Minimizing Tocopherol-Mediated Radical Phase Transfer in Low-Density Lipoprotein Oxidation with an Amphiphilic Unsymmetrical Azo Initiator

Sean M. Culbertson,[†] Melinda R. Vinqvist,[‡] L. Ross C. Barclay,[‡] and Ned A. Porter*[,§]

Contribution from the Department of Chemistry, Duke University, Durham, North Carolina 27708, Department of Chemistry, Mount Allison University, Sackville, New Brunswick, Canada E4L 1G8, and Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37235

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Abstract: The antioxidant α-tocopherol (α-TOH) has been found to act as a pro-oxidant under many in vitro conditions. The observed tocopoherol-mediated peroxidation (TMP) is dependent on two primary factors. (1) Chain transfer: α-TO• radical reacts with lipid to form lipid peroxyl radicals. (2) Phase transfer: α-TOH can transport radical character into the lipoprotein. Given the limitations of existing initiators, there is a need for new compounds that avoid the requirement for α-TOH to act as a phase-transfer agent. We report here a study showing that the new unsymmetrical azo compound, **C-8**, initiates LDL lipid peroxidation without requirement for α-TOH. This initiator provides a steady source of free amphiphilic peroxyl radicals that efficiently initiates oxidation of α-TOH-depleted LDL at a rate comparable to that reported for the very reactive hydroxyl radical (•OH). With other initiators tested, unsymmetrical **C-12** and **C-16** and symmetrical **C-0** and MeOAMVN, α-TOH-depleted LDL displayed significant resistance to oxidation. Results indicate that the amphiphilic nature of the unsymmetrical initiators increases their partitioning into lipoprotein depending on the hydrocarbon chain length, and the symmetrical azo initiators **C-0** and MeOAMVN primarily remain in the aqueous phase. Evidence suggests that even when the phase-transfer activity of α-TOH is limited, with the use of an initiator such as **C-8**, the mechanism of peroxidation remains controlled by TMP chain-transfer activity.

Introduction

Oxidative modification of lipoproteins has been increasingly implicated as an early event in the pathogenesis of atherosclerosis.[1,2] Low-density lipoprotein (LDL)[3] is the primary cholesterol transport protein in human blood plasma. Oxidatively modified LDL, including lipid hydroperoxides formed in the LDL core, have been found to possess atherogenic properties.[4,5] However, it is still unknown how and where LDL gets oxidized in vivo.[1,6] Studies of initiation and antioxidant defenses are of key importance to understanding the mechanism(s) of lipid peroxidation and their potential involvement in atherosclerosis.

The naturally abundant and potent antioxidant α-tocopherol (α-TOH) has attracted significant attention as a peroxidation chain breaker in lipoproteins.[7] However, there is growing evidence to suggest that this powerful antioxidant may also act as a pro-oxidant under many in vitro experimental conditions.[8,9] The mechanism by which α-TOH can exert a pro-oxidant activity in LDL oxidations, tocopherol-mediated peroxidation (TMP), occurs when the unreactive α-tocopheroxyl radical (α-TO•) acts as a chain-transfer agent propagating lipid peroxidation within the lipoprotein.[10] This unusual activity of α-TOH is increased by the unique phase separation a lipoprotein imposes.[9,11] Namely, the LDL core confines and concentrates the reactants so that the TMP reaction between α-TO• and bis-allylic hydrogens of lipids (LH) ($k_{TMP} = 0.01-0.1\ M^{-1}\ s^{-1}$)[12] occurs to produce lipid hydroperoxides (LOOH) mediated by tocopherol. Oxidation conditions that allow the α-TO• radical to have an extended lifetime in the lipoprotein (e.g., 17 min) give α-TOH the opportunity to act as a pro-oxidant. Antioxidant activity of α-TOH can be accomplished in two ways that involve shortening the resident lifetime of α-TO• in a LDL particle. First, increasing radical generation provides more radical−radical

* To whom correspondence should be addressed. Phone: (615) 343-2695. Fax: (615) 343-5478. E-mail: n.porter@vanderbilt.edu.
† Duke University.
‡ Mount Allison University.
§ Vanderbilt University.

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(3) Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AH, ascorbic acid; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); α-TOH, α-tocopherol; α-TO•, α-tocopheroxyl radical; **C-0**, 2,2′-azobis[2-(2-imdazolin-2-yl)propane] dihydrochloride; Ch18:2-OOH, cholesteryl linoleate hydroperoxide; EDTA, ethylenediaminetetraacetic acid; IA, isoascorbic acid; LDL, low-density lipoprotein; LH, lipid with bis-allylic hydrogen, LOOH, lipid hydroperoxide; MeOAMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); MPA, metaphosphoric acid; •OH, hydroxyl radical; PBS, phosphate-buffered saline; PC, phosphatidylcholine; ROO•, peroxyl radical; TMP, tocopherol-mediated peroxidation; UA, uric acid.
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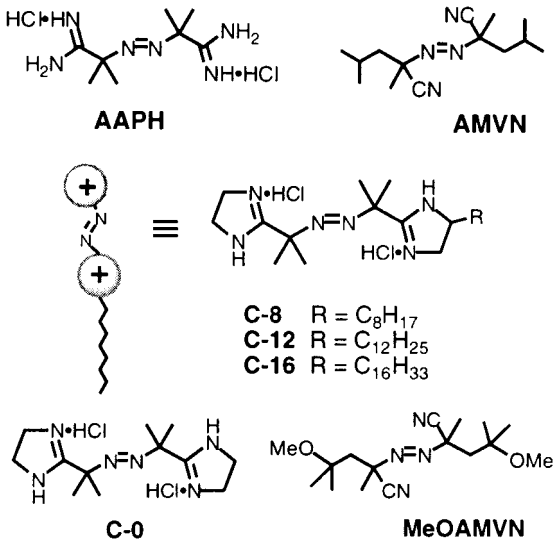
termination events and consumes α-TO• before TMP can occur. Second, addition of a coantioxidant such as water-soluble ascorbic acid reduces the α-TO• radical at the aqueous−lipid interface and exports radical character out of the confined lipid regions of LDL.[11] At this time, it is unknown whether α-TOH has pro-oxidant activity in vivo; however, lipoprotein fractions isolated from advance human atherosclerotic plaques have shown coexistence of α-TOH and oxidized lipids.[6]

The initiation process of lipoprotein oxidation is of crucial importance, and α-TOH can play a significant role in modulating lipoprotein susceptibility to oxidant stresses. Surprisingly, it was found that in vivo or in vitro α-TOH-depleted LDL were highly resistant to peroxidation initiation compared to native LDL when oxidants were formed at low rates.[13] This unexpected resistance of α-TOH-depleted LDL was true for a variety of oxidants including water- and lipid-soluble peroxyl radicals (ROO•), hydroxyl radicals (•OH), Cu^{2+}, the transition metal-containing Ham's F-10 medium, soybean 15-lipoxygenase, and horseradish peroxidase. Furthermore, LDL lipid oxidizability was found to be dependent on the fractional content of the lipoprotein's α-TOH. However, under high radical generation conditions, even α-TOH-depleted LDL could be oxidized by the highly reactive •OH and to a much lesser extent by water-soluble ROO•. These results strongly suggest that there is a requirement of α-TOH for efficient initiation of lipoprotein lipid peroxidation and that α-TOH is more pro-oxidant the lower the initiating radical's reactivity and radical flux.[11,14] Thus, another important aspect of the LDL oxidation model involves phase transfer of aqueous radical character into the lipid environment via α-TOH at the LDL surface.

Unfortunately, the requirement of α-TOH for efficient initiation of lipoprotein oxidation complicates attempts to study the kinetics of α-TOH in an intact LDL particle. Azo initiators, such as 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), are often employed to produce a slow, steady source of free radicals by known chemical decomposition mechanisms.[28] However, the increased viscosity of the lipid environment and the macromolecular "cage" nature of a lipoprotein significantly prevents the cage escape of the lipophilic radicals formed in the decomposition of lipophilic AMVN. Studies of the peroxidation of LDL include the use of high concentrations of AMVN to produce initiation at a reasonable rate, and it has been reported that only 4−5% of radicals generated from AMVN actually escape the cage to initiate peroxidation in LDL.[10] Such high initiator concentrations may compromise the integrity of the lipoprotein and, combined with the poor cage escape, limit the attainable radical flux. While the hydrophilic radicals derived from AAPH exhibit efficient cage escape in water, the transfer of these radicals into the lipid region of a lipoprotein is largely dependent upon phase-transfer mechanism(s) via α-TOH or even LOOH.[13,15] There is an increasing body of evidence suggesting that subtle changes in the initiator source, rate of decomposition, reactivity, or location of radical formation can influence mechanistic pathways of lipoprotein peroxidation.[9]



Given the limitations of existing initiators, there is a need for new compounds that efficiently generate radicals in lipid regions of lipoproteins and avoid the requirement for α-TOH to act as a phase-transfer agent. We report here a study showing that the new unsymmetrical azo compound, **C-8**, initiates LDL lipid peroxidation without a requirement for α-TOH.[16,17] This initiator provides a steady source of free amphiphilic peroxyl radicals that efficiently initiate oxidation of α-TOH-depleted LDL at a rate comparable to that reported for the very reactive •OH.[13] With other initiators tested, unsymmetrical **C-12** and **C-16**, and symmetrical 2,2′-azobis[2-(2-imdazolin-2-yl)propane] dihydrochloride (**C-0**) and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN), α-TOH-depleted LDL displayed significant resistance to oxidation. Results indicate that the amphiphilic nature of the unsymmetrical initiators increases their partitioning into lipoprotein depending on the hydrocarbon chain length, and the symmetrical azo initiators **C-0** and MeOAMVN primarily remain in the aqueous phase. Initiator structure and location of decomposition can impact oxidation mechanism and favor α-TOH activity as a pro-oxidant when initiation relies heavily on α-TOH as a phase-transfer agent. However, evidence also supports the notion that even when the phase-transfer activity of α-TOH is limited, with the use of an initiator such as **C-8**, the mechanism of peroxidation remains controlled by TMP chain-transfer activity.

Experimental Procedures

Materials. Phosphate-buffered saline (PBS; pH 7.4, 50 mM) was placed over Chelex-100 resin for 24 h. The resin was removed and 100 μM ethylenediaminetetraacetic acid (EDTA) was added to chelate any remaining trace metal contaminants.[18] All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without further purification, unless otherwise noted. The azo initiators **C-0** (VA-044) and MeOAMVN (V-70) were generously donated by Wako Chemicals USA Inc. (Richmond, VA). Unsymmetrical azo initiators **C-8**, **C-12**, and **C-16** were synthesized as described in detail previously.[16] The antioxidants, α-tocopherol and δ-tocopherol, were purchased from Sigma and purified by preparative HPLC, 0.5% 2-propanol in hexanes with UV detection at 292 nm. Solvents were HPLC quality and purchased from commercial sources.

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Analytical HPLC was carried out on a Waters model 600E HPLC, Waters model 717 plus autosampler, with a Waters model 996 photodiode array detector connected to a Waters Millennium 32 chromatography station, or a Bioanalytical Systems Inc. LC-4 electrochemical detector connected to a Hewlett-Packard 3396 Series III integrator. A single Beckman 5-μm Ultrasphere C-18 column (4.6 mm $\times$ 25 cm) was used for reversed-phase conditions, and two tandem Beckman 5 μm Ultrasphere columns (4.6 mm $\times$ 25 cm) were used for normal-phase conditions.

Oxidation of LDL. Low-density lipoprotein was isolated from normolipidemic healthy subjects as reported previously.[16] Lipoprotein concentrations were adjusted with PBS to give final concentrations of 0.75 mg of protein/mL and allowed to equilibrate to 37 °C for 5 min. When required, water-soluble antioxidant, uric acid (UA) in PBS or ascorbic acid (AH) in PBS, was added to the LDL suspension before azo initiator addition. Initiators were added as solutions in methanol (except for **C-0** in PBS) to provide final initiator concentrations from 0.25 to 3.0 mM and $\leq$3% (v/v) methanol. Following addition of initiator (time zero), aliquots for α-TOH analysis (200 μL), cholesterol linoleate oxidation (Ch18:2-OOH) analysis (200 μL), UA analysis (50 μL), and AH analysis (100 μL) were removed at various intervals, placed directly on ice, and treated as described below.

To the α-TOH aliquot was added butylated hydroxytoluene (BHT) and internal standard, δ-tocopherol. Extraction of all the aliquots was performed with ice-cold methanol (1 mL) and hexanes (5 mL). The hexane phase was removed and concentrated under argon and then stored at $-$78 °C until analysis. The UA aliquots were simply stored at $-$78 °C until analysis. Analysis for α-TOH and UA was by reversed-phase HPLC with electrochemical detection described previously.[16] The analysis method for AH was modified from Kutnink et al. and Motchnik et al. and is reported in Supporting Information along with the experimental results from AH containing experiments.[19,20] To the 200-μL aliquot of cholesteryl linoleate was added BHT, the internal standard, and 13-hydroxyoctadecane-*cis*-9,*trans*-11-dienoate, and the hydroperoxides were reduced to alcohols with the addition of triphenylphosphine ($\sim$1 μmol). These reduced cholesteryl linoleate oxidation (Ch18:2-OH) samples were then extracted the same as the α-TOH samples. However, analysis was by normal-phase HPLC using 0.5% isopropyl alcohol in hexanes with ultraviolet detection (234 nm) and provided separation of the four isomeric Ch18:2-OH products as described previously.[21] Local concentrations were calculated using the oxidation protein concentration, assuming apoprotein B100 was 550 kDa and total lipid volume in a LDL particle was 3.2×10^{-21} L.[10] The local rate of radical initiation in the lipid (R_i) was calculated from the local rate of consumption of α-TOH ($R_i = 2 \cdot d[\alpha\text{-TOH}]/dt$).[22]

Preparation of α-TOH-Depleted LDL.[13] Freshly isolated unoxidized native LDL ($\sim$2.4 mg of protein/mL, final concentration) was incubated with AAPH (110 mM, final concentration) at 37 °C in PBS. Under these conditions of high radical generation, LDL's α-TOH was consumed in approximately 16$-$18 min with formation of only small amounts of lipid hydroperoxides, as reported.[13] Briefly, oxidation was stopped by placing the LDL samples on ice followed by two passages through PD-10 gel filtration columns to remove the added AAPH. The α-TOH-depleted LDL was then incubated for 30 min at 37 °C in the presence of glutathione ($\sim$1 mM) added in PBS and ebselene ($\sim$40 μM) added in dimethyl sulfoxide (DMSO) ($\sim$1% DMSO (v/v)).[23] After reduction of any preformed hydroperoxides, the LDL sample was passed through two consecutive gel filtration columns to remove the added reductants. Typically, α-TOH-depleted LDL contained $\leq$6 molecules of preformed Ch18:2-OH products and no detectable Ch18:2-OOH, and $\sim$95% of the α-TOH was consumed compared to the native LDL. Protein concentration was redetermined[24] and α-TOH-depleted oxidations were completed using the same conditions as described above for native LDL.

Initiator Incorporation into LDL. Hydroperoxide-free native LDL (2.0 mg of protein/mL, final concentration) was incubated for 30 min at room temperature with a variety of initiators (2.6 mM **C-8**, 1.3 mM **C-12**, 1.3 mM **C-16**, 2.6 mM **C-0**, or 0.6 mM MeOAMVN, all final concentrations). Half of the initiator-treated LDL was placed on ice and used as a positive control. However, the remaining half was passed through two consecutive PD-10 columns to remove any unassociated initiator.[13] After protein concentrations were redetermined[24] for the postfiltration samples, UA was added to both the positive control and postfiltration LDL samples, which were adjusted to a final concentration of 0.75 mg of protein/mL containing added 60 μM UA. Oxidations were begun by incubation at 37 °C.

Determinations for **C-8**, **C-12**, and **C-16** critical micelle concentrations were made using a Sigma 70 automated tensiometer applying the Wilhelmy method[25] at 23 °C. Initiator stock solutions were 0.10$-$0.16 M in methanol and were diluted with deionized water and methanol to give a range of concentrations of 0.12$-$5 mM initiator and 3% methanol (v/v).

Determination of Rate Constants for Azo Decomposition (k_d). The k_d values were measured by following the evolution of nitrogen gas in a pressure-sensitive apparatus with an automatic recording device described previously.[26] The sample and reference cells contained identical nitrogen-purged (to remove oxygen that could be consumed) mixtures except for the presence of initiator in the sample cell. Before addition of initiator to the sample cell, the system was equilibrated until a steady baseline was achieved. However, experiments in phoshatidylcholine (PC) liposomes contained initiators added when the liposomes were made, so there was no equilibration step. Initiators were added as a concentrated solution in a minimal volume of PBS ($\leq$5% total volume) providing final concentrations from 1.0 to 3.0 mM. Reaction mixtures contained UA ($\sim$125 μM) for all the aqueous media, and liposome suspensions contained added α-TOH ($\sim$140 μM). Rate analysis was made by applying the Guggenheim method using intervals of 20, 30, and 40 h.[27] Determinations for k_d in methanol were by loss of the azo chromophore at 366 nm, described previously.[16]

Results

Initiation of α-TOH-Depleted LDL Oxidation with C-8. The addition of azo initiators to lipid suspensions, such as LDL, generates free peroxyl radicals at constant rates by spontaneous thermal decomposition.[28] In LDL, however, the antioxidant α-TOH has been shown to act as an effective phase-transfer agent for aqueous free radicals, and this complicates the fraction of radicals actually delivered (radical flux)[14] to the lipid regions.[9,11] In other words, the presence of α-TOH in a LDL particle makes the lipids in the LDL core more susceptible to oxidation from both water-soluble and lipid-soluble free radical azo initiators than is the case if α-TOH is absent from the LDL. In fact, it was previously demonstrated that peroxyl radicals derived from AAPH in the aqueous phase did not efficiently initiate LDL oxidation in the absence of α-TOH, unless the radical generation rate was very high.[13] The capability of the unsymmetrical azo initiator **C-8** to circumvent the phase-transfer activity of α-TOH was tested by initiating LDL oxidation in the presence and absence of α-TOH.

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(22) The induction method ($R_i = 2\,[\alpha\text{-TOH}]_0/\tau$, where τ is length of the lag phase) is a better way to determine R_i when analysis for α-TOH at complete consumption is available. This is especially true with initiators that rely on α-TOH phase transfer to access LDL lipids because as [α-TOH] changes so does R_i.

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Native LDL was depleted of endogenous α-TOH without the formation of significant oxidation products by exposing LDL to AAPH-derived peroxyl radicals formed from high concentrations of initiator as described in Experimental Procedures. The validity of this α-TOH-depletion procedure was extensively evaluated by Stocker et al., and our studies confirm their observations[13]

We find that the unsymmetrical amphiphilic initiator **C-8** efficiently initiates oxidation of α-TOH-depleted LDL. Oxidation of native and α-TOH-depleted LDL was initiated in experiments in which the concentration of **C-8** was systematically varied (Figure 1).[29] Evaluation of the rate of product formation in these lipid peroxidations leads to several important observations. (1) In α-TOH-depleted LDL, initiation with **C-8** shows significant lipid oxidation that is initiator concentration dependent. (2) In native LDL, the oxidation rates observed when α-TOH was present were independent of initiator concentration. However, after consumption of α-TOH, the oxidation was observably dependent on initiator concentration. (3) At **C-8** concentrations above 1 mM, the oxidation rate in the absence of α-TOH exceeds the rate in the presence of α-TOH (Figure 1A,B) (These oxidation rates are more clearly compared in Table 1, where the rate of initiation is R_i, the rate of cholesteryl linoleate oxidation in native LDL with α-TOH present is $R_{p,inh}$, and the rate of cholesteryl linoleate oxidation in α-TOH-depleted LDL is R_p^-.) But, when [**C-8**] = 0.5 mM, the oxidation rate was greater with tocopherol present in the lipoprotein (Figure 1C). (4) The lag phase (time period of α-TOH consumption) was also dependent on initiator concentration.

C-8-induced oxidation of α-TOH-depleted LDL provides an advantage over initiation with the symmetrical azo initiators MeOAMVN and **C-0**[16,17,30] These initiators are known to have different rates of decomposition at 37 °C: MeOAMVN (in acetonitrile) $k_d = 3.2 \times 10^{-5}$ s^{-1}, $\tau_{1/2} = 6$ h;[30] **C-0** (in methanol) $k_d = 7.1 \times 10^{-6}$ s^{-1}, $\tau_{1/2} = 27$ h; **C-8** (in methanol) $k_d = 8.3 \times 10^{-6}$ s^{-1}, $\tau_{1/2} = 23$ h.[16] Initiator concentrations were chosen to provide comparable radical generation rates. Thus, we assumed that the rate of radical generation from 0.25 mM MeOAMVN was approximately equal to that from 1.0 mM **C-8** and that from 1.0 mM **C-0** approximately equal to that from 1.0 mM **C-8**. (For more information concerning comparisons of initiator decomposition rates and radical generation efficiencies, see results later in this section.) A comparison of **C-8** with the symmetrical lipid- and water-soluble initiators demonstrated the increased effectiveness that **C-8** provides for lipoprotein oxidation in the absence of α-TOH (Figure 2). With either the lipophilic MeOAMVN or the hydrophilic **C-0**, α-TOH-depleted LDL was more resistant to lipid oxidation than was native LDL. In α-TOH-depleted LDL **C-8** provides approximately 4−5-fold more efficient oxidation than either of the symmetrical initiators. Surprisingly, the unsymmetrical azo initiators **C-12** and **C-16** were not very effective initiators in α-TOH-depleted LDL (Table 1).

The profile of oxidized cholesteryl linoleate geometric isomers confirms that peroxidation occurs in the absence of α-TOH in the depleted LDL (Figure 3).[21,31] In the presence of α-TOH, the hydrogen atom-donating ability of the lipid environment is relatively high. Thus, the kinetic cis,trans and trans,cis cholesteryl linoleate oxidation products were predominately trapped (Figure 3A).[32] As the tocopherol concentration drops so does the hydrogen atom-donating ability. The poorer hydrogen atom donors, lipid bis-allylic hydrogen, support formation of primarily

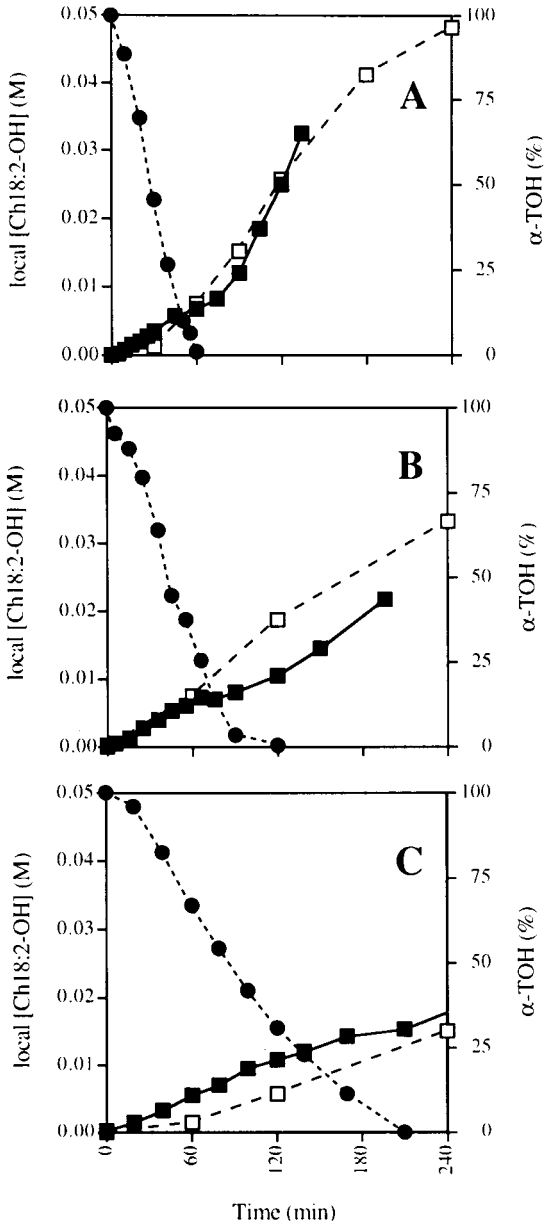

Figure 1. Oxidation of native and α-TOH-depleted LDL with the unsymmetrical azo initiator **C-8**. Native LDL (closed symbols) and α-TOH-depleted LDL (open symbols) was initiated at 37 °C in the presence of initiator **C-8**: 2.0 (A), (B), or 0.5 mM (C). Cholesteryl linoleate oxidation products (total of all four isomers) (squares) were determined by normal-phase HPLC with UV detection at 234 nm as described in the Experimental Procedures and reported as local concentration (all oxidations [LDL] = 0.75 mg of protein/mL). The α-TOH-depleted LDL experiments were corrected for Ch18:2-OH (∼0.002 M) formed during the depletion process. The percent of α-TOH remaining (circles) was determined by reversed-phase HPLC with electrochemical detection. The initial local α-TOH concentrations for native LDL and α-TOH-depleted LDL were ∼4.5 and 0.2 mM, respectively.

(29) The concentrations of the reduced cholesteryl linoleate oxidation products (Ch18:2-OH) and initial α-TOH have been reported as local concentrations to provide a more accurate representation of their surroundings within a lipoprotein particle.

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Table 1. Cholesteryl Linoleate Oxidation Rates in Native[a,b] and α-TOH-Depleted LDL[a,c]

initiator	concn, mM	R_i [d]	$R_{p,inh}$	R_p^-	R_{i+UA}	$R_{p,inh+UA}$	$R_p^{-}{}_{+UA}$
C-8	0.5	7.8 (2.7)	15.2 (0.9)	12.2	5.1 ± 2.4	14.9 ± 1.3	4.4 ± 0.3
	1.0	15.4 ± 2.5	18.7 ± 0.4	26.9 (7)	9.2	15.5	5.2
	2.0	25.0 (0.7)	19.8 (1.2)	45.7	17.0 (5.2)	22.8 (2.5)	8.3
C-12	0.5	3.2	8.8	2.1 (0.7)	2.2 (0.5)	6.3 (6.3)	1.8
C-16	0.5	1.8 (0.2)	7.4 (3.4)	0.9 (0.8)	2.2 ± 0.6	5.6 ± 1.5	0.8
C-0	0.5				1.2	9.8	0.7 (0.1)
	1.0	7.3	9.4	5.6 (3.5)	2.0	9.6	2.1
MeOAMVN	0.25	4.5	11.3	7.3	6.7 (6.3)	16.3 (8.2)	2.0 (0.9)

[a] All rates are given in units $\times 10^{-7}$ M s^{-1} and are means with the range given in parentheses when $n = 2$ or ±SD when $n = 3-5$. LDL concentrations were 0.75 mg of protein/mL. [b] Local rates of Ch18:2-OH formation in oxidizing native LDL containing endogenous α-TOH ($R_{p,inh}$) (~4.5 mM, local concentration), or α-TOH with added UA (~60 μM, bulk concentration) ($R_{p,inh+UA}$). [c] Local rates of Ch18:2-OH formation in oxidizing α-TOH-depleted LDL (~95% α-TOH consumed) (R_p^-), or α-TOH-depleted LDL with added UA ($R_p^{-}{}_{+UA}$). [d] R_i calculated as described in Experimental Procedures.

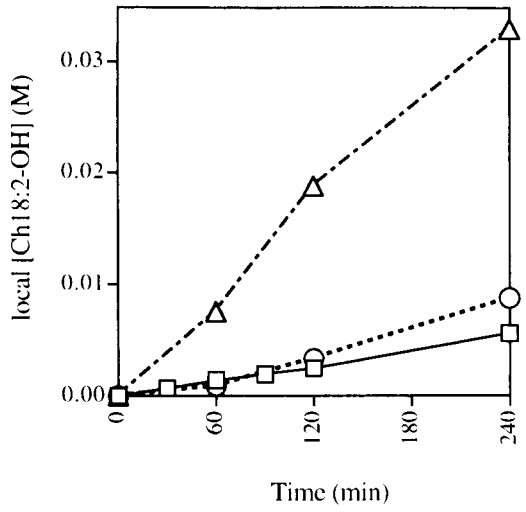


Figure 2. Symmetrical azo initiator oxidation of α-TOH-depleted LDL. Oxidation of α-TOH-depleted LDL at 37 °C in the presence of initiator 0.25 mM MeOAMVN (circles) or 1.0 mM **C-0** (squares). Cholesteryl linoleate oxidation products were determined as indicated in Figure 1. Oxidiations in α-TOH-depleted LDL using 1.0 mM **C-8** (Δ) are also shown to compare similar rates of radical generation from **C-8** and the two symmetrical initiators MeOAMVN (0.25 mM) and **C-0** (1 mM).

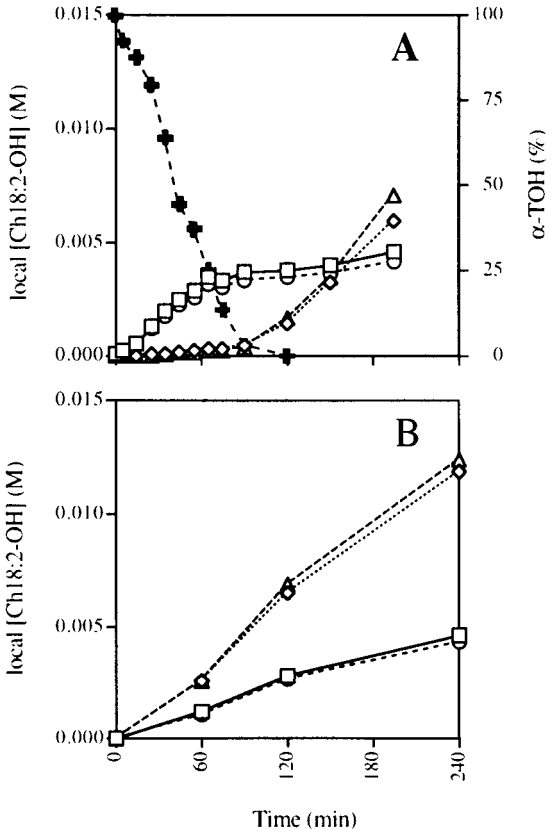


Figure 3. Ch18:2-OH isomer profiles in **C-8**-initiated native and α-TOH-depleted LDL. Native LDL (A) and α-TOH-depleted LDL (B) was initiated at 37 °C in the presence of initiator **C-8** (1.0 mM). Cholesteryl linoleate oxidation products were reduced with PPh_3 and determined by normal-phase HPLC, 0.5% isopropyl alcohol in hexanes with UV detection ($\lambda = 234$ nm), as described in the Experimental Procedures. Oxidation products were *trans,cis*-13-OH Ch18:2 (□); *trans,trans*-13-OH Ch18:2 (◇); *cis,trans*-9-OH Ch18:2 (○); *trans,trans*-9-OH Ch18:2 (Δ). The percent of α-TOH remaining (crosses) was determined by reversed-phase HPLC with electrochemical detection.

the thermodynamic trans,trans cholesteryl linoleate oxidation products. Oxidation in the α-TOH-depleted LDL displayed the expected predominance of the thermodynamic trans,trans products (Figure 3B). One may note that the cis,trans and trans,cis isomers accumulate at a higher rate in α-TOH-depleted LDL (Figure 3B) than in the post-tocopherol phase in native LDL (Figure 3A). The decomposition of linoleic acid hydroperoxides has recently been reported to be rather rapid,[33] and cholesteryl hydroperoxides are expected to decompose in similar pathways. Thus, decomposition of the cis,trans and trans,cis hydroperoxide pool at the beginning of the post-tocopherol phase is likely competing with hydroperoxide formation to decrease the observed rate compared to the α-TOH-depleted experiment.

The effect of aqueous antioxidants in α-TOH-depleted LDL oxidation was also studied and is reported in Supporting Information. Inclusion of the aqueous antioxidant UA decreases lipid peroxidation in α-TOH-depleted LDL but not native LDL (Figure SI-1, Supporting Information). The primary effect of UA on oxidation of native LDL was to increase the induction period. The powerful aqueous coantioxidant AH prevents the oxidation of **C-8**-initiated LDL (Figure SI-2, Supporting Information).

Initiator Partitioning between Aqueous and Lipid Phases. To provide a better understanding of α-TOH's involvement in radical phase transfer, it was important to try to determine the location where different initiators generated free radicals. Ideally, water-soluble azo initiators would generate radicals in the aqueous phase, lipid-soluble azo initiators would produce radicals in the lipid phase, and unsymmetrical amphiphilic initiators would decompose at the interface, providing radicals in both the lipid and aqueous phases.[16,17] The initiators having lipophilic character (**C-8**, **C-12**, **C-16**, and especially MeOAMVN) were expected to associate with the lipoprotein particle.[30]

(33) Schneider, C.; Tallman, K. A.; Porter, N. A.; Brash, A. R. *J. Biol. Chem.* **2001**, *276*, 20831−20838.

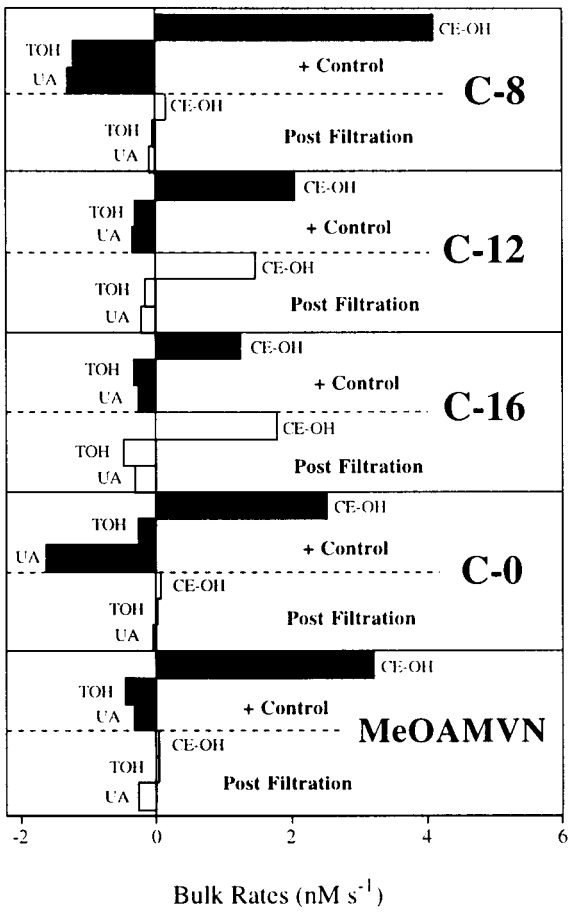


Figure 4. Initiator incorporation into LDL. Native LDL (2 mg of protein/mL) was treated with initiator **C-8** (2.7 mM**), C-12** (1.3 mM), **C-16** (1.3 mM), **C-0** (2.7 mM), or MeOAMVN (0.7 mM) (initiator/LDL ratios were 1, 0.5, 0.5, 1, or 0.25 mM per 0.75 mg of protein/mL, respectively) for 30 min at room temperature. A portion of each initiator LDL mixture was set aside and stored at 4 °C for positive control oxidations. The remaining initiator-treated LDL samples were each passed through two consecutive PD-10 size exclusion columns to remove any unassociated initiator from the LDL. Protein concentrations were redetermined for the postfiltration samples. Both sets of LDL samples, + control (solid bars) and postfiltration (open bars), were adjusted to 0.75 mg of protein/mL containing endogenous α-TOH (12 μM, bulk concentration) and added UA (60 μM). The oxidations were begun by incubation at 37 °C. During the first 5 h of oxidation, bulk rates of cholesteryl linoleate oxidation product formation and rates of α-TOH or UA consumption were determined as presented in Experimental Procedures. Surprisingly, the lipid-soluble initiator MeOAMVN was not retained in the postfiltration LDL mixture.

To examine the incorporation of the initiators into the lipoprotein particle, native LDL was incubated with initiator for a short time at room temperature, followed by size exclusion chromatography (through PD-10 gel filtration columns). This filtration process removed initiators or any other compounds that were partitioned into the aqueous phase of the LDL mixture (Figure 4).[13] After PD-10 treatment, UA was added and the isolated LDL were heated to 37 °C to determine whether initiator was present. For the unsymmetrical azo initiators, the length of the hydrocarbon chain affected the partitioning of initiator into the LDL particle. The imidazoline derivative initiators with no chain or a short chain (**C-0** and **C-8**, respectively) were effectively removed by filtration, as signified by the absence of oxidation products and antioxidant consumption when the reisolated LDL were heated to 37 °C. The longer chain, **C-12** and **C-16**, initiators showed effective initiation of LDL oxidation, comparable to the positive control, even after filtration. Unexpectedly, the lipophilic initiator MeOAMVN produced no significant lipid oxidation or α-TOH consumption in LDL after the size exclusion filtration.[30] MeOAMVN, as well as the unsymmetrical azo initiators, was added to LDL as a concentrated solution in methanol (≤3% v/v, final concentration) and showed no signs of precipitating initiator or disruption of the LDL suspension. These results support the notion that the initiators **C-0**, **C-8**, and MeOAMVN partition mostly to the aqueous phase; however, the initiators **C-12** and **C-16** were significantly incorporated into the lipoprotein particle.

Agarose gel electrophoresis confirms association of the unsymmetrical azo initiators **C-12** and **C-16** with LDL, by altering electrophoretic mobility as reported previously.[17] However, the electrophoresis experiments (shown in Supporting Information) suggest that concentrations above 0.5 mM for **C-12** or **C-16** (per 0.75 mg of protein/mL) may disrupt the LDL particles. Critical micelle concentrations were determined at 23 °C by the Wilhelmy plate method ([**C-8**] > 5 mM; [**C-12**] > 4 mM; [**C-16**] = 0.8 mM).[25]

The more water-soluble unsymmetrical initiator **C-8** did not show a significant shift in LDL electrophoretic mobility even at 3.0 mM.[34] Addition of other positively charged water-soluble initiators such as AAPH and **C-0** also do not alter the migration compared to the untreated LDL (data not shown). This suggests that even though **C-8** has an amphiphilic structure similar to **C-12** and **C-16**, the shorter hydrocarbon chain allows **C-8** to partition primarily to the aqueous phase in a lipoprotein suspension.

Lipophilic and hydrophilic radical scavenging studies suggest that there is efficient lipophilic radical generation efficiency in LDL with the unsymmetrical azo initiator **C-8**. The lipophilic radical scavenger used was the endogenous α-TOH and the hydrophilic radical scavenger used was UA. As noted previously, these efficient antioxidants work independently of each other and trap two peroxyl radicals, with α-TOH presumably being biased toward reacting with lipid-soluble radicals, and UA presumably reacting with water-soluble radicals.[35,36] To evaluate the radical generation in different regions of a LDL suspension, the rate of consumption of both α-TOH and UA was determined by HPLC.[16] Scheme 1 gives a representation of unsymmetrical azo initiator decomposition in a lipoprotein providing peroxyl radicals that would be scavenged in different regions based on the resulting peroxyl radical's solubility. Total, lipophilic, and hydrophilic ek_d values (e_Tk_d, e_Lk_d, and e_Hk_d, respectively) were calculated by dividing the consumption rate of the appropriate antioxidant by the initiator concentration (eq 1).[36,37] Radical generation was determined in oxidizing LDL

$$e_Tk_d = e_Lk_d + e_Hk_d = \frac{d[\alpha\text{-TOH}]/dt}{[\text{initiator}]} + \frac{d[\text{UA}]/dt}{[\text{initiator}]} \quad (1)$$

containing both antioxidants α-TOH and UA, as well as in oxidizing LDL containing only α-TOH (Figure 5). These data represent oxidations conducted in LDL from three different donors. The low lipophilic scavenging of **C-0** suggests that the aqueous peroxyl radicals it generates do not have efficient access to the lipid core of LDL. Each of the unsymmetrical initiators

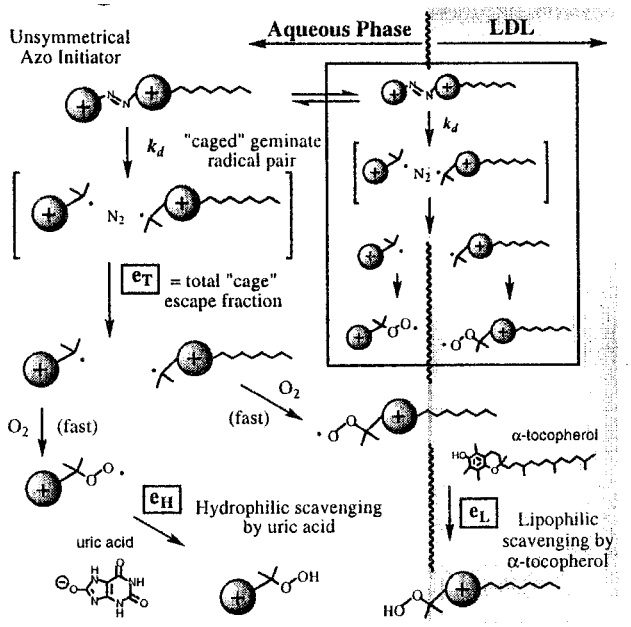
(34) **C-8** is readily soluble in water and methanol; however, **C-12** and **C-16** are only sparingly soluble in water unless a lipid domain is present.

(35) Simic, M. G.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1989**, *111*, 5778−5782.

(36) Niki, E.; Saito, M.; Yoshikawa, Y.; Yamamoto, Y.; Kamiya, Y. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 471−477.

(37) Barclay, L. R. C.; Locke, S. J.; MacNeil, J. M.; VanKessel, J.; Burton, G. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1984**, *106*, 2479−2481.

Scheme 1. Unsymmetrical Initiator Decomposition and Radical Scavenging



tested displayed significantly greater proportion of lipophilic scavenging (e_Lk_d/e_Tk_d) than did **C-0** (**C-8**, **C-12**, **C-16**, ~0.4; **C-0**, ~0.13). The radical scavenging for MeOAMVN was significantly greater in LDL suspensions than had been found for either micelles or liposomes reported previously.[16] However, the filtration experiments presented in Figure 4 demonstrate that MeOAMVN likely decomposes significantly in the aqueous phase where macromolecular cage escape does not participate as in the lipoprotein.

Decomposition Rate Constants for Azo Initiators Determined by Nitrogen Evolution. Typically, azo initiator decomposition rate constants (k_d) have been estimated from initiator decomposition in organic solvents or buffer.[38,39] For decomposition of water-soluble initiators, such as AAPH or **C-0**, this assumption of k_d values from organic solvents or buffer likely provides an accurate estimate of the decomposition rate in liposome and lipoprotein suspensions.[10] However, when the initiator decomposition may occur significantly in the lipid regions of molecular aggregate structures, the k_d value assumed from solvent decomposition is less certain. Previously, we reported decomposition rate constants for the initiators **C-0**, **C-8**, **C-12**, and **C-16** determined at 37 °C in methanol by monitoring loss of the azo absorption in the UV at 366 nm (Table 2).[16] This UV analysis was also applied to measure the k_d for **C-0** in PBS pH 7.4 and some micellar media. Rate constants for unsymmetrical azo initiators could not be determined by the UV method in buffer, micelles, liposomes, or lipoproteins.

Decomposition rate constants for the initiators presented here have been determined at 37 °C in several appropriate media (buffer pH 7.4, PC liposomes, or LDL suspensions[40]) by measuring the nitrogen gas evolved over ≥3 half-lives (Table 2).[39] The evolution of nitrogen gas was monitored in a pressure-sensitive apparatus that included a reference and sample cell separated by a pressure transducer that was connected to a recording device.[26] This method provides the advantage that initiator decomposition can be measured directly in the system of interest, i.e., LDL.[41] The k_d values determined by nitrogen evolution for the initiators tested were actually similar to the values estimated from the UV method in methanol.[16] For **C-0** decomposition in PBS pH 7.4, the UV method underestimated the k_d compared to nitrogen evolution. Surprisingly, the rate constant for decomposition of the lipid-soluble MeOAMVN in PC liposomes was only about half the value reported for decomposition in organic solvent.[30] (However, considering that partitioning experiments presented here suggest MeOAMVN decomposes in the aqueous phase, we assume that the k_d measured in acetonitrile gives a better approximation of decomposition in free solution.) Overall radical generation

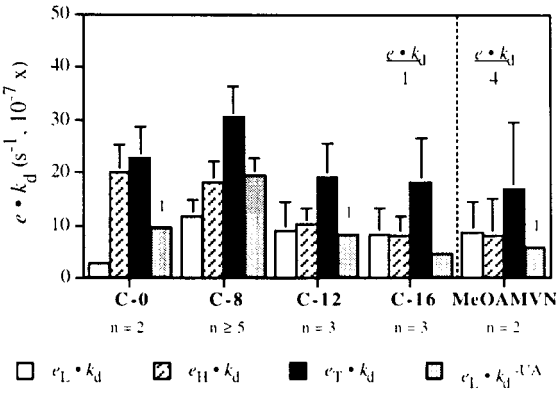


Figure 5. Lipophilic/hydrophilic radical scavenging by α-tocopherol and uric acid in LDL. Radicals scavenged by the lipophilic (e_Lk_d) antioxidant α-TOH (~12 μM, bulk concentration) and the hydrophilic (e_Hk_d) antioxidant UA (60 μM). LDL concentrations were 0.75 mg of protein/mL and initiated at 37 °C by **C-0** (0.5−1.0 mM), **C-8** (0.5−2.0 mM), **C-12** (0.5 mM), **C-16** (0.5 mM), or MeOAMVN (0.25 mM). Lipophilic scavenging in the absence of UA was also determined ($e_Lk_d^{-UA}$). Because of the ~4-fold faster decomposition rate of the initiator MeOAMVN, the values of ek_d were divided by 4 to place all initiators on a comparable scale. The mean values ±SD are given for the number of experiments indicated (unless marked above the specific column).

Table 2. Decomposition Rate Constants for Azo Initiators Determined by Nitrogen Evolution[a]

	rate const, k_d			
initiator	MeOH[b]	PBS	X-100	PC
C-8	8.3	8.5 ± 1	8.5 ± 0.8	7.0 ± 1
C-16[e]	7.4		7.3 ± 0.9	6.8 ± 1
C-0	7.1	6.8 ± 0.4[d]		
MeOAMVN[e]	31.8[c]			18 ± 5

[a] Rate constants for decomposition (k_d, units × 10^{-6} s^{-1}) of azo initiators at 37 °C determined by evolution of nitrogen gas in PBS (50 mM, pH 7.4), Triton X-100 (0.1 M in PBS), or PC liposomes (10.3 mM in PBS).[51] [b] Decomposition in methanol at 37 °C was determined by loss of azo absorption in the UV at 366 nm and included for comparison (**C-12** (methanol) $k_d = 8.0 \times 10^{-6}$ s^{-1}). [c] MeOAMVN decomposition as reported in acetonitrile.[35] [d] Determination of k_d for **C-0** in PBS by the UV method reported previously was 4.7×10^{-6} s^{-1}.[18] [e] Rates for the decomposition of lipid-soluble initiators **C-16** and MeOAMVN in PBS or LDL could not be determined even with nitrogen evolution because their required addition in methanol solutions created significant background drift.

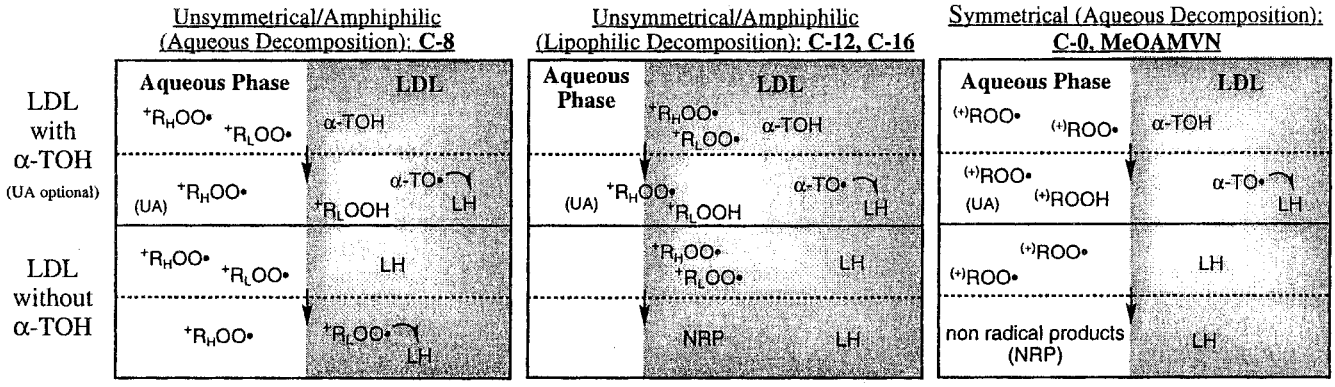
(38) Fujie, H.; Shiraki, K.; Miyagawa, T.; Minamii, N. *Pure Appl. Chem.* **1992**, *A29*, 741−751.

(39) Hammond, G. S.; Neuman, R. C. *J. Am. Chem. Soc.* **1963**, *85*, 1501−1508.

(40) Determination of **C-8** k_d was attempted in LDL suspension; however, the reproducibility of results was poor and did not allow for a reasonable estimate directly in LDL.

(41) This apparatus has typically been used to measure oxygen consumption during lipid peroxidations. However, peroxidation events causing loss of gas pressure were eliminated, by purging the solutions with nitrogen. Inclusion of both a sample and a reference cell allow contributions from vapor pressure to be corrected during the experiment, but when initiators required delivery in methanol there was too much reequilibration drift to yield accurate rate determinations.

Scheme 2. Apparent Azo Initiator Initiation Pathways in LDL



efficiencies for each initiator can be calculated by dividing the radical scavenging ability presented in Figure 5 by the appropriate k_d from Table 2 (e.g., **C-8**: $e_T = (e_T k_d)/k_d = 32 \times 10^{-7}/\sim 8.5 \times 10^{-6} = 0.38$).

Discussion

Two important factors contribute significantly to tocopherol-mediated peroxidation in LDL. They are as follows. (1) Chain transfer: the fairly unreactive α-TO• radical, locked and concentrated within the LDL particle, if given enough time reacts with lipid to form lipid peroxyl radicals. and (2) Phase transfer: oxidation processes within a lipoprotein are dependent on entry of radicals, or oxidants, into the lipid regions, and α-TOH can be an effective means for transporting radical character into the lipoprotein.[8,11] Oxidation studies in LDL are made difficult because of two fundamental shortfalls of the currently used initiators AAPH and AMVN.[10,16,17] First, AAPH-derived peroxyl radicals are water-soluble and rely on α-TOH to transfer aqueous radical character into the lipid via α-TO•[13] (and likely LOO• as well).[15] Second, efficient formation of initiator peroxyl radicals within the lipoprotein, as expected for AMVN, is severely limited by both poor cage escape and concentration maximums.[30]

The results presented here demonstrate that the unsymmetrical amphiphilic initiator **C-8** substantially circumvents the requirement of α-TOH for efficient initiation of LDL oxidation. In α-TOH-depleted LDL, generation of a **C-8**-derived amphiphilic peroxyl radical provided increased lipophilic character that enabled delivery of initiating radicals directly to LDL lipids (Figure 1). The delivery of initiating radicals to lipoprotein lipids without the aid of α-TOH phase transfer was ∼5-fold lower for the symmetrical initiators **C-0** and MeOAMVN (Figure 2, Table 1).

This advantage of **C-8** to effectively initiate oxidation of α-TOH-depleted LDL appears to be based on two important properties of **C-8**. (1) Generation of freely diffusing radicals in the aqueous phase free from confinements of the LDL macromolecular "cage": **C-8** could be removed from LDL suspensions by simple size exclusion chromatography, and addition of up to 3.0 mM **C-8** did not alter LDL's electrophoretic mobility (Figure 4, Figure SI-3). Furthermore, inclusion of aqueous radical scavengers, such as UA or AH, diminished (UA) or even halted (AH) **C-8**-induced oxidation of α-TOH-depleted LDL (Figure SI-1, Figure SI-2). (2) Increased partitioning of the resulting free radicals into the lipoprotein core lipids or phospholipid monolayer: The longer chain unsymmetrical initiators **C-12** and **C-16** could not be removed from LDL suspensions by size exclusion gel filtration (Figure 4). As expected, initiators bearing long hydrocarbon tails had an apparent increased solubility in the lipoprotein particle (Figure SI-3). Finally, the location of radical scavenging when both a hydrophilic antioxidant (UA) and a lipophilic antioxidant (α-TOH)[16] were used shows that **C-8** yields a higher portion of scavenged radicals trapped by the lipophilic α-TOH when compared to **C-0** (Scheme 1, Figure 5). ($e_L k_d/e_T k_d$ for **C-8** ∼0.4 and **C-0** ∼0.13) Scheme 2 provides a comparative representation of the apparent initiation pathways observed for **C-8** and the other initiators tested in this work.

The other initiators tested here with the imidazoline structure, **C-0**, **C-12**, and **C-16**, were not capable of efficiently oxidizing LDL without the requirement for α-TOH.[42] The unsymmetrical initiators **C-12** and **C-16** displayed unexpected resistance to oxidizing α-TOH-depleted LDL (Table 1). Interestingly, even though **C-12** and **C-16** significantly partitioned into the lipoprotein, they both showed substantial radicals scavenged in aqueous and lipophilic regions (Figure 5). It is reasonable to believe **C-16**, or even **C-12**, would decompose primarily in the lipoprotein particle and produce one hydrophilic peroxyl radical (that could escape to the aqueous phase) and one amphiphilic peroxyl radical that would remain in the lipoprotein of origin ($e_L k_d/e_H k_d \approx 1$).[43] It should be noted that **C-12** and **C-16** likely disrupt LDL structure at concentrations greater than 0.5 mM per 0.75 mg of protein/mL.

The lack of efficient initiation of oxidation in α-TOH-depleted LDL with **C-12** and **C-16** is not well understood. However, we speculate that **C-12** and **C-16** decompose in the phospholipid membrane to produce a pair of charged peroxyl radicals that remain near the lipoprotein surface. We suggest that in the absence of α-TOH, radical termination within the macromolecular cage ($[^+R_HOO^\bullet]$ 5.2×10^{-4} M × $[^+R_LOO^\bullet]$ 5.2×10^{-4} M × k_t ($1 \times 10^5 - 1 \times 10^7$ M^{-1} s^{-1}) ≈ 0.027−2.7 M s^{-1}) competes effectively with escape of the hydrophilic peroxyl radical ($^+R_HOO^\bullet$) (Scheme 2). However, when α-TOH is present in the lipoprotein, scavenging by α-TOH ($[^+R_{H(L)}OO^\bullet]$ 1.04×10^{-3} M × [α-TOH] $5.2 \times 10^{-4} - 6.2 \times 10^{-3}$ M × k_{inh} 1×10^6 M^{-1} s^{-1} ≈ 0.54−6.4 M s^{-1}) can compete effectively with radical termination of the initial radical pair ($^+R_HOO^\bullet$ and $^+R_LOO^\bullet$). This creates a new radical pair within the lipoprotein of $^+R_{H(L)}$-$OO^\bullet$ and α-TO•, which could reasonably result in an increased phase separation of the radical pair. For example, the uncharged and very lipophilic α-TO• radical is expected to rapidly diffuse into the core regions of the LDL. The positively charged

(42) Results using water-soluble symmetrical **C-0** were found comparable to the reported resistance of α-TOH-depleted LDL to AAPH-induced oxidation (Figures 2, 6, and 8).

(43) In α-TOH-depleted LDL, the inclusion of UA did not increase the effectiveness of the **C-12** or **C-16** (scavenging a majority of the hydrophilic peroxyl radicals was expected to decrease termination events from interparticle radical transfer through the aqueous phase).

initiator-derived radical $^{+}R_{H(L)}OO^{\bullet}$ could then either escape from the lipoprotein or be scavenged by an aqueous antioxidant (such as UA) near the surface before radical termination within the macromolecular cage.

A rather unexpected result of these studies was the apparent inability of the lipophilic symmetrical azo initiator MeOAMVN to incorporate into the lipoprotein lipid regions.[44] MeOAMVN has the shortest half-life for decomposition of any of the initiators studied and this should help overcome poor cage escape for this initiator.[30] LDL depleted of α-TOH was resistant to lipid peroxidation in the presence of MeOAMVN, despite comparable radical generation and radical scavenging to **C-8** (0.25 mM MeOAMVN ≈ 1 mM **C-8**) (Figure 2, Table 1, Figure 5). Size exclusion gel filtration resulted in removal of the initiator and prevention of lipid and α-TOH oxidation of MeOAMVN LDL (Figure 4). These findings support the conclusion that MeOAMVN primarily decomposes to produce free radicals in the aqueous phase and the derived "lipophilic" peroxyl radicals require α-TOH for effective transfer of radical character into the lipoprotein (Scheme 2).

Several factors could be contributing to this behavior of MeOAMVN in LDL. (1) The methoxy substituent significantly increases the initiator's partitioning into the aqueous phase compared to AMVN.[45] In the same respect, formation of the peroxyl moiety significantly increases the polarity of the "lipophilic" MeOAMVN derived radicals.[12,45] (2) MeOAMVN may indeed incorporate into the LDL to some extent, but the rate of decomposition and cage escape efficiency may be significantly less in the lipoprotein lipid.[10,46] (3) Diffusion of the "lipophilic" MeOAMVN initiator or the derived "lipophilic" peroxyl radicals across the phospholipid monolayer may be significantly less than expected. The initiator AMVN was not tested here, but these results with MeOAMVN indicate the traditional view[30] of both these "lipophilic" initiators producing free radicals within the lipoprotein lipid may be questionable.

We conclude that the specific locations of initiator decomposition and free radical production are critical factors of the LDL oxidation mechanism. The important aspects of **C-8** that make it the most effective initiator tested in α-TOH-depleted LDL are largely based on solubility of both the parent azo initiator and the derived peroxyl radicals.[47]

Even though the initiator **C-8** circumvents the requirement of α-TOH activity as a peroxyl radical phase-transfer agent, our results suggest that the mechanism of oxidation remains controlled by TMP,[12] largely via α-TOH's chain-transfer activity. It is generally viewed that α-TOH-inhibited peroxidation can proceed through two fundamentally different oxidation mechanisms, conventional and tocopherol-mediated.[9] These models have previously been mathematically simulated, and the results suggest that the conventional mechanism should display a dependence of the rate of LOOH formation on the concentration of initiator, during both the presence and the absence of the antioxidant α-TOH.[12] For the TMP mechanism, the rate of LOOH formation in the presence of α-TOH was simulated to be independent of initiator concentration. (For a more detailed discussion of initiator concentration dependence on the conventional and TMP models see the Supporting Information.) Kinetic analysis of rates of LOOH formation in **C-8** initiated LDL oxidation with α-TOH present ($R_{p,inh}$) or absent ($R_{p,uninh}$) demonstrates that $R_{p,uninh}$ but not on $R_{p,inh}$ shows a significant concentration dependence on this new and effective initiator (Table 1, Figure SI-4). This lack of initiator concentration dependence in the presence of α-TOH ($R_{p,inh}$) strongly suggest a TMP chain-transfer mechanism.

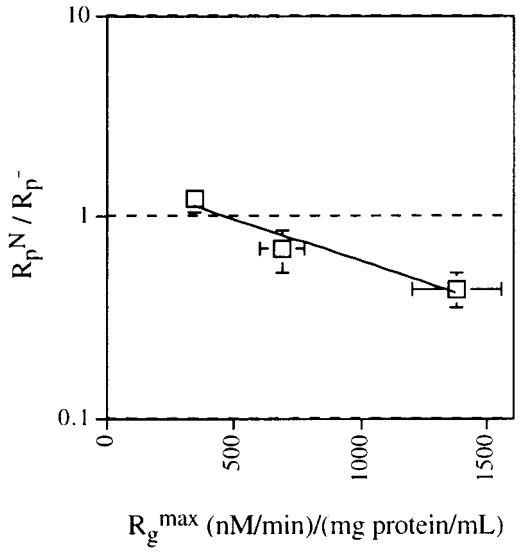


Figure 6. Antioxidant and pro-oxidant activity of a-TOH in LDL initiated with **C-8**. The ratio of the rates of cholestryl linoleate oxidation propagation in native LDL (R_p^N) to α-TOH-depleted LDL (R_p^-) compares the oxidizability of LDL in the presence and absence of α-TOH. Values above 1 indicate pro-oxidant activity and values below 1 indicate antioxidant activity. Oxidations were initiated with **C-8**, 0.5, 1.0, or 2.0 mM in LDL (0.75 mg of protein/mL). Radical generation was calculated by assuming no correction for efficiency and $k_d \approx 8.6 \times 10^{-6}$ s^{-1}. **C-8** provided neutral activity for α-TOH ($R_p^N/R_p^- = 1$) when the radical generation was 470 ± 60 nM/min per mg of protein/mL.

Depending on the degree of radical generation or the reactivity of the oxidant, α-TOH has been shown to exhibit anti- or pro-

(44) It has been previously observed that α-TOH-depleted LDL was resistant to oxidation by the very similar analogue AMVN. Both initiators are lipophilic organic-soluble compounds that are believed to readily incorporate into the lipoprotein lipid.

(45) The calculated values for log *P* of MeOAMVN and AMVN were 3.15 ± 0.62 and 4.12 ± 0.58, respectively. The calculated values for log *P* of the derived hydroperoxides ((1/2R)-OOH) of MeOAMVN and AMVN were 1.25 ± 0.56 and 1.74 ± 0.53, respectively. The predicted value of log *P* was obtained using the ACD/I-Lab Web service.

(46) That fraction of initiator that decomposes in the aqueous phase may therefore initiate radical chains more efficiently than does the lipid-bound initiator.

(47) LDL oxidation initiation with **C-8** substantially avoids the requirement for α-TOH phase transfer and delivers peroxyl radicals directly to the lipoprotein lipids, which favors an antioxidant activity for α-TOH at significantly lower radical generation rates. This should also present the lipoprotein lipids a more constant rate of initiation than oxidants dependent on phase transfer via α-TOH, because as the concentration of α-TOH diminishes so does the rate of radicals entering the lipoprotein. It would be of interest, for example, to probe the extent that **C-8** avoids α-TOH phase-transfer effects by varying the levels of α-TOH within the lipoprotein (by partial depletion or enrichment)[13] and kinetic isotope experiments (by oxidizing LDL in D_2O). (Witting, P. K.; Bowry, V. W.; Stocker, R. *FEBS Lett.* **1995**, *375*, 45−49.) It would also be interesting to evaluate phospholipid oxidation products in comparison to cholesteryl ester oxidation products. The more consistent entry of radicals into the lipoprotein may also be of value for examining the kinetics of lipid peroxidation and antioxidant activity directly in lipoprotein suspensions. (Culbertson, S. M.; Antunes, F.; Havrilla, C. M.; Lohr, G. J.; Porter, N. A., manuscript in preparation.) It should be kept in mind that experiments longer than a few hours result in a decrease in radical production from **C-8** ($k_d = 8.5 \times 10^{-6}$ s^{-1} in LDL, or $t_{1/2} = 22.6$ h). Considering that the synthesis and purification of the initiator **C-8** is a significant undertaking, we point out that similar amphiphilic unsymmetrical azo initiators may be significantly easier to make than **C-8**. For example, we have reported the synthesis and limited study of initiators having structures similar to AAPH but which bear one stearyl side chain.[17] The research presented here indicates that shorter hydrocarbon chains (likely anywhere from 6 to 12 carbons) on these unsymmetrical substituted amidine azo initiators could be potentially effective lipoprotein initiators that circumvent the requirement for α-TOH-mediated phase transfer.

oxidant effects.[11] Interestingly, results presented here show that the initiator **C-8** provides free amphiphilic peroxyl radicals that circumvents α-TOH pro-oxidant activity at a level comparable to that reported for the very reactive •OH.[13] These effects can be evaluated by comparing oxidation rates in LDL with (R_p^N) and without (R_p^-) α-TOH (Figure 6). A ratio of R_p^N/R_p^- above 1 indicates pro-oxidant activity, and a ratio below 1 indicates antioxidant activity. The point at which α-TOH activity switched from a pro-oxidant to an antioxidant ($R_p^N/R_p^- = 1$) for **C-8** was reached at a maximal radical generation rate (R_g^{max})[14] of 470 ± 60 nM/min per mg of protein/mL LDL. In comparison, the switching point for •OH and AAPH was reported to be reached at a maximal radical generation of 130 and 250 nM/min per 0.25 mg of protein/mL LDL, respectively[13] (or •OH 520 nM/min per mg of protein/mL, and AAPH 1000 nM/min per mg of protein/mL). The difference in the maximal radical generation required for neutral α-TOH activity of •OH compared to AAPH-derived peroxyl radicals has been explained on the basis of the reactivity differences between the two radicals.[11] The fact that **C-8** amphiphilic peroxyl provides antioxidant activity at a radical generation comparable to the very reactive •OH suggests that radical access, rather than reactivity, significantly controls the antioxidant behavior of α-TOH in a lipoprotein. Thus, when initiating radicals depend largely on α-TOH-mediated phase transfer, the activity of α-TOH favors a pro-oxidant result.

The unsymmetrical amphiphilic azo initiators studied here were designed to improve the in vitro tools for initiating controlled lipoprotein oxidations and to provide a better understanding of oxidation and antioxidant defenses. We believe the new initiators show that radical structure and transfer into lipoprotein lipid are complicated and important aspects influencing the apparent activity of α-TOH in LDL. The oxidants responsible for in vivo oxidation of lipoproteins are not fully characterized.[1,11] However, it is interesting to compare the effective **C-8** initiation of α-TOH-depleted LDL to recent work using soybean lipoxygenase-1 and pancreatic (type I) phospholipase A_2.[48] It is noted that α-TOH-depleted LDL was resistant to oxidation by lipoxygenase alone but overcome by addition of lipase. The authors conclude that inclusion of lipase and lipoxygenase together produces free fatty acid peroxyl radicals (FFA-OO•), which may escape the lipoprotein particle and transfer free radical chain reactions to other lipoprotein particles. It is probable that peroxyl radicals of amphiphilic nature may be generated in vivo and their presence could have a substantial impact on lipoprotein oxidation.

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Supporting Information Available: Additional information as noted in text. This material is available free of charge on the Internet at http://pubs.acs.org.

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