPH); 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN); α-tocopherol (α-TOH); cholesteryl linoleate hydroperoxides (Ch18:2-OOH); 2,4-dinitrophenylhydrazine (DNPH); electron paramagnetic resonance (EPR); hydroxylamine ($R_2NOH$); lipid peroxyl radicals ($LOO^\cdot$); low-density lipoprotein (LDL); nitroxide ($R_2NO^\cdot$); oxidized LDL ($LDL_{ox}$); native LDL ($LDL_n$); partially oxidized LDL ($LDL_{pox}$); 2,2,5,5- tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl (TPI); 2,2,5,5-tetramethyl-4-phenyl-3-oxide-1-hydroxylimidazoline (TPHI); thiols ($RS^-$); Trolox C (TxOH); ubisemiquinone radical ($QH^\cdot$).

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## EXPERIMENTAL

### Materials

Phosphate buffer (pH 7.4, 50 mM in phosphate) was prepared from Nanopure water and stored over Chelex 100 (Bio-Rad, Richmond, CA, USA) at 4 °C for at least 24 h. This treatment removed contaminating transition metals, as verified by the ascorbate autoxidation method.[15] 2,2′-Azobis(2-amidopropane) dihydrochloride (AAPH) and 2,2′- azobis(2,4- dimethylvaleronitrile) (AMVN) were purchased from Polysciences (Warrington, PA, USA) and were dissolved in freshly prepared phosphate buffer and ethanol, respectively. Trolox C (TxOH) and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Aldrich (Sydney, Australia). Solutions of DNPH were prepared as a 2% (w/v) solution in 3 M hydrochloric acid and were treated with Chelex 100 as described above. α-Tocopherol (α-TOH) was

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obtained as a gift from Henkel (La Grange, IL, USA) and prepared as an 18 mM stock solution in DMSO. Cholesteryl linoleate, EDTA, reduced glutathione and hexacyanoferrate(III) were purchased from Sigma Chemicals (Sydney, Australia). 13-[*S*, (*E*,*Z*)]-hydroperoxyocta-9,11-decadienoic acid (18:2-OOH, purity >98%) was obtained from Sapphire Bioscience (Sydney, Australia). 6-Acrolyl-2-dimethylamino-naphthalene (acrylodan) was purchased from Molecular Probes (Eugene, OR, USA) and prepared as a 1 mM stock solution in dimethylformamide. Ebselen (2-phenyl-1,2-benzoisoselenazole) was a gift from Dr N. Hunt (University of Sydney) and was prepared as a 10 mM stock solution in absolute ethanol. The spin label 2,2,5,5-tetramethyl-4-phenylimidazolin-3-oxide-1-oxyl (TPI) and its corresponding hydroxylamine, 2,2,5,5-tetramethyl-4-phenyl-3-oxide-1-hydroxyimidazoline (TPHI), were gifts from Dr V. Roginsky (Russian Academy of Sciences, Moscow, Russia); see Fig. 1 for compound structures. Centriprep-30 concentrator tubes (molecular weight cut-off 30 000 Da) were purchased from Amicon (Beverly, MA, USA). Authentic samples of cholesteryl linoleate hydroperoxide (Ch18:2-OOH) were prepared as described[16] and stored as a stock solution in ethanol at −20 °C.

## Preparation of native and partially oxidized LDL

Plasma was prepared by centrifuging freshly obtained human-heparinzed blood at 900 × *g* at 4 °C for 15 min. For partially oxidized LDL ($LDL_{pox}$) fresh plasma (10 ml) was enriched with α-TOH by incubating with 400 µl of α-TOH in DMSO (18 mM) at 37 °C for 6 h.[17] LDL was isolated from both control and α-TOH-enriched plasma by rapid density-gradient ultracentrifugation as described.[18] Excess KBr and remaining low molecular weight water-soluble antioxidants were removed by gel filtration chromatography using a PD-10 column (Pharmacia, Uppsala, Sweden). Where required, LDL was concentrated by centrifugation using Centriprep-30 tubes. $LDL_{pox}$ was obtained by allowing the freshly isolated, α-TOH-supplemented LDL to stand at room temperature for 2 h, followed by storage at 4 °C for 48 h. α-TOH-enriched LDL affords LDL, which autoxidizes more readily than native LDL[19] in accordance with lipid peroxidation proceeding via a tocopherol-mediated mechanism.[20] Samples of $LDL_{pox}$ obtained in this fashion were devoid of endogenous ubiquinols and typically contained molar ratios of Ch18:2-OOH/apo protein B of 10–20. The apo protein B content of all LDL preparations was obtained as described.[21]

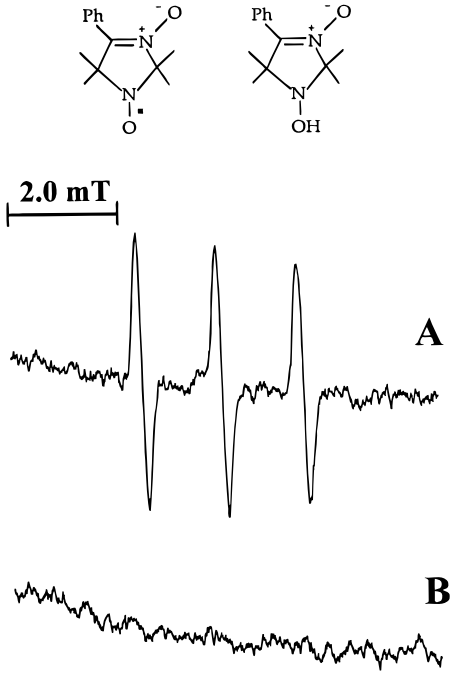


**Figure 1.** EPR signal obtained from addition of 20 µM TPI to hydroperoxide-free LDL (0.7 mg apo protein B $ml^{-1}$) (A) before and (B) after consecutive passage through two gel filtration columns. Hydroperoxide-free LDL was used in this case to minimize the nitroxide decay due to interaction with the lipoprotein. For reference, the structures of TPI and TPHI are shown. EPR spectral parameters: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 12.5 kHz; time constant, 163 s; sweep time, 20 s; number of accumulations, 2.

## Preparation of oxidized, α-tocopherol-free LDL

Oxidized LDL, free of α-TOH ($LDL_{ox}$), was prepared by incubating freshly isolated $LDL_n$ (0.8 mg apo protein B $ml^{-1}$) with the peroxyl radical generator AAPH (50 mM, 37 °C, 1 h). The use of a relatively high peroxyl radical flux affords rapid α-TOH consumption with relatively little lipid peroxidation and only minor alterations to the protein moiety of LDL.[20] Following oxidation, $LDL_{ox}$ was reisolated using ultracentrifugation as described above. Excess KBr and residual AAPH were removed by size-exclusion chromatography. Complete consumption of α-TOH was confirmed by HPLC analysis (see below). LDL oxidation performed using $Cu^{2+}$ was carried out as described.[17]

## Lipid hydroperoxide-free LDL

To obtain hydroperoxide-free LDL, $LDL_{pox}$, containing significant amounts of Ch18:2-OOH, was incubated with ebselen (20 µM) and reduced glutathione (60 mM) at 37 °C for 15 min and subsequently passed through three consecutive PD-10 columns as described by Lynch and Frei.[22] The resulting hydroperoxide-free, α-TOH-containing LDL samples were used immediately upon preparation.

## Sulfhydryl (SH) group capping in LDL

The free thiol groups present in apolipoprotein B-100 were derivatized by incubating LDL (0.3 mg apo protein B $ml^{-1}$) with acrylodan (5 molar excess with respect to apo protein B concentration) at 37 °C, as described by Sommer *et al.*;[23] this treatment may modify lysine residues as well as free SH groups.[24]

## Carbonyl assay of $LDL_{pox}$, $LDL_{ox}$ and $LDL_n$

$LDL_{pox}$, $LDL_{ox}$ and $LDL_n$ (0.2 mg apo protein B $ml^{-1}$) were treated separately with DNPH (final concentration 0.2%, w/v) and analysed for hydrazone adducts ($\varepsilon_{360\,nm}$ 22 000 l $mol^{-1}$ $cm^{-1}$) as described.[25] Solutions of DNPH were carefully treated with Chelex 100 since exposure of $LDL_{pox}$ or $LDL_{ox}$ to untreated DNPH solutions resulted in consumption of hydroperoxides with concomitant formation of an adventitious adduct.

## Analysis of lipid hydroperoxides, α-tocopherol and α-tocopherylquinone

The lipid-soluble components (Ch18:2-OOH, α-TOH and unesterified cholesterol) present in the various LDLs were analysed by HPLC as described.[18] Unesterified cholesterol was employed as the internal stan-

dard for all lipid-soluble components analysed. Determination of α-tocopherylquinone was carried out by HPLC analysis.[26]

### EPR assays

Stock solutions of TPI (100 μM) and LDL (at concentrations given in the figure legends) were mixed rapidly and transferred to a variable-temperature flat cell (Wilmad Glass, Buena, NJ, USA), warmed to 37 °C, and EPR spectra were recorded as a function of time. Unless indicated otherwise, the initial concentration of TPI was 20 μM for all EPR assays. Under these conditions no spin–spin interactions were observed. EPR spectra were obtained at 9.41 GHz using a Bruker ESP 300 EPR spectrometer fitted with an X-band cavity. Temperature control was obtained using a Bruker Temperature Control Unit and temperatures were accurate to ±0.5 °C. Irrespective of whether experiments were performed in aqueous or ethanolic solutions, a sharp, symmetric triplet signal was obtained with nitrogen hyperfine splitting $A^N = 1.51$ mT. Since line height measurements were in good agreement with intensity values as determined by double integration, and the peak widths at half-height remained essentially constant throughout the experiments, line heights of the low-field signal were measured routinely and used as a first approximation of signal intensity.[27] Decay data was normalized against a 20 μM TPI standard (in the absence of LDL) obtained under identical spectrometer conditions, and expressed as a percentage of signal intensity. Peak heights measured from standard samples showed no sign of decay over consecutive accumulations over a 90 min period. EPR analyses were performed at least three times (using different samples of LDL on each occasion) and the figures included are of representative experiments.

## RESULTS

The extent of interaction of TPI with freshly isolated, hydroperoxide-free LDL and oxidized LDL was assessed by following the decay of the nitroxide triplet signal [Fig. 1(A)] by EPR spectroscopy. No other EPR signal was detected during the period the various reaction mixtures were monitored and the triplet signal, assigned to TPI, remained completely symmetrical throughout these studies with nitrogen hyperfine coupling $A^N = 1.51$ mT. No EPR signal was detected after percolating the TPI-treated, hydroperoxide-free LDL through two successive size exclusion columns [Fig. 1(B)], indicating that the water-soluble nitroxide was not incorporated into the lipoprotein.

The rate of decay of EPR signal intensity for TPI in the presence of $LDL_{pox}$ at 37 °C was rapid in the initial stages and slowed over the time period monitored, as shown in Fig. 2. Up to 98% of the initial EPR signal was restored on treatment of $LDL_{pox}$ samples with

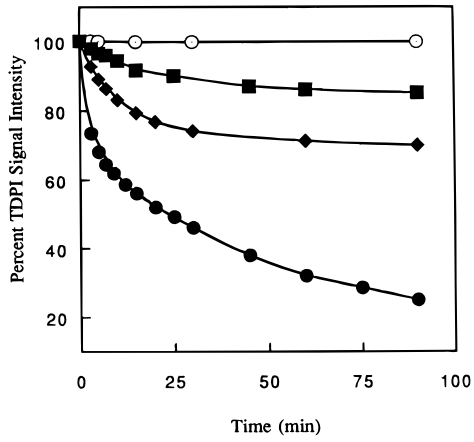


**Figure 2.** EPR signal decay of the spin label TPI (20 μM) at 37 °C in the presence of (○) phosphate buffer alone, (■) $LDL_{ox}$ (1 mg apo protein B $ml^{-1}$, 61 μM Ch18:2-OOH and α-TOH free), (◆) hydroperoxide-free LDL (1 mg apo protein B $ml^{-1}$, 22 μM) α-TOH) and (●) $LDL_{pox}$ (1.1 mg apo protein B $ml^{-1}$, 39 μM α-TOH and 26 μM Ch18:2-OOH). EPR parameters as in Fig. 1. The results shown are representative of 3–5 separate experiments using different LDL preparations.

hexacyanoferrate(III)[28] after 90 min of incubation. Hexacyanoferrate(III) readily converted TPHI into TPI (not shown). Addition of 500 μM EDTA to $LDL_{pox}$ prior to the addition of TPI did not affect either the rate or extent of signal decay compared with TPI treated with $LDL_{pox}$ alone, indicating that contaminating free transition metals in the buffer of the LDL solution did not play a role in the decay of TPI. Furthermore, incubation of TPI in 50 mM phosphate buffer in the absence of $LDL_{pox}$, under identical conditions, did not result in signal decay (Fig. 2). Both the initial rate of decay and the extent of decay were affected by the degree of oxidation of the LDL sample. Thus, freshly isolated, hydroperoxide-free LDL showed intermediate rates and extents of signal decay compared with that of $LDL_{pox}$, while $LDL_{ox}$, completely free of α-TOH and containing 61 μM Ch18:2-OOH, showed extremely slow decay with 85–95% of signal intensity remaining after 90 min (Fig. 2). Under all conditions tested, both the initial rate and the extent of signal decay decreased with decreasing concentrations of LDL (data not shown).

The decay of EPR signal intensity was titrated readily with successive additions of 20 μM aliquots of TPI, as shown in Fig. 3, suggesting that the interaction of TPI with $LDL_{pox}$ was irreversible in nature. Each addition of TPI resulted in the reduction of both the initial rate of decay of EPR signal intensity and the overall extent of decay. LDL free of lipid hydroperoxides and thiol capped showed similar initial rates of signal decay compared with lipid hydroperoxide-free LDL that contained unaltered thiol groups, with the overall extent of decay after 90 min differing by only *ca.* 6%, as shown in Fig. 4. Treatment of samples of $LDL_{pox}$, $LDL_{ox}$ or $LDL_n$ with DNPH gave no resultant DNPH adduct absorbing at 355–370 nm.[25] In contrast, LDL incubated with $Cu^{2+}$ at 37 °C for 8 h, at a molar ratio of $Cu^{2+}$:LDL ≈ 17,[17] gave a distinct DNPH adduct with $\lambda_{max} \approx 360$ nm (data not shown). Together, these results indicated that carbonyl compounds were not present in

 

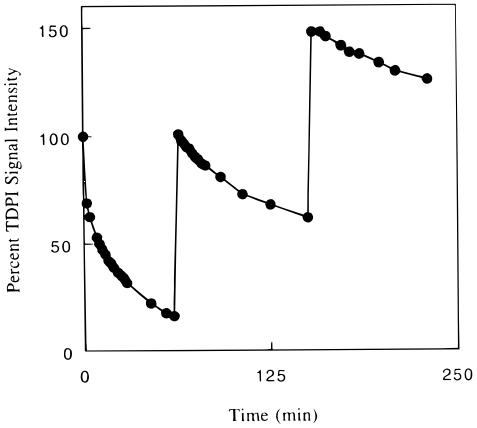


**Figure 3.** Effect of repeated addition of TPI (20 μM) to $LDL_{pox}$ (1.1 mg apo protein B $ml^{-1}$, 39 μM α-TOH and 26 μM Ch18:2-OOH) at 37 °C. EPR parameters as in Fig. 1. The results shown are representative of three separate experiments using different LDL preparations.

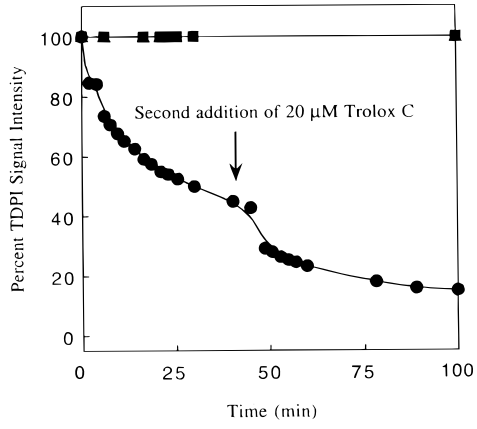


**Figure 5.** EPR signal decay for the spin label TPI (20 μM) at 37 °C in homogeneous solution. TPI was dissolved in phosphate buffer and (●) treated with two successive equimolar additions of Trolox C, or treated separately with ethanolic solutions of either (▲) 45.5 μM Ch18:2-OOH or (■) 40 μM 18:2-OOH. EPR parameters as in Fig. 1. A symmetric triplet signal was obtained for the nitroxide in both aqueous and ethanolic solutions. Note that the data points for ▲ and ■ superimpose. The results shown are representative of three separate experiments using different LDL preparations.

$LDL_{pox}$, $LDL_{ox}$ or $LDL_n$ preparations, and that carbonyl compounds were detected only in LDL oxidized by prolonged exposure to $Cu^{2+}$. The latter is consistent with the transition metal-mediated degradation of lipid hydroperoxides leading to the formation of both aqueous and lipophilic aldehydic lipid products.[29] Collectively, these results suggest that α-TOH was required to afford reduction of the nitroxide and neither free thiol nor carbonyl compounds contributed significantly to the decay of TPI, where the nitroxide was exposed to $LDL_{pox}$, $LDL_{ox}$ or $LDL_n$.

To obtain a further insight into a possible mechanism for the observed decay of the nitroxide, we exposed TPI to individual components of $LDL_{pox}$ in homogeneous solutions as summarized in Fig. 5. TPI treated with an equimolar amount of the water-soluble analogue of α-TOH, Trolox C (TxOH), resulted in a decay profile similar to that observed for TPI treated with $LDL_{pox}$. In contrast, no decay was observed when TPI was treated with either Ch18:2-OOH (45.5 μM) or 18:2-OOH (40 μM) in ethanol.

The initial rate of signal decay for TPI in the presence of 10 μM TxOH increased with increasing pH as shown in Fig. 6. The effect of pH, on the initial rate of reduction of TPI indicates a $pK_a$ value of 11.6 for TxOH, which is in good agreement with the reported value of 11.7–11.9.[30] TxOH was employed at 10 μM in these experiments to slow the initial rate of signal decay, allowing more accurate determination of initial decay rates. In the absence of TxOH, the EPR signal intensity remained constant for TPI at the various pH values indicated (data not shown). No significant decay of signal intensity was observed for TPI treated with a 1–5

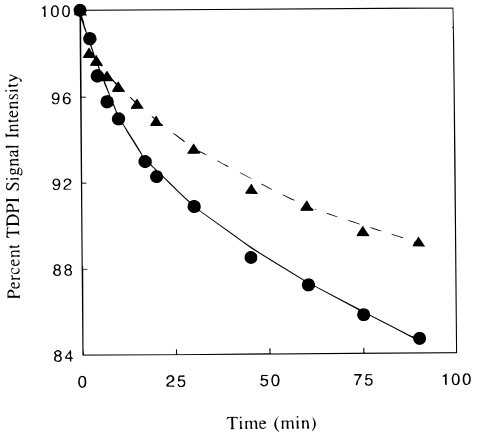


**Figure 4.** Effect of SH capping on EPR signal decay of the spin label TPI (20 μM) at 37 °C in the presence of hydroperoxide-free LDL (1 mg apo protein B $ml^{-1}$, 15 μM α-TOH), (●) not treated and (▲) hydroperoxide-free LDL treated with acrylodan to cap sulfhydryl groups (1 mg apo protein B $ml^{-1}$, 13 μM α-TOH). EPR parameters as in Fig. 1. Note the different scale of the ordinate compared with Fig. 2. The results shown are representative of three separate experiments using different LDL preparations.

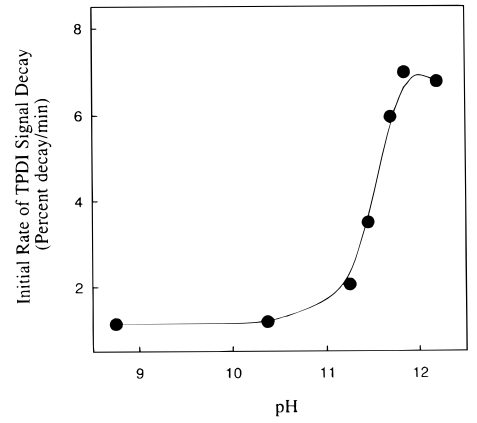


**Figure 6.** Trolox C (10 μM)-induced change in the initial rate of EPR signal decay of TPI (20 μM) is dependent on the pH. The experiments were carried out in buffered solutions at the pH conditions indicated at 37 °C. EPR parameters as in Fig. 1. The results from a single series of experiments are shown.

molar excess of reduced glutathione, used as a model compound for the thiol groups of the apo protein B in $LDL_{pox}$, where the molar ratio of TPI to thiol groups is *ca.* 6:1. However, a slow decay, resulting in the complete consumption of TPI, was achieved by treatment of the spin label with a 1000-fold molar excess of reduced glutathione (data not shown).

In all cases where treatment of the nitroxide resulted in the decay of TPI, no other free radical was observed in the same region of the field, indicating that the treatments employed either did not result in the formation of a $g \approx 2$ radical derived from the reductant, or that the putative radical was below the limit of detection. In an attempt to determine if other radicals were formed during the decay of TPI in the presence of LDL, TPI was treated with α-TOH-enriched and initially lipid hydroperoxide-free LDL. Thus, addition of TPI to LDL (3.1 mg apo protein B $ml^{-1}$ and containing 120 μM α-TOH) resulted in a 38–43% loss of the nitroxide signal intensity during the 90 min period the decay was monitored but, again, no other free radical was detected (data not shown). Experiments using lower initial concentrations of TPI (2–5 μM), employed to reduce the residual nitroxide signal, failed to detect the formation of other organic free radicals.

Incubation of hydroperoxide-free LDL (1 mg apo protein B $ml^{-1}$) with TPI at 37 °C resulted in both increased rates of α-TOH consumption and overall production of Ch18:2-OOH when compared with a corresponding control sample in the absence of TPI (data not shown). Overall, 1.23 μM of α-TOH was consumed, and approximately 0.5 μM Ch18:2-OOH was produced over the 90 min incubation period. Concurrent EPR experiments using identical LDL resulted in 23% decay of signal intensity (data not shown).

Addition of 20 μM TPI to $LDL_{pox}$ resulted in the consumption of 10 μM α-TOH over the time period studied shown in Fig. 7, with less than 4% of the vitamin consumed being converted to α-tocopherylquinone (latter data not shown).

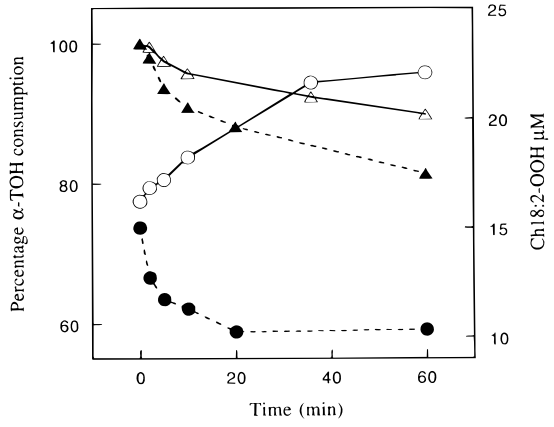


**Figure 7.** Consumption of α-TOH and Ch18:2-OOH during incubation of $LDL_{pox}$ (1.1 mg apo protein B $ml^{-1}$) at 37 °C in the presence (broken line) or absence (solid line) of 20 μM TPI. At the time points indicated aliquots were removed, lipids extracted and analysed for C18:2-OOH (circle), and α-TOH (triangle) as described.[18] The initial α-TOH concentration was 41.2 μM. The results shown are representative of three separate experiments using different LDL preparations.

The fate of the majority of unaccounted α-TOH was not investigated further. In addition to α-TOH, approximately 10 μM Ch18:2-OOH was consumed within the first 20 min of the incubation. Subsequently, the concentration of Ch18:2-OOH remained constant over the period monitored. In the corresponding control sample, incubation of $LDL_{pox}$ in the absence of the TPI resulted in consumption of only 5 μM α-TOH and formation of 6 μM Ch18:2-OOH over the same time period.

## DISCUSSION

Several reactions of nitroxides ($R_2NO^{\bullet}$) in biological systems may result in reduction to the corresponding hydroxylamine product ($R_2NOH$) [e.g. reactions (1)–(4)]. Thus, nitroxides can react with ubisemiquinone radicals ($QH^{\bullet}$),[31] ascorbate ($AH^{-}$),[32,33] thiols ($R'S^{-}$)[28,34] or aldehydes [R′C(O)H], whereby ubiquinone (Q), ascorbyl radical ($A^{\bullet -}$), disulfide (R′SSR′) or reactive carbonyl species [RC(O)•] are formed, respectively:

$$QH^{\bullet} + R_2NO^{\bullet} \rightarrow Q + R_2NOH \quad (1)$$

$$AH^{-} + R_2NO^{\bullet} \rightarrow A^{\bullet -} + R_2NOH \quad (2)$$

$$R'S^{-} + R_2NO^{\bullet} + H^{+} \rightarrow 1/2R'SSR' + R_2NOH \quad (3)$$

$$R'C(O)H + R_2NO^{\bullet} \rightarrow RC(O)^{\bullet} + R_2NOH \quad (4)$$

However, none of these reactions appeared important in the reduction of TPI in the presence of $LDL_{pox}$ since neither ubiquinol, ascorbate nor carbonyls were present in the LDL preparations, and apo protein B's thiols only marginally affected the consumption of the nitroxide (Fig. 4).

The fact that no significant EPR signal decay was observed when $LDL_{ox}$, devoid of α-TOH, was treated with TPI indicated that the nitroxide decay was dependent on the vitamin. In support of this, nitrogen dioxide ($NO_2^{\bullet}$) has been shown to be reduced by the water-soluble vitamin E analogue TxOH [reaction (5)].[30] This reaction is pH dependent, with a faster rate of reaction at higher pH,[30] similar to that of the TxOH-induced decay of TPI shown in Fig. 6. Together these results suggest that the $LDL_{pox}$-induced decay of TPI was most likely initiated by the slow reduction of the nitroxide by α-TOH, with subsequent formation of α-tocopheroxyl radicals (α-$TO^{\bullet}$) and TPHI [reaction (6)].

$$NO_2^{\bullet} + TxOH \rightarrow HNO_2 + TxO^{\bullet} \quad (5)$$

$$TPI + \alpha\text{-}TOH_{LDLpox} \rightarrow TPHI + \alpha\text{-}TO^{\bullet}_{LDLpox} \quad (6)$$

α-$TO^{\bullet}$ produced by reaction (6), could conceivably react with Ch18:2-OOH present in $LDL_{pox}$, resulting in the formation of corresponding lipid peroxyl radicals ($LOO^{\bullet}$) with the regeneration of α-TOH [reaction (−7)], i.e. the reverse of the classical inhibition reaction (7) where α-TOH scavenges $LOO^{\bullet}$ generated *in situ*. Alternatively, $LOO^{\bullet}$ may degenerate to non-radical products (NRP) [reactions (8) and (9)], which would

explain the observed loss of Ch18:2-OOH *and enhanced* consumption of α-TOH during exposure of $LDL_{pox}$ to TPI. A rapid decay of α-TO$^{\cdot}$ in $LDL_{pox}$ exposed to TPI may explain why the chromanoxyl radical was not detected. As the levels of Ch18:2-OOH decreased rather than increased, the termination reactions (8) and (9) would appear to dominate over the lipid peroxidation chain propagating reaction (10) despite the high concentration of polyunsaturated lipid (LH). Perhaps the latter is explained by entrapment of two radicals within an LDL particle favouring bimolecular termination.

$$\alpha\text{-TO}^{\cdot} + \text{LOOH} \rightarrow \alpha\text{-TOH} + \text{LOO}^{\cdot} \quad (-7)$$

$$\alpha\text{-TOH} + \text{LOO}^{\cdot} \rightarrow \alpha\text{-TO}^{\cdot} + \text{LOOH} \quad (7)$$

$$\text{LOO}^{\cdot} + \alpha\text{-TO}^{\cdot} \rightarrow \text{NRP} \quad (8)$$

$$2\text{LOO}^{\cdot} \rightarrow \text{NRP} \quad (9)$$

$$\text{LOO}^{\cdot} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\cdot} \quad (10)$$

The mechanism proposed above is also consistent with the observed net formation of Ch18:2-OOH upon exposure of TPI to hydroperoxide-free LDL (not shown), since α-TO$^{\cdot}$ in LDL, in the absence of biological reductants, can cause lipid peroxidation according to reaction (11).[19,20,35] In the case of $LDL_{pox}$, reaction (−7) (with an estimated rate constant[36] $k_{-7} \approx 0.5$ l mol$^{-1}$ s$^{-1}$ may compete successfully with reaction (11) ($k_{TMP} \approx 0.01$–$0.1$ l mol$^{-1}$ s$^{-1}$),[20,37] leading to the net consumption of Ch18:2-OOH.

$$\alpha\text{-TO}^{\cdot} + \text{LH} \xrightarrow{k_{TMP}} \xrightarrow{O_2} \alpha\text{-TOH} + \text{LOO}^{\cdot} \quad (11)$$

One final pathway may explain the decay of the nitroxide in the presence of the various LDL preparations. Nitroxides are known to react with simple carbon-centred radicals (R$^{\cdot}$) leading to the formation of EPR-silent products [reaction (12)], but are relatively unreactive toward peroxyl radicals.[1]

$$\text{R}^{\cdot} + \text{R}_2\text{NO}^{\cdot} \rightarrow \text{R}_2\text{NOR} \quad (12)$$

The decay of TPI on exposure to either $LDL_n$ or $LDL_{pox}$ is, however, unlikely to be a result of the scavenging of carbon-centred lipid radicals (L$^{\cdot}$), for several reasons. First, the nitroxide must compete with oxygen (present at concentrations *ca.* 200 μM) for the L$^{\cdot}$ produced from autoxidation of esterified polyunsaturated fatty acids either at the surface or in the core of LDL [suggestive that the rate of reaction (12) is close to the diffusion limit]. Second, this mechanism is inconsistent with both the enhanced rate of consumption of α-TOH observed on treatment of TPI with $LDL_{pox}$ (Fig. 7), and the regeneration of up to 98% of the nitroxide signal observed on addition of hexacyanoferrate(III) to $LDL_{pox}$ treated with TPI. Finally, the inability of the water-soluble nitroxide to incorporate into LDL indicates that TPI is most likely incapable of participating in lipid-phase redox reactions.

## CONCLUSION

Our finding that the water-soluble nitroxide TPI decayed in the presence of partially oxidized LDL led us to propose a novel reaction of the nitroxide TPI with the lipoproteins α-TOH, resulting in consumption of both the vitamin and lipid hydroperoxides. If these reactions hold true for other water-soluble nitroxides, it is conceivable that they could contribute to the *in vivo* protective activities observed by these compounds under conditions of oxidative stress.[2–8] A reaction of nitroxides with α-TOH in LDL, and perhaps other vitamin E-containing lipoproteins, could contribute to the reduction of nitroxides in circulation in addition to the well documented reduction of nitroxides to their corresponding hydroxylamine products by ascorbate in plasma.[32,33] This would add to the complexity of the decay of nitroxide spin labels *in vivo*.

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