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Quantitative Studies on the Peroxidation of Human Low-Density Lipoprotein Initiated by Superoxide and by Charged and Neutral Alkylperoxyl Radicals[1]

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Abstract: Rates of peroxidation of human LDL and rates of consumption of the LDL's α-tocopherol (TocH) have been measured at 37 °C. Peroxidation was initiated by radicals generated in the aerated aqueous phase at known rates by thermal decomposition of appropriate precursors: superoxide ($O_2^{\bullet-}$/HOO$^\bullet$) from a hyponitrite and alkylperoxyls (ROO$^\bullet$, two positively charged, one negatively charged and one neutral) from azo compounds. The efficiencies of escape from the solvent cage of the geminate pair of neutral carbon-centered radicals was found to be 0.1, but it was 0.5 for the three charged radicals, a result attributed to radical/radical Coulombic repulsion within the cage. All four alkylperoxyls initiated and terminated tocopherol-mediated peroxidation (TMP) with about equal efficiency and essentially all of these radicals that were generated were consumed in these two reactions. TMP is a radical chain process, and when initiated by the alkylperoxyls, the rate of LDL peroxidation was faster in the early stages while TocH was present than later, after all of this "antioxidant" had been consumed. In contrast, only about 3−4% of the generated superoxide radicals reacted in any measurable fashion with TocH-containing LDL at pH's from 7.6 to 6.5 and peroxidation was much slower than with a similar rate of generation of alkylperoxyls. After all the TocH had been consumed, LDL peroxidation was negligible at pH 7.6 and 7.4, but at pH 6.8 and 6.5, the peroxidation rates showed a large increase over the rates while the TocH had been present. That is, endogenous TocH behaves as an antioxidant in LDL subjected to attack by the physiologically relevant superoxide radical, whereas TocH behaves as a prooxidant in LDL subjected to attack by the probably far less physiologically important alkylperoxyls. Rates of LDL peroxidation initiated by superoxide increased as the pH was decreased, and the results are consistent with the initiation of peroxidation of fresh LDL occurring via H-atom abstraction from TocH by HOO$^\bullet$ to form the Toc$^\bullet$ radical and termination by reaction of $O_2^{\bullet-}$ with Toc$^\bullet$, a process that occurs partly by addition leading to TocH consumption and partly by electron plus proton transfer leading to the regeneration of TocH.

The primary step in the cascade of pathobiological processes that lead to atherosclerosis is generally agreed to be the uptake of oxidatively damaged low-density lipoprotein (LDL) by macrophages with consequent conversion of the macrophages into foam cells.[3−8] A typical LDL particle contains ∼1200 readily peroxidizable polyunsaturated fatty acid (PUFA) moieties, mainly linoleate, and between 5 and 12 molecules of

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α-tocopherol (TocH).[4,9] TocH is the most active form of vitamin E and is, quantitatively, the major lipid-soluble, radical-trapping antioxidant in human blood[10] and in LDL.[9] In all early studies of LDL peroxidation, the rate at which free radicals were generated, R_g (by, e.g., addition of Cu(II)[4]) was unknown and uncontrolled. It was simply assumed that TocH functioned as a peroxyl radical trapping antioxidant, breaking two oxidation chains as is the case in homogeneous solutions of lipids[10] and in aqueous lipid dispersions such as micelles[11] and liposomes,[12,13] reactions 1−6. In these equations, LH represents a

$$\text{initiator} \rightarrow \text{R}^{\bullet} \xrightarrow{\text{O}_2} \text{ROO}^{\bullet} \ (\text{rate} = R_g) \tag{1}$$

$$\text{ROO}^{\bullet} + \text{LH} \rightarrow \text{ROOH} + \text{L}^{\bullet} \ (\text{rate} = R_i) \tag{2}$$

$$\text{L}^{\bullet} + \text{O}_2 \xrightarrow{\text{fast}} \text{LOO}^{\bullet} \tag{3}$$

$$\text{LOO}^{\bullet} + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}^{\bullet} \tag{4}$$

$$\text{LOO}^{\bullet} + \text{TocH} \xrightarrow{k_{inh}} \text{LOOH} + \text{Toc}^{\bullet} \tag{5}$$

$$\text{LOO}^{\bullet} + \text{Toc}^{\bullet} \xrightarrow{\text{fast}} \text{nonradical products} \tag{6}$$

bisallylic methylene moiety, $(-CH{=}CH)_2CH_2$, in a PUFA and R_i is the rate of initiation of peroxidation chains. In homogeneous systems, under most conditions, 50% of the radicals generated ($ROO^{\bullet}$ or $LOO^{\bullet}$) will react with the TocH to produce $Toc^{\bullet}$ radicals and the other 50% will be trapped by the $Toc^{\bullet}$ to form nonradical products and destroy TocH. Thus, R_g and R_i will be equal and can be determined by measuring the rate of consumption of TocH, i.e.,

$$R_g = R_i = 2\ \text{d[TocH]/d}t \tag{7}$$

The rate of a TocH inhibited homogeneous peroxidation, R_p^{inh}, is given by

$$R_p^{inh} = \text{d[LOOH]/d}t = R_i(k_p/2k_{inh})[\text{LH}]/[\text{TocH}] = R_g(k_p/2k_{inh})[\text{LH}]/[\text{TocH}] \tag{8}$$

and the kinetic chain length in the presence of TocH, ν_{TocH}, by

$$\nu_{TocH} = R_p^{inh}/R_i = R_p^{inh}/R_g = (k_p/2k_{inh})[\text{LH}]/[\text{TocH}] \tag{9}$$

The two rate constants are known: $k_p \sim 30\ M^{-1}\ s^{-1}$;[14] $2k_{inh} \sim 3 \times 10^6\ M^{-1}\ s^{-1}$.[10] In an LDL particle there are ~1450 LH moieties and ⩾5 TocH molecules.[9] The expected chain length would therefore be

$$\nu_{TocH} \leq (30/3 \times 10^6)(1450/5) \leq 0.003 \tag{10}$$

That is, LDL is not expected to undergo peroxidation in a chain reaction since, by definition, a chain reaction has $\nu > 1$.

This "expectation" is not fulfilled, as was discovered when a water-soluble azo compound, 2,2′-azobis(amidinopropane) dihydrochloride, ABAP (or AAPH), was used to initiate LDL peroxidation,[9,15−18] reaction 11. For this compound, R_g could

$$\underset{\text{ABAP(AAPH)}}{[(H_2N)_2{}^{+}CC(CH_3)_2N{=}]_2} \xrightarrow[k_{11}]{\Delta} 2e\ \underset{2e^{+}\text{A}^{\bullet}}{(H_2N)_2{}^{+}CC(CH_3)_2{}^{\bullet}} \xrightarrow{\text{O}_2} \underset{2e^{+}\text{AOO}^{\bullet}}{2e\ (H_2N)_2{}^{+}CC(CH_3)_2OO^{\bullet}} \tag{11}$$

be calculated via the general equation

$$R_g = 2ek_i[\text{RN}{=}\text{NR}] \tag{12}$$

where e is the efficiency of cage escape of the geminate pair of $^{+}A^{\bullet}$ radicals and $k_i = k_{11}$ for [RN=NR] = [ABAP], since e and k_i were known for ABAP (see below). Surprisingly, the peroxidation of LDL at low R_g was found to proceed by a chain reaction since the chain lengths based on R_g, i.e., R_p^{inh}/R_g, were >1. Even more surprisingly, the rates of peroxidation were faster in TocH-containing LDL than in the same LDL particles after all the TocH had been consumed and they were also faster the richer the starting LDL was in TocH. The occurrence of a chain reaction was attributed[15] to the reaction of $Toc^{\bullet}$ radicals with LH:

$$\text{Toc}^{\bullet} + \text{LH} \xrightarrow{k_{TMP}} \text{TocH} + \text{L}^{\bullet} \tag{13}$$

and the increase in R_p^{inh} with increasing TocH in the LDL was attributed to the ability of TocH to transfer radical character from the aqueous phase ($^{+}AOO^{\bullet}$) into the LDL particle ($Toc^{\bullet}$). The three-step peroxidation chain, which sequentially involves reactions 13, 3, and 5, has been christened tocopherol-mediated peroxidation (TMP), and in the simplest kinetic analysis, the rate of TMP is given by[9]

$$R_{TMP} = R_p^{inh} = (n/2)k_{TMP}[\text{LH}] \tag{14}$$

where n is the number of LDL particles, [LH] refers to the concentration of bisallylic methylene groups in an LDL particle (~0.8M),[9] and $k_{TMP} \sim 0.1\ M^{-1}\ s^{-1}$.[15]

ABAP(AAPH) was introduced in 1984 for quantitative kinetic studies of lipid peroxidation in aqueous dispersions.[19,20] All studies of TMP in LDL using a source of water-soluble peroxyl radicals to initiate the reaction with known and controlled R_g would appear to have relied on this same azo initiator. However, $^{+}AOO^{\bullet}$ is not a biomimetic peroxyl radical because it is *positively* charged whereas most peroxyl radicals likely to be formed in vivo will be negatively charged or neutral.[21] Furthermore, we recently demonstrated that the charge on a peroxyl radical can have profound consequences in biological systems: positively charged water-soluble peroxyls readily cleave double-stranded DNA whereas neutral and negatively charged water-soluble peroxyls do not induce strand scission.[21] This difference was attributed to Coulombic attraction between the DNA polyanion and the positively charged peroxyls. Since

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LDL has a negative ζ-potential, the initiation of TMP in LDL by $^{+}$AOO$^{\bullet}$ might be much more facile than by negatively charged and neutral peroxyls, just as is the case for DNA strand cleavage. An investigation of this matter is reported herein using ABAP, 3,3′-azobis(3-cyano-1-butanesulfonic acid) disodium salt as a source of negatively charged water-soluble *tert*-alkylperoxyls, $^{-}$BOO$^{\bullet}$ (reaction 15),

$$[^{-}O_3SC_2H_4C(CH_3)(CN)N{=}]_2 \xrightarrow[k_{15}]{\Delta} \xrightarrow{O_2} {}^{-}O_3SC_2H_4C(CH_3)(CN)OO^{\bullet} \quad {}^{-}BOO^{\bullet} \qquad (15)$$

2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide] as a source of neutral water-soluble *tert*-alkylperoxyls, COO$^{\bullet}$ (reaction 16),

$$[HOC_2H_4NHC(O)C(CH_3)_2N{=}]_2 \xrightarrow[k_{16}]{\Delta} \xrightarrow{O_2} HOC_2H_4NHC(O)C(CH_3)_2OO^{\bullet} \quad COO^{\bullet} \qquad (16)$$

and 2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine) dihydrochloride as a second source of positively charged *tert*-alkylperoxyls, $^{+}$DOO$^{\bullet}$ (reaction 17):

$$[\overline{HN(CH_2)_2NH}CC(CH_3)_2N{=}]_2^{2+} \xrightarrow[k_{17}]{\Delta} \xrightarrow{O_2} [\overline{HN(CH_2)_2NH}CC(CH_3)_2OO^{\bullet}]^{+} \quad {}^{+}DOO^{\bullet} \qquad (17)$$

Of course, very few water-soluble alkylperoxyl radicals will be formed in vivo relative to the amounts of the superoxide radical anion, $O_2^{\bullet-}$, and its conjugate acid, HOO$^{\bullet}$. In quantitative terms, these are the major radicals produced in living organisms (estimates suggest ∼10 kg/adult per year). Previous studies on superoxide-mediated LDL peroxidation in cell-free systems have employed γ-radiolysis[22−25] and the aerobic xanthine oxidase (XO)/hypoxanthine[26] or XO/xanthine[27,28] couples to generate this radical. In none of this work was TMP observed. Furthermore, in the three publications that addressed the matter,[22−24] TocH was found to act as an antioxidant giving a well-defined lag phase prior to the accumulation of lipid oxidation products. These results could have been due to high R_g in these experiments since TMP induced by ABAP is only observed at low R_g.[9] Indeed, TocH becomes an antioxidant at high R_g.[9] However, under physiological conditions, the superoxide dismutases (SODs) ensure that steady-state concentrations of $O_2^{\bullet-}$/HOO$^{\bullet}$ are extremely low, and therefore, if these radicals can oxidize TocH to Toc$^{\bullet}$ in LDL, then TMP might occur and the TocH would function as a prooxidant. For thermodynamic reasons, only HOO$^{\bullet}$ could oxidize the TocH and start TMP.

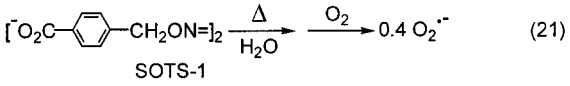
$$HOO^{\bullet} + TocH \rightarrow H_2O_2 + Toc^{\bullet} \quad (\Delta H \approx -11 \text{ kcal/mol}) \qquad (18)$$

$$O_2^{\bullet-} + TocH \not\rightarrow HOO^{-} + Toc^{\bullet} \quad (\Delta H \approx 17 \text{ kcal/mol}) \qquad (19)$$

However, we considered it possible that $O_2^{\bullet-}$ might, at least to some extent, reduce Toc$^{\bullet}$ and regenerate TocH (reaction 20),

$$O_2^{\bullet-} + Toc^{\bullet} + H^{+} \rightarrow O_2 + TocH \qquad (20)$$

in which case superoxide would be more likely to serve as an anti-TMP agent than as a TMP promotor. Thus, the physiologically significant question remains: At low R_g, does superoxide induce TMP in LDL or does it (together with TocH) function as an LDL antioxidant? To answer this question, we have employed our novel thermal source of superoxide, bis(4-carboxybenzyl) hyponitrite (SOTS-1). This compound generates



SOTS-1 (21)

superoxide at a known, slow and steady rate under physiological conditions, pH ∼7, 37 °C.[29, 30]

Results

Efficiencies of *tert*-Alkylperoxyl Radical Generation Formed by Thermolysis of the Azo Initiators in Aerated Water at 37 °C. A proper comparison of any effect of charge on the ability of an alkylperoxyl radical to initiate TMP in LDL requires that the radicals be generated at identical rates from each of the four azo compounds we employed. We previously reported[21] the rate constants for decomposition of all four azo compounds at 37 °C in D_2O (pD∼7), i.e., k_{11}, k_{15}, k_{16}, and k_{17}, and in initial experiments on LDL it was simply assumed that the efficiency of cage escape of the geminate radical pairs from each azo compound, *e*, would be 0.5, a value that is well establish for ABAP, reaction 11.[19] However, as the experiments progressed, it became clear that *e* for the azo compound giving neutral peroxyls (reaction 16) was considerably smaller than the *e* values for azo compounds giving charged peroxyls, for which the *e* values appeared to be similar (see, for example, Figure S1, Supporting Information). The obvious possibility was that the neutral azo compound partitioned into the LDL where *e* would be much reduced relative to its value of ∼0.5 in a homogeneous, low-viscosity solvent; e.g., at 37 °C the lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) has an *e* of only ∼0.05 in LDL[9] and ∼0.1 in multilamellar vesicles.[31] This possibility was, however, eliminated by showing that detectable (UV−visible) amounts of the neutral azo compound could not be extracted from aqueous solutions into hexane, toluene, chloroform, diethyl ether, or olive oil (data not shown).

The efficiencies of cage escape were therefore directly determined by monitoring the consumption of *p*-cresol using azo-initiator concentrations that were roughly "matched" to ensure that roughly the same amount of each compound decomposed per unit period of time. The rates of consumption of *p*-cresol were essentially identical for the three charged azo compounds, but consumption was 5 times slower with the neutral azo compound (see Figure 1). Since *e* for ABAP is 0.5,[19] it must also

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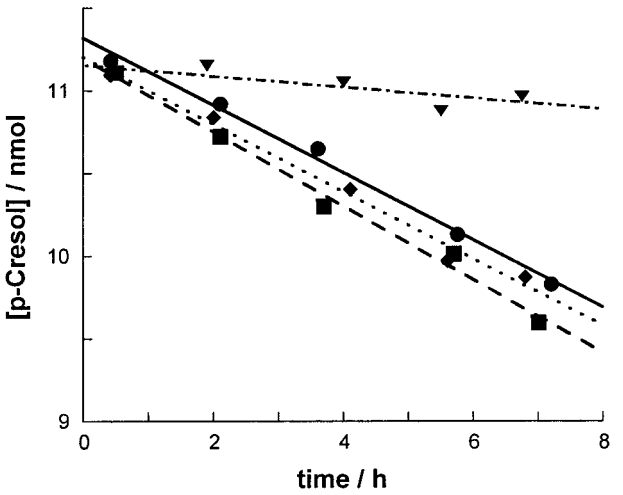

Figure 1. Consumption of *p*-cresol during incubation at 37 °C in PBS (50 mM, pH 7.4) with different azo initiators. Key: (●, —) $^{+}$AOO$^{\bullet}$ from 8.2 mM ABAP; (◆, •••) $^{+}$DOO$^{\bullet}$ from 1.7 mM (2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine); (■, ---) $^{-}$BOO$^{\bullet}$ from 34 mM 3,3′-azobis-(3-cyano-1-butanesulfonic acid); (▼, •-•-) COO$^{\bullet}$ from 60 mM 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide]. Lines are linear regression of the data points.

Table 1. Kinetic Data for the Decomposition of Some Azo Compounds Yielding Water-Soluble Peroxyl Radicals at 37°C in D_2O (pD ~7)

initiator (peroxyl)	$10^6 k_i$ / s^{-1} [a]	e [b]
$[(H_2N)_2^+CC(CH_3)_2N=]_2$ ($^{+}$AOO$^{\bullet}$) (ABAP)	k_{11} = 1.3	0.5[c]
$[^-O_3SC_2H_4C(CH_3)(CN)N=]_2$ ($^{-}$BOO$^{\bullet}$)	k_{15} = 0.35	0.5[d]
$[HOC_2H_4NHC(O)C(CH_3)_2N=]_2$ (COO$^{\bullet}$)	k_{16} = 0.18	0.1[d]
$[HN(CH_2)_2NHCC(CH_3)_2N=]_2^{2+}$ ($^{+}$DOO$^{\bullet}$)	k_{17} = 5.2	0.5[d]
$[^-O_2C$–C$_6$H$_4$–$CH_2ON=]_2$ ($^{-}$OO$^{\bullet}$/HOO$^{\bullet}$) (SOTS-1)	k_{21} = 150	0.2[e]

[a] See ref 21. [b] Efficiency of cage escape of the geminate radical pair. [c] Reference 19. [d] This work. [e] References 29 and 30.

be 0.5 for the other two charged azo compounds, but it must be only 0.1 for the neutral compound. The kinetic data required to carry out correctly "matched" LDL peroxidation experiments with the azo compounds are summarized in Table 1.

TMP of LDL Initiated by *tert*-Alkylperoxyl Radicals. The theory of TMP predicts that the rate of LDL peroxidation in the presence of TocH, R_p^{inh}, should be independent of the rate of radical generation, R_g, and rate of initiation, R_i.[9,18,32] For analytical simplicity, we measured only the rate of formation of cholesteryl ester hydroperoxides, CEOOH. Since CEOOH represents ~75% of the hydroperoxides formed in TocH-containing LDL during TMP,[9] values of R_p^{inh} have been calculated throughout via eq 22.

$$R_p^{inh} = (4/3)(d[CEOOH]/dt) \qquad (22)$$

In preliminary experiments, using various LDL preparations in aerated phosphate-buffered saline (PBS, 50 mM, pH 7.4) at 37 °C, it was found that all four azo compounds induced TMP

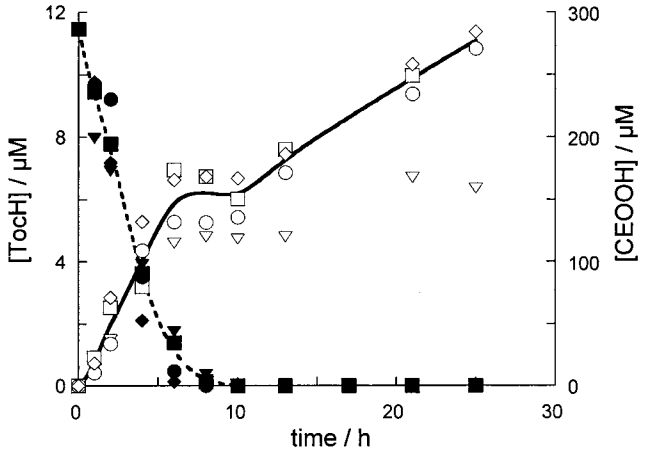

Figure 2. Formation of CEOOH (open symbols) and TocH depletion (filled symbols) during the incubation of LDL (1 μM) with different azo initiators at 37 °C in aerated PBS (50 mM, pH 7.4). Key: (●, ○) 1 mM ABAP, calculated R_g ($^{+}$AOO$^{\bullet}$) = 1.3 nM s^{-1}; (◆, ◇) 0.23 mM of the other positively charged azo compound, calculated R_g ($^{+}$DOO$^{\bullet}$) = 1.2 nM s^{-1}; (■, □) 4.2 mM of the negatively charged azo compound, calculated R_g ($^{-}$BOO$^{\bullet}$) = 1.5 nM s^{-1}; (▼, ▽) 43 mM of the neutral azo compound, calculated R_g (COO$^{\bullet}$) = 1.5 nM s^{-1}. Lines are only a visual aid and do not include the data for the incubation of LDL with the neutral azo compound.

because peroxidation occurred via a chain reaction at low R_g's, i.e., $\nu_{TocH} > 1.0$. Furthermore, provided R_p^{inh} reached its maximum value within the incubation time (which did not happen at very low R_g even with a 160-min incubation), the values of R_p^{inh} for a particular azo compound and particular LDL preparation were equal within experimental error. However, in these preliminary experiments, the (limiting) R_p^{inh} values normalized to 1.0 μM LDL for the four azo compounds ranged from ~3 to ~17 nM s^{-1}. (These and some of the other kinetic data obtained using *tert*-alkylperoxyl initiators are summarized in Table S1, Supporting Information.)

Since the theory of TMP predicts that R_p^{inh} values for the same LDL preparation should be independent of R_g, it would be expected that R_p^{inh} would be the same for all four azo compounds. The large range of R_p^{inh} values found in these preliminary experiments seemed likely to be due to the use of different LDL preparations. We therefore carried out "matched" experiments using the same LDL preparation in aerated PBS (pH 7.4) at 37 °C and approximately the same R_g for all four azo compounds. These results are presented graphically in Figure 2. The (four) CEOOH formation curves all display an early rate maximum which declines precipitously when all the TocH has been consumed, and this, in turn, is followed by a faster rate but a rate that is not as high as when TocH was present. These [CEOOH] versus time profiles are characteristic of TMP of LDL.[9] More interestingly, the rates of CEOOH accumulation while the TocH was present were equal and the rates of TocH consumption were equal for the four azo compounds, at least within our likely experimental errors. Under the conditions of these four experiments, the calculated values of R_g (eq 12) ranged from 1.2 to 1.5 nM s^{-1} (see Figure 2 caption and Table S1) and an initial concentration of TocH equal to 11.5 μM was consumed in a zero-order reaction in ~5.8 h (based on extrapolation of the linear portion of the TocH decay curve shown in Figure 2). The calculated value of R_i (eq 7) is:

$$R_i \approx 2 \times 11.5 \times 10^{-6}/5.8 \times 60 \text{ x } 60 \text{ M s}^{-1} \approx 1.1 \text{ nM s}^{-1}$$

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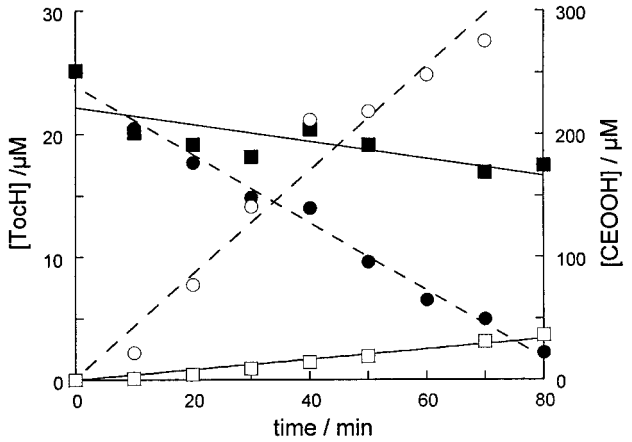


Figure 3. Formation of CEOOH (open symbols) and TocH depletion (filled symbols) during the incubation of LDL (3.7 μM) with (■, □) 0.2 mM SOTS-1, calculated R_g ($O_2^{\bullet-}$) = 12 nM s^{-1}, or (●, ○) 10 mM ABAP, calculated R_g ($^+AOO^\bullet$) = 13 nM s^{-1}, at 37 °C in aerated PBS (50 mM, pH 7.4). Lines are only a visual aid.

and hence R_i/R_g varies from ~0.7 to ~0.9; cf., lower values of 0.28 to 0.55 reported earlier for this ratio.[9] We have no explanation for this difference. However, for practical purposes, we can write

$$R_i \approx 0.8R_g \tag{23}$$

for LDL peroxidation (TMP) initiated by all four water-soluble alkylperoxyls (whereas $R_i \approx R_g$ for the peroxidation of lipids containing TocH in homogeneous solution, eq 7). During TMP, the rate of accumulation of CEOOH (taken from the line shown in Figure 2) is ~7.1 nM s^{-1} which yields (via eq 22)

$$R_p^{inh} = 4 \times 7.1/3 = 9.5 \text{ nM s}^{-1}$$

Thus, the chain lengths, R_p^{inh}/R_i during TMP in these four experiments was ~9.5/1.1 = 8.6.

Peroxidation of LDL Initiated by *tert*-Alkylperoxyl Radicals in the Post-TMP Period. After consumption of TocH in the four matched experiments described above, i.e., $t > 10$ h, the rates of CEOOH formation were approximately equal for initiation with the three charged alkylperoxyls and were significantly greater than for initiation with the uncharged peroxyl (see Figure 2).

Peroxidation of LDL Initiated with Superoxide. Preliminary experiments at pH 7.4 revealed that SOTS-1-derived superoxide was very much less efficient at promoting the peroxidation of TocH-containing LDL than were the four *tert*-alkylperoxyl radicals. Because the half-life of SOTS-1 (80 min) is much shorter than the half-lives of any of the four azo compounds used in this work to generate *tert*-alkylperoxyls,[33] an experiment was carried out using the same LDL preparation with SOTS-1 and ABAP concentrations "matched" to produce roughly the same initial R_g of ~12 nM s^{-1}. This experiment was run in PBS (pH 7.4) at 37 °C for just 80 min, and the results are presented in Figure 3. The rate of CEOOH formation is roughly 12 times greater with ABAP than with SOTS-1, and the rate of TocH consumption is also much larger for ABAP than for SOTS-1.

The LDL used in the experiment shown in Figure 3 was relatively concentrated (3.7 μM), and as a consequence, the initial TocH concentration was high (25 μM). Since a low concentration of SOTS-1 (0.2 mM) was employed in this experiment, only a small fraction of the initial TocH was consumed, too small an amount to estimate R_i reliably. Therefore, more dilute LDL (0.6 μM) was employed in some subsequent experiments which had an initial TocH concentration of 5.5 μM. The reaction of this LDL with SOTS-1 (0.6 μM initially and made to that concentration again at 80 min) produced a complete, or almost complete, consumption of TocH at various pH's in ≥150 min (see Figure 4, bottom panel). Similar experiments were carried out at various pH's using 0.6 μM SOTS-1 and another sample of LDL (0.6 μM) containing 4.5 μM TocH (Figure 4, top panel). From the *initial* rates of TocH consumption in these and other experiments, values of R_i were determined (eq 7). From the readily calculated values for R_g (see Table 1), the mean values of R_i/R_g were calculated to be 0.033, 0.034, 0.037, and 0.039 at pH 7.6, 7.4, 6.8, and 6.5, respectively. Thus, only *about 3−4% of the superoxide radicals that were generated reacted in any measurable fashion with the LDL* in the pH range from 7.6 to 6.5, and over this range of pH's, R_i/R_g increased by a factor of 1.2.

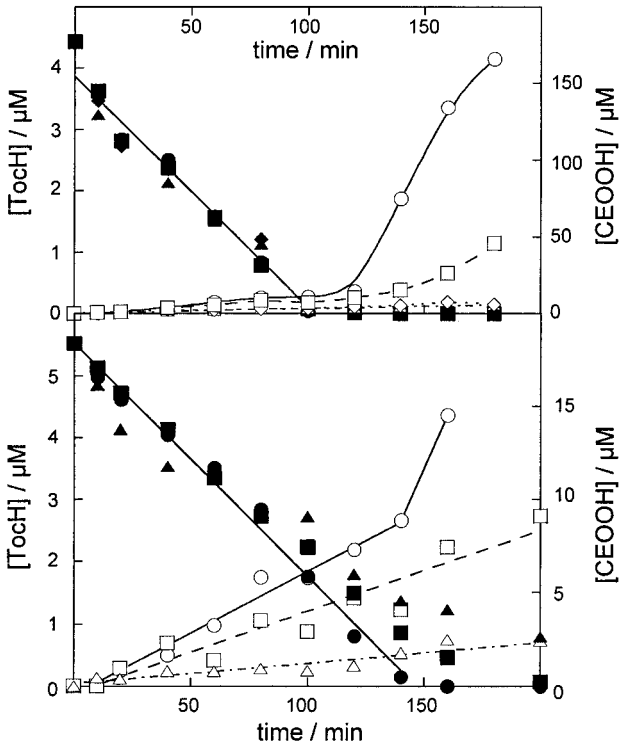


Figure 4. Formation of CEOOH (open symbols) and TocH depletion (filled symbols) during the incubation of with SOTS-1 (0.6 mM) at 37 °C in aerated PBS (50 mM) at different pHs of two different samples of LDL (both 0.6 μM) containing initial TocH concentrations of 4.5 (top panel) and 5.5 μM (bottom panel). After 80-min incubation the reaction solution was diluted 1:1 with a "fresh" SOTS-1 solution (0.9 mM) to obtain a [SOTS-1] identical to the initial concentration. Key: (▲, △) pH 7.6; (◆, ◇) pH 7.4; (■, □) pH 6.8; (●, ○) pH 6.5. Lines are only a visual aid.

Over the pH range 7.6−6.5 there was also an increase in R_p^{inh} as the pH decreased; e.g., R_p^{inh}/nM s^{-1} = 0.24, 0.35, 0.97, and 1.5 at pH 7.6, 7.4, 6.8, and 6.5, respectively, (Figure 4, bottom panel) corresponding to an overall increase by a factor of 6.3. In the other, similar set of experiments, R_p^{inh}/nM s^{-1} = 0.67, 0.69, 1.9, and 1.9 at the same pH's for an overall increase by a factor of 4.0 (see Figure 4, top panel and Table S2, Supporting Information). In all experiments at pH 7.6 and 7.4, the initial chain lengths in the presence of TocH, ν_{TocH}(=R_p^{inh}/

(33) Half-lives (h): ABAP 148; azobis(3-cyano-1-butanesulfonic acid) 550; azobis[2-methyl-*N*−(2-hydroxyethyl)propionamide] 1070; azobis-(dimethyleneisobutyramidine) 37.

R_i) were <1.0, but at lower pH's, ν_{TocH} rose to slightly above 1.0, e.g., 1.2 and 1.9 in two experiments at pH 6.5 (see Table S2). Despite the fact the ν_{TocH} was <1.0 at pH 7.4, values of R_p^{inh}/nM s^{-1} were 0.33, 0.43, and 0.41 at R_g/nM s^{-1} = 12, 26, and 44, respectively. That is, R_p^{inh} appears to be independent of R_g, which accords with the theory of TMP (eq 14) and not with the conventional picture of TocH-inhibited lipid peroxidation (eq 8).

Confirmation that superoxide was initiating LDL peroxidation was obtained in experiments using a single LDL preparation and a SOTS-1 concentration that gave an initial R_g = 33 nM s^{-1}. Incubation at 37 °C for 3 h (to ensure that most of the SOTS-1 had decomposed) gave 2.4 μM CEOOH. Carrying out the same experiment in the presence of 25 units of SOD gave only 0.06 μM CEOOH (i.e., 2.5% of the no-SOD yield). A similar experiment with 4560 units of catalase (CAT) gave 0.23 μM CEOOH (i.e., 9.6% of the no-CAT yield). Blank experiments with no SOTS-1 present gave no CEOOH in the presence of the same amount of SOD and 0.15 μM CEOOH in the presence of the CAT.

A related set of experiments were carried out using the traditional aerobic xanthine oxidase/acetaldehyde (XO/AA) couple to generate superoxide at the same initial R_g (33 nM s^{-1}). After a 3-h incubation, the yield of CEOOH was 1.0 μM. This yield was much less influenced by the addition of 25 units of SOD (0.55 μM, 55%) or 4560 units of CAT (0.61 μM, 61%) than was the case for superoxide generated from SOTS-1.

In the experiments with LDL and SOTS-1, it was observed that after the TocH had been consumed the rate of formation of CEOOH also increased as the pH was decreased from 6.8 to 6.5. To explore this phenomenon over a wider pH range than was possible with LDL,[34] experiments were carried out using 0.46 mM SOTS-1 to initiate the peroxidation of 0.72 mM 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC)/ 0.97 mM sodium cholate mixed micelles. After 80-min incubation at 37 °C, the yields of phosphatidylcholine hydroperoxides (PCOOH) were 0.0, 1.2, 5.6, 8.8, 10.8, and 16.4 μM at pH's of 10, 9, 8, 7.4, 7, and 6, respectively. The yields of PCOOH at pH 7.4 were reduced to 0.32 (3%) and 0.48 (4%) μM in the presence of 8 units of SOD and 3890 units of CAT, respectively.

In those SOTS-1/LDL experiments at pH 6.8 and 6.5, which were continued until all the TocH had been completely consumed, the rate of CEOOH formation became much greater than it had been while the TocH was still present (see Figure 4 and Table S2). Thus, *TocH behaves as an antioxidant for the superoxide-initiated peroxidation of LDL*, which contrasts sharply with its role as a prooxidant in alkylperoxyl radical-initiated LDL peroxidation. Furthermore, in these SOTS-1/LDL experiments, the rate of formation of CEOOH after the TocH-induced induction period increased as the pH decreased (see Figure 4 and Table S2).

The difference in the behavior of $^{+}AOO^{\bullet}$ and $O_2^{\bullet-}$ was also observed using LDL that had been depleted of >95% of its initial TocH. With 10 mM ABAP, the [CEOOH] increased from its initial 13 μM to 151 μM after 80-min incubation at pH 7.4 and 37 °C, whereas incubation with 0.23 mM SOTS-1 for the same length of time produced little or no increase in [CEOOH] (see Figure 5).

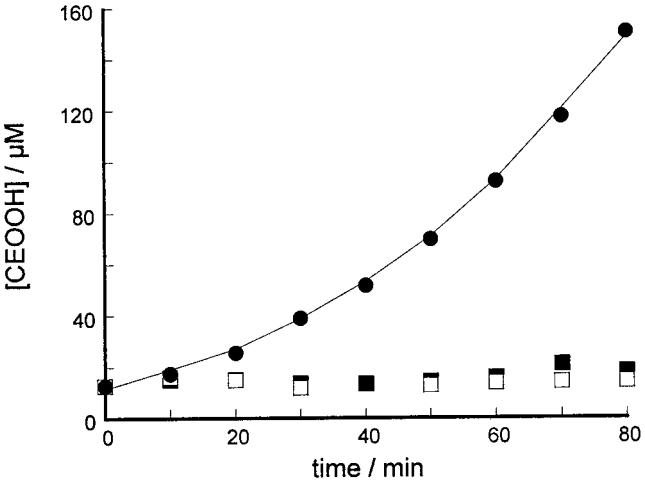
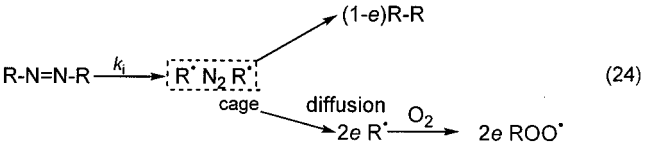


Figure 5. Formation of CEOOH during the incubation of LDL (0.3 μM) artificially depleted of TocH (□) alone, with (■) SOTS-1 (0.23 mM) or (●) ABAP (10 mM) at 37 °C in aerated PBS (50 mM, pH 7.4).

Discussion

LDL Peroxidation Initiated by *tert*-Alkylperoxyl Radicals. The importance of electrostatic charge on alkylperoxyl radical reactions in water was established when we demonstrated that two positively charged peroxyls ($^{+}AOO^{\bullet}$ and $^{+}DOO^{\bullet}$) cleaved double-stranded DNA and that a negatively charged peroxyl ($^{-}BOO^{\bullet}$) and a neutral peroxyl ($COO^{\bullet}$) were ineffective in this regard.[21] We have now discovered that the presence of charge, whether positive or negative, greatly encourages the geminate pair of carbon-centered radicals formed during the thermal decomposition of an azo compound to escape from the solvent cage in which they were generated. The overall process can be represented with $R_g = 2e\ k_i$[RN=NR] (eq 12). When both $R^{\bullet}$



(24)

are neutral, i.e., $C^{\bullet}$, e = 0.1. However, when both $R^{\bullet}$ are positively charged, i.e., $^{+}A^{\bullet}$ and $^{+}D^{\bullet}$, and when both $R^{\bullet}$ are negatively charged, i.e., $^{-}B^{\bullet}$, e = 0.5 (see Figure 1 and Table 1). These large increases in e relative to the value for the neutral geminate radical pair are clearly a consequence of Coulombic repulsion between the two similarly charged $R^{\bullet}$ in the solvent cage.

In "matched" experiments in which a single preparation of LDL was subjected to attack by the four peroxyl radicals, $^{+}AOO^{\bullet}$, $^{-}BOO^{\bullet}$, $COO^{\bullet}$, and $^{+}DOO^{\bullet}$, generated at roughly the same rate it was found (Figure 2) that all four azo initiators gave hydroperoxide (CEOOH) accumulation curves that had the shape characteristic of TMP in LDL. In these experiments, while the TocH was still present, the rates of CEOOH accumulation and the rates of TocH depletion were the same for all four azo initiators. During this time the lipid hydroperoxides were formed in a chain reaction (chain length, 8.6; see Results) which is a second characteristic of TMP. It is clear that all four peroxyls are equally capable of promoting TMP despite the negative surface charge on LDL particles. We suggest that these results can best be accommodated by assuming that the negative surface charge is distributed heterogeneously and is largely associated with apoprotein B-100. Peroxyl radicals in the aqueous phase initiate TMP by abstracting the phenolic hydrogen atom from TocH present in the phospholipid coat of the LDL particle, where it is probably well separated physically from the apoprotein with its regions of

(34) The pH range was severely limited because the water solubility of SOTS-1 declines dramatically at pH <6.5, while light-scattering measurements showed that LDL particles were not stable at pH ~8.

negative charge. At all events, it is clear that the expected Coulombic repulsion between $^{-}$BOO$^{\bullet}$ and LDL is not sufficient to prevent TMP. Furthermore, the fact that in the matched experiments the rates of TocH consumption and CEOOH growth were the same for all four azo compounds necessarily implies that the rate constants for TMP initiation (reaction 25) *and* the

$$(ROO^{\bullet})_{aq} + (TocH)_{LDL} \rightarrow (ROOH)_{aq} + (Toc^{\bullet})_{LDL} \quad (25)$$

rate constants for TMP termination must each be roughly equal for all four alkylperoxyl radicals (reaction 26 and/or reaction 25 followed by reaction 27 where both Toc$^{\bullet}$ are in the same LDL particle).

$$(ROO^{\bullet})_{aq} + (Toc^{\bullet})_{LDL} \rightarrow \text{nonradical products} \quad (26)$$

$$(Toc^{\bullet})_{LDL} + (Toc^{\bullet})_{LDL} \rightarrow \text{nonradical products} \quad (27)$$

An interesting and surprising result (cf. ref 9) is that while TocH is present in the LDL there is almost complete consumption of each of the four alkylperoxyl radicals by the LDL, i.e., $R_i/R_g \sim 0.7-0.9$ (see Results). The bimolecular self-reaction of these *tert*-alkylperoxyls (reaction 28) must be simply too slow to compete with reaction 25 at the R_g's and

$$(ROO^{\bullet})_{aq} + (ROO^{\bullet})_{aq} \rightarrow \text{nonradical products} \quad (28)$$

(TocH-containing) LDL concentrations used in the present work (see below).

The four alkylperoxyl radicals do exhibit differences in the rate of CEOOH accumulation after complete consumption of TocH. These rates are very similar for the three charged azo compounds, but they are significantly larger than the rate for the uncharged initiator (Figure 2). Neutral *tert*-alkylperoxyl radicals undergo very slow bimolecular self-rections in comparison to most radical/radical reactions, i.e., $2k_{28} \sim 10^4$ M^{-1} s^{-1}.[35] Although reaction 28 is comparatively unimportant while the LDL contains TocH, we suggest that it does become important once the LDL has been depleted of its TocH. We furthermore suggest that the rates of destruction of charged *tert*-alkylperoxyl radicals by reaction 28 in the aqueous phase will be retarded by Coulombic repulsion relative to the rate for otherwise comparable, but neutral *tert*-alkylperoxyls. As a result, the steady-state concentrations of the charged peroxyls will be higher at the same R_g than the steady-state concentration of the neutral peroxyl. The higher concentration of the charged peroxyl radicals will enhance the initiation of peroxidation, probably via a roughly thermoneutral hydrogen atom abstraction from the already peroxidized PUFA in the LDL particle:

$$(ROO^{\bullet})_{aq} + (LOOH)_{LDL} \rightarrow (ROOH)_{aq} + (LOO^{\bullet})_{LDL} \quad (29)$$

LDL Peroxidation Initiated by Superoxide. The superoxide-mediated peroxidation of LDL exhibits a number of noteworthy features:

(1) The accumulation of CEOOH in the SOTS-1-initiated reaction could be almost completely prevented by the addition of SOD, which proves that the active agent is superoxide. The fairly strong (~90%) reduction in CEOOH accumulation produced by the addition of CAT must therefore be attributed to the known ability of CAT to react with superoxide.[36−38] In contrast, the accumulation of CEOOH in "matched" XO/AA-initiated reactions was retarded by less than 50% by the same quantities of SOD and CAT. This can be attributed to transition metal contamination of the XO, which leads to Fenton chemistry and some HO$^{\bullet}$ radical-initiated reactions.[21,39,40] (Both H_2O_2 and $O_2^{\bullet-}$ are formed in the XO/AA reaction.)[41,42] The yield of CEOOH after 3 h with XO/AA (1.0 μM) was lower than in the (initially) matched SOTS-1 experiment (2.4 μM) because XO is deactivated during turnover even more rapidly than SOTS-1 decays.[42] The apparently different behavior of "superoxide" generated from SOTS-1 and XO/AA has been observed previously during DNA strand-cleavage experiments.[21] The present results re-emphasize the SOTS-1 advantage: it is a "clean" chemical source of superoxide.

(2) The experiment at pH 7.4 in which R_g with ABAP and R_g with SOTS-1 were made equal using the same LDL preparation (Figure 3) demonstrated that $^{+}$AOO$^{\bullet}$ radicals are much more efficient at initiating LDL peroxidation and consuming TocH than superoxide radicals.

(3) In the LDL/SOTS-1 experiments, at pH 6.8 and 6.5, the rate of formation of CEOOH increases once all the TocH has been consumed (see Figure 4). Thus, *when LDL is subjected to attack by superoxide, the endogenous TocH acts as an antioxidant*. This stands in sharp contrast to the situation in which LDL is subjected to attack by water-soluble alkylperoxyl radicals in which the *TocH acts as a prooxidant*.

(4) During the TocH-induced induction period in the LDL/SOTS-1 experiments, the chain lengths, R_p^{inh}/R_i, were generally less than 1.0 (range, 0.24−1.9; see Table S2). Thus, superoxide-induced, TocH-inhibited LDL peroxidation does not, generally, involve much of a chain reaction. The occurrence of a chain reaction has been considered, heretofore, as one of the criteria associated with TMP. However, TMP can also occur in a nonchain process (vide infra).

(5) Both during and after the induction period, the rate of the SOTS-1induced peroxidation of LDL increased as the pH was reduced (see Figure 4 and Table S2), as has previously been observed with radiolytically generated superoxide.[22−24] For example, during the induction period, the rate of CEOOH formation increased by a factor of between 4 and 6 when the pH was decreased from 7.6 to 6.5 (vide supra), but the rate of TocH consumption increased only marginally (factor of 1.2, vide supra) with this change in pH (see Figure 4). A larger pH range, 6−10, could be covered with PLPC/cholate mixed micelles, and again the rate of the SOTS-1-induced peroxidation increased as the pH was decreased (see Results).

Clearly there are very large differences in the kinetics of LDL peroxidation initiated with SOTS-1 compared with peroxidation initiated with ABAP and other sources of water-soluble alkylperoxyl radicals. It was the use of ABAP that led to the discovery of TMP in LDL,[15,16] and since that early work, ABAP has been used almost exclusively in studies of LDL peroxidation under conditions where R_g was known and controlled, despite

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the obvious fact that $^{+}AOO^{\bullet}$ is never formed in vivo. In aerated aqueous solutions at near-neutral pH, ABAP yields only $^{+}AOO^{\bullet}$ radicals, and in the presence of LDL, these radicals both initiate and terminate the TMP process. In contrast, in aerated aqueous solutions at near-neutral pH, SOTS-1 yields a pH-dependent equilibrium mixture of the superoxide radical anion and its conjugate acid:

$$O_2^{\bullet-} + H^+ \rightleftarrows HOO^{\bullet} \quad (pK_a \sim 4.7) \tag{30}$$

For thermodynamic reasons (see Introduction), TMP could be initiated by $HOO^{\bullet}$ (reaction 18) but not by $O_2^{\bullet-}$ (reaction 19). However, both $HOO^{\bullet}$ and $O_2^{\bullet-}$ would be expected to react with $Toc^{\bullet}$ at rates equal, or close, to the diffusion-controlled limit because both processes involve the reactions of two unlike radicals. Therefore, both $HOO^{\bullet}$ and $O_2^{\bullet-}$ should be effective at terminating TMP.

At normal pH's there is a vast excess of the superoxide radical anion relative to its conjugate acid (reaction 30), e.g., ~99.84% $O_2^{\bullet-}$:0.16% $HOO^{\bullet}$ at pH 7.5 and 98.44% $O_2^{\bullet-}$:1.56% $HOO^{\bullet}$ at pH 6.5. Such a 1 unit decrease in pH therefore produces a roughly 10-fold increase in the $[HOO^{\bullet}]/[O_2^{\bullet-}]$ ratio. Under conditions where all superoxide is destroyed in the bimolecular self-reaction 31

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{2k_{31}} H_2O_2 + O_2 \tag{31}$$

the total steady-state concentration of superoxide, is given by

$$([O_2^{\bullet-}] + [HOO^{\bullet}])_{ss} = (R_g/2k_{31})^{1/2} \tag{32}$$

where $2k_{31}$ is pH-dependent.[43] A decrease in pH from 7.5 to 6.5 causes $2k_{31}$ to increase by a factor of 10 (from 2×10^5 to 2×10^6 M^{-1} s^{-1}).[43] Thus, at constant R_g, this 1-unit decrease in pH causes a decrease in the total steady-state concentration of superoxide by a factor of $(10)^{1/2} \approx 3$ as well as an increase in the $[HOO^{\bullet}]/[O_2^{\bullet-}]$ ratio by a factor of 10. This means that the steady-state concentration of $HOO^{\bullet}$ will *increase* at constant R_g by a factor of $10/(10)^{1/2} \approx 3$ as the pH is decreased from 7.5 to 6.5.

The foregoing analysis is relevant to the LDL/SOTS-1 system. TMP must be initiated by $HOO^{\bullet}$ (reaction 18; see Introduction). We suggest that TMP is terminated mainly by $O_2^{\bullet-}$

$$HOO^{\bullet} + TocH \rightarrow H_2O_2 + Toc^{\bullet} \tag{18}$$

(because is $O_2^{\bullet-}$ present in overwhelming excess over $HOO^{\bullet}$) via reactions 20 and 33.

$$O_2^{\bullet-} + Toc^{\bullet} + H^+ \rightarrow O_2 + TocH \tag{20}$$

$$O_2^{\bullet-} + Toc^{\bullet} \rightarrow {}^{-}OOToc \tag{33}$$

Reaction 33 is analogous to the known addition of $O_2^{\bullet-}$ to various phenoxyl radicals [44−46] (e.g., the tyrosyl radical,[44a] where 90% of the overall reaction is addition[44b]). This reaction is required because it "consumes" $Toc^{\bullet}$ radicals and hence consumes TocH as is observed experimentally[47] (see Figures 3 and 4). Reaction 20, by contrast, terminates TMP by regenerating TocH, so that TocH is not consumed. This is analogous to the reaction between $O_2^{\bullet-}$ and the phenoxyl radical derived from a water-soluble analogue of TocH, Trolox[45a] (where 91% of the overall reaction occurs by electron transfer[45b]). Because of the structural similarities between $Toc^{\bullet}$ and the Trolox-derived radical, reaction 20 would be expected to be more important than reaction 33, which is consistent with the fact that only ~3−4% of the superoxide radicals generated were found to react in any measurable fashion with the LDL (see Results). That is, not all 97% of the unaccounted for superoxide radicals were necessarily consumed in the aqueous phase via bimolecular self-reaction 31. It should be noted that reaction 18 followed by reaction 20 is simply a *catalytic* version of reaction 31, and therefore, the reaction pair, 18 + 20, will influence neither the $HOO^{\bullet}/O_2^{\bullet-}$ ratio nor the rate at which TocH is consumed via reactions 18 + 33. Indeed, it can be argued that most of the unaccounted for superoxide is not destroyed via reaction 31 because, if it were, then at a constant R_g a decrease in pH from 7.5 to 6.5 would result in the following: (i) increase $[HOO^{\bullet}]_{ss}$ by a factor of ~3 (see above) leading to an increase in R_i by roughly the same factor and (ii) decrease $([HOO^{\bullet}] + [O_2^{\bullet-}])_{ss}$ by a factor ~3 (see above) leading to a decrease in the TMP termination rate, R_t^{inh} by roughly the same factor. Since $R_p^{inh} \propto R_i / R_t^{inh}$, this decrease in pH would be expected to increase R_p^{inh} by a factor of ~10 if most superoxide were destroyed in reaction 31, whereas the experimental increase in R_p^{inh} on changing the pH from 7.6 to 6.5 was only a factor of 4−6 (see Results and Figure 4). Similarly, the rate of TocH consumption must depend on R_i and hence on $[HOO^{\bullet}]_{ss}$, and if, therefore, most superoxide was destroyed in reaction 31, a decrease in pH by 1 unit would be expected to increase d[TocH]/d*t* by a factor of ~3. The experimental increase in d[TocH]/d*t* as the pH is decreased from 7.6 to 6.5 was considerably smaller than this (vide supra and Figure 4), which also leads us to conclude that reaction 31 does not control the steady-state concentration of superoxide in our LDL/SOTS-1 experiments.

During the induction period, the SOTS-1-induced peroxidation of LDL occurred with a chain length, R_p^{inh}/R_i, that was generally less than 1.0 (see Table S2); i.e., peroxidation was not a chain reaction. Nevertheless, that small amount of peroxidation that did occur must, for kinetic reasons, have involved hydrogen atom abstraction from a polyunsaturated fatty acid moiety by a $Toc^{\bullet}$ radical (reaction 13), i.e., by the reaction that distinguishes TMP from "ordinary" lipid peroxidation in the absence or presence of TocH. To illustrate, consider an LDL particle that has just reacted with an $HOO^{\bullet}$ radical and now contains a $Toc^{\bullet}$ radical. Let this $Toc^{\bullet}$ radical abstract a hydrogen atom (reaction 13) and then be regenerated via reactions 3 and 5 before it is destroyed by $O_2^{\bullet-}$(reaction 33). One molecule of lipid hydroperoxide has been produced by two superoxide radicals. Thus, *TMP occurred but the chain length was only 0.5*; i.e., *the occurrence of a chain reaction is not necessarily diagnostic for TMP*.

A more reliable diagnostic criterion for the occurrence of TMP in LDL is that R_p^{inh} should be independent of R_g. The range of SOTS-1 concentrations was severely limited because (i) a steady-state concentration of $Toc^{\bullet}$ in the LDL particles and hence the appropriate limiting R_p^{inh} cannot be established

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(46) The partitioning, of $O_2^{\bullet-}$+ $ArO^{\bullet}$ reactions between electron transfer (cf. reaction 20) and addition (cf. reaction 33) is very dependent on the structure of $ArO^{\bullet}$; see refs 44b and 45b.

(47) Alternatively, TocH may be consumed by the double occurrence of reaction 18 to produce two $Toc^{\bullet}$ radicals in a single LDL particle, followed by reaction 27.

at low [SOTS-1] before too much of the SOTS-1 has decomposed and (ii) the maximum concentration of SOTS-1 is determined by its limited solubility in water. However, within experimental error, R_p^{inh} was found to be approximately independent of [SOTS-1] and hence of R_g at pH 7.4 (vide supra). Thus, superoxide does induce TMP in LDL at physiological pH's although the chain lengths were very short (often <1.0) and the TocH in the LDL actually functions not as a prooxidant (cf., ABAP) but as an antioxidant because peroxidation at pH 6.8 and 6.5 becomes much faster after all the TocH has been consumed (see Figure 4).[48]

Conclusion

Water-soluble, but not physiologically relevant, positively charged, negatively charged, and neutral *tert*-alkylperoxyl radicals promote peroxidation chain reactions of human low-density lipoprotein containing α-tocopherol with about equal efficiencies. The rates of these tocopherol-mediated peroxidations are greater than the rates of peroxidation after all the TocH has been consumed; i.e., TocH functions as a pro-oxidant for LDL subjected to attack by water-soluble alkylperoxyl radicals. In contrast, the physiologically relevant superoxide radical is not only very much less efficient at promoting TMP in LDL than alkylperoxyls but also, and more importantly, the rate of peroxidation increases after all the TocH has been consumed. That is, for LDL subjected to attack by superoxide, the TocH in the LDL fulfills its traditional role as an antioxidant. The differences in the peroxidation of human LDL containing TocH when it is subjected to attack by superoxide and by *tert*-alkylperoxyls are ascribed to two phenomena: (i) superoxide initiates TMP with a lower efficiency than the alkylperoxyls; (ii) superoxide terminates TMP with a higher efficiency than the alkylperoxyls.

The present work raises, yet again, [7,18,32,49,50] the question as to whether TMP is likely to be of any importance in vivo. In superoxide-mediated LDL peroxidation, R_p^{inh} increases as the pH decreases, hence TMP is more likely to be important in regions of low pH (<6.5). Such low pH's may be encountered by LDL particles that have undergone phagocytosis by a macrophage.[51] However, it is generally believed that phagocytosis via the macrophage scavenger receptor involves LDL particles that have already been oxidatively modified. Thus, while TMP of LDL might occur in vivo, it may well be irrelevant to the formation of oxidatively modified LDL and hence to the initiation of atherosclerosis.

Experimental Section

Materials. 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP), 2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine) dihydrochloride, and 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl) propionamide] were obtained from Wako Chemicals, USA Inc. (Richmond, VA). 3,3′-Azobis(3-cyano-1-butanesulfonic acid) disodium salt[52] and SOTS-1[30] were synthesized according to literature procedures. Isoluminol, cholesteryl benzoate, and all enzymes were purchased from Sigma and used as received. Sodium cholate (Aldrich) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidyl choline (PLPC) (Avanti Polar Lipids, Alabaster, AL) were also used as received. Acetaldehyde (AA) (Aldrich) was distilled immediately before use. α-Tocopherol (TocH) (Aldrich) was purified by chromatography on silica gel (eluent: hexane/ethyl acetate, 92:8 v/v). The HPLC solvents, methanol, and 2-propanol (EM Science, Gibbstown, NJ) were stored over 4-Å molecular sieves for at least 1 week to decompose adventitious hydroperoxides. Phosphate-buffered isotonic saline (PBS, pH 7.4, 50 mM in phosphate) was prepared from Millipore water and the highest purity reagents commercially available. All buffers were stored over Chelex-100 for at least 24 h prior to use.

Methods. (i) Efficiencies, *e*, of Cage Escape of Geminate Radical Pairs Formed by Thermolysis of the Azo Initiators. The rate constants for thermal decomposition of the four azo compounds at 37 °C have been reported.[21] To determined R_g, it is necessary to determine the efficiency, *e*, of cage escape of the initially produced geminate radical pairs. To this end, air-saturated solutions of each azo compound in PBS containing 1 mM *p*-cresol (Aldrich) were incubated at 37 °C in matched experiments in which the amounts of the azo compounds decomposing per unit time were roughly the same: 8.2 mM ABAP, 1.7 mM azobis(dimethyleneisobutyramidine), 34 mM azobis(3-cyano-1-butanesulfonic acid), and 60 mM azobis [2-methyl-*N*-(2-hydroxyethyl)propionamide]. The rates at which the *p*-cresol was consumed by the peroxyl radicals formed from these initiators were determined by HPLC on a Spherisorb ODS 2 column using 3:2 (v/v) 20 mM aqueous NaH_2PO_4/methanol as eluent at a flow rate of 1 mL/min (retention time, ∼11 min, *p*-cresol quantified by its 278-nm absorption). Each molecule of *p*-cresol will capture two alkylperoxyl radicals.[10,13]

(ii) Isolation of LDL. The procedure of Chung et al.[53] was followed using a slightly modified ultracentrifuge protocol. Briefly, blood plasma was separated from the venous blood of a healthy male collected in lithium heparin vacuum containers (Becton Dickinson, Franklin Lakes, NJ) using a clinical centrifuge. After adjusting the density of the plasma to 1.21 g/mL with KBr and establishing a density gradient in Quick-Seal centrifuge tubes, a Beckman L8-70 ultracentrifuge equipped with a 70.1 Ti rotor was used for 4 h at 4 °C and 55 000 rpm. The well-separated, orange LDL band was carefully removed through the side of the centrifuge tube using a syringe with a 15-gauge needle and stored at 4 °C.

(iii) Peroxidation of LDL and HPLC Analyses. Immediately prior to an experiment, water-soluble antioxidants such as ascorbate and urate were removed from the LDL by size exclusion chromatography on Sephadex PD-10 columns (Amersham Pharmacia, Uppsala, Sweden) using PBS as eluent. This LDL preparation was mixed with an appropriate amount of an azo initiator stock solution prepared in the same buffer. SOTS-1 and the aerated XO/AA couple were both employed in studies of superoxide-mediated LDL peroxidation; the rates of superoxide production from both systems were measured using the Fe^{III} cytochrome *c* assay.[42,54,55] The XO concentration was adjusted so that the initial rate of $O_2^{\bullet-}$ formation from XO/AA was equal to that from SOTS-1. SOD, CAT, or both were added where indicated. For studies of the pH dependence of the $O_2^{\bullet-}$-mediated peroxidation, the LDL preparation was mixed with a SOTS-1 solution in phosphate buffer (pH 7.4) and HCl (0.1 M) or NaOH (0.1 M) was added to obtain the desired pH. After a reaction time of 80 min (1 half-life of SOTS-1), a freshly prepared SOTS-1 solution was added to bring the SOTS-1 concentration back to its initial value.

Reactions were carried out at 37 °C in polyethylene tubes, and 200-μL aliquots were withdrawn periodically for analysis. Sample workup involved extraction into hexane following the procedure of Sattler et al.[56] Cholesteryl ester hydroperoxides (CEOOH) were analyzed by HPLC (HP 1090, series II) on a Supelcosil LC-18-DB column eluted with methanol/2-propanol (1:1, v/v) at a flow rate of 1 mL/min. The

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retention time of CEOOH was ~9.1 min. The hydroperoxides were detected by postcolumn chemiluminescence using a 1:1 (v/v) methanol/borate buffer (pH 10, 100 mM) solution containing 2 mM isoluminol and 5 mg/L microperoxidase (MP-11). This solution (1 mL/min) joined the efflux from the HPLC in a mixing T-joint and then passed through a single-photon-counting chemiluminescence detector (Shodex CL-2). The chemiluminescence signal was calibrated with *tert*-butyl hydroperoxide making the appropriate correction for the different response factors for this hydroperoxide and CEOOH.[57] TocH was quantified by HPLC of the hexane extract of the LDL on the same Supelcosil column using an electrochemical detector (HP 1049 programmable electrochemical detector) and elution with a 2 mM LiCl solution in methanol/2-propanol (1:1, v/v) at a flow rate of 1 mL/min. The TocH retention time was ~5.2 min, and its detection was achieved in the oxidative mode with a 600-mV potential. The extraction efficiency into hexane was determined using cholesteryl benzoate as a standard.[56] LDL concentrations were estimated by quantifying the free cholesterol in the hexane extracts and assuming that each LDL particle contained 550 cholesterol molecules.[58]

(iv) Depletion of TocH.[49] LDL in PBS (pH 7.4) was incubated with 100 mM ABAP for 12 min at 37 °C; the ABAP was then removed by size exclusion chromatography (PD-10 column). HPLC analysis on this LDL (0.3 μM) showed that >95% of the TocH had been consumed and that 13 μM CEOOH had been produced which indicates that ~5% of the cholesterol esters had been peroxidized (based on 270 μM PUFA esters of cholesterol, or 326 μM LH moieties present as cholesteryl esters in the LDL preparation).[9] Further oxidation studies on this TocH-depleted LDL were carried out as described above.

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(v) Peroxidation of PLPC/Cholate Micelles. PLPC (25 mg) was dissolved in deaerated chloroform (1.0 mL) in a N_2-purged glovebag. The $CHCl_3$ was evaporated from an aliquot (170 μL) under a N_2 stream, and PBS (281 μL) and a sodium cholate solution in PBS (30 mg/mL, 109 μL) were added. This mixture was vortex stirred and then passed through a polycarbonate membrane (pore diameter, 100 nm) 25 times in a hand-held extrusion apparatus (Avestin, Ottawa, ON, Canada). This solution, with added enzymes where indicated, was then subjected to matched $O_2^{\bullet-}$ rates of formation from SOTS-1 and XO/AA at 37 °C for 3 h. Following which, a 200-μL aliquot was mixed with 1 mL of methanol, dried under N_2, and redissolved in 200 μL of methanol. HPLC analysis for phosphatidylcholine hydroperoxide was performed as described for CEOOH but with methanol (1 mL/min) as eluent, retention time 6.3 min.

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Supporting Information Available: Formation of CEOOH and TocH depletion during incubation of LDL with two charged and one uncharged azo initiator at concentrations where k_i [RN=NR] ~ constant (Figure S1) and some kinetic data for LDL peroxidation initiated with four water-soluble peroxyl radicals (Table S1) and with superoxide (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

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