Fatty Acid Composition of Low-Density Lipoprotein Influences Its Susceptibility to Autoxidation†

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ABSTRACT: Low-density lipoprotein (LDL) oxidation was studied using copper or the water-soluble initiator azobis(2-amidinopropane) dihydrochloride (ABAP) to catalyze the reaction. These studies were carried out with purified, native LDLs that had a well-defined composition and which contained different concentrations of polyunsaturated fatty acids (PUFA) and α-tocopherol. The LDL was obtained from nonhuman primates fed diets enriched in cholesterol and one of four types of fatty acids: saturated (Sat), monounsaturated (Mono), ω-6 (ω-6FA), or ω-3 (ω-3FA) fatty acids. The PUFA concentration of the LDLs depended upon the diet and had the following order: ω-6FA > Sat ≈ Mono ≈ ω-3FA. Linoleic acid was the predominant PUFA in all of the LDLs. The rates of oxidation were linearly dependent upon the concentration of PUFA. When ABAP was used to initiate oxidation the lag time was linearly related to the amount of α-tocopherol. However, with copper catalysis no linear correlation was evident. If the different enrichments were analyzed independently, it was found that copper-catalyzed oxidation of LDLs enriched with ω-6 and ω-3 fatty acids showed a linear correlation between the lag time and the amount of α-tocopherol but that LDLs enriched with Sat or Mono fatty acids did not show a correlation. These results demonstrate that the rate of oxidation is dependent upon PUFA concentration and that the ability of α-tocopherol to inhibit oxidation depends upon the lipid environment and the mode of initiation.

Free-radical chain oxidation of low-density lipoprotein (LDL)[1] has been suggested as a possible *in vivo* mechanism for modifying LDL and thereby facilitating its uptake by macrophages (Steinberg et al., 1989) during atherogenesis. LDL has been oxidized *in vitro* by incubating native LDL with several types of cultured cells (Henriksen et al., 1981; Henriksen et al., 1983; Hiramatsu et al., 1983; Morel et al., 1984); leukocytes (Cathcart et al., 1985), lipoxygenases (Parthasarathy, et al., 1989; Rankin et al., 1991), transition-metal ions (Steinbrecher et al., 1984; Dousset et al., 1990), UV radiation (Dousset et al., 1990), or azo initiators (Sato et al., 1990) or by simply letting the LDL sit for several months (Liao et al., 1991). Free-radical oxidation of LDL produces fatty acid hydroperoxides from the polyunsaturated fatty acids (PUFA) (Lenz et al., 1990), oxidized cholesterol derivatives (Bhadra et al., 1991), and aldehydes from the decomposition of the hydroxyperoxides and the chemical and structural modification of apoprotein B (apoB) (Steinbrecher, 1987). The amounts and types of products depend upon the conditions under which the oxidation is performed. Mild oxidation like that initiated by UV irradiation gives mostly hydroperoxides (Dousset et al., 1990). Copper oxidation generates lipid hydroperoxides and aldehydes, and causes the loss of amino moieties from apoB and the fragmentation of apoB (Steinberg et al., 1989; Esterbauer et al., 1992). When the oxidation process does not significantly change the charge on apoB, oxidized LDL is taken up through the LDL receptor (Negre-Salvayre et al., 1990). In contrast, LDLs that have been subjected to vigorous oxidation are taken up via the scavenger receptor (Steinberg et al., 1989). Irrespective of how oxidized LDL is transported into the cell, the toxicity of oxidized LDL appears to be related to the amount of hydroperoxide that is carried by the oxidized LDL (Cathcart et al., 1985; Negre-Salvayre et al., 1990; Morel et al., 1983).

Autoxidation is a complex process that involves the chain reaction of radical species giving hydroperoxides as the major product. Although all of the olefinic compounds carried by LDL will undergo autoxidation the PUFAs, constituents of more complex lipids like the cholesteryl esters, phospholipids, and triglycerides are more susceptible to free-radical chain autoxidation than are other fatty acids or cholesterol. Therefore, PUFA hydroperoxides are the major products at the earliest stages of autoxidation.

The first step of autoxidation is the initiation of the free-radical chain by a preformed radical species. In this study, two different types of water soluble initiators, Cu^{2+} or azobis-(2-amidinopropane) dihydrochloride, were used to initiate LDL autoxidation. ABAP was chosen because it initiates autoxidation with a hydroperoxyl radical that is similar in some respects to the chain-carrying radical of PUFA autoxidation, $PUFAO_2^{\bullet}$. A simplified mechanism for the ABAP initiated autoxidation of PUFA is shown in Scheme 1, steps 1–6. Copper catalysis was employed because of its widespread use in the preparation of oxidized LDL for biological studies. The scheme for copper is similar to that for ABAP except that steps 1 and 3, the generation of the initiating radical species, are not as well defined and may involve the participation of an alkoxy radical. Several lines of evidence suggest that preformed hydroperoxide carried by LDL may be required for copper to initiate autoxidation (Thomas & Jackson, 1991).

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[1] ABAP, azobis(2-amidinopropane) dihydrochloride; LDL, low-density lipoprotein; Mono, monounsaturated fatty acids; PUFA, polyunsaturated fatty acid; Sat, saturated fatty acids; ω-6FA, ω-6 fatty acids; ω-3FA, ω-3 fatty acids.

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Scheme 1

$$\text{ABAP} \rightarrow 2\text{R}^{\bullet} + \text{N}_2 \quad \text{step 1}$$

$$\text{R}^{\bullet} + \text{O}_2 \rightarrow \text{RO}_2^{\bullet} \quad \text{step 2}$$

$$\text{RO}_2^{\bullet} + \text{PUFA} \rightarrow \text{RO}_2\text{H} + \text{PUFA}^{\bullet} \quad \text{step 3}$$

$$\text{PUFA}^{\bullet} + \text{O}_2 \rightarrow \text{PUFAO}_2^{\bullet} \quad \text{step 4}$$

$$\text{PUFAO}_2^{\bullet} + \text{PUFA} \rightarrow \text{PUFAO}_2\text{H} + \text{PUFA}^{\bullet} \quad \text{step 5}$$

$$2\text{PUFAO}_2^{\bullet} \rightarrow \text{nonradical products} \quad \text{step 6}$$

$$\text{PUFAO}_2^{\bullet} + \alpha\text{-tocopherol} \rightarrow \text{PUFAO}_2\text{H} + \alpha\text{-tocopherol}^{\bullet} \quad \text{step 7}$$

$$\alpha\text{-tocopherol}^{\bullet} + \text{PUFAO}_2^{\bullet} \rightarrow \alpha\text{-tocopherol–O}_2\text{PUFA} \quad \text{step 8}$$

The chain reaction depicted in steps 4 and 5 is responsible for the formation of the hydroperoxides characteristic of autoxidation. Step 6 shows how the chain reaction is terminated by the spontaneous reaction of two hydroperoxyl radicals giving nonradical products. Radical chain reactions are also stopped by antioxidants like α-tocopherol. This phenolic antioxidant reacts with the hydroperoxyl radical, yielding a second radical species that does not readily abstract a hydrogen atom from PUFA. LDL carries several antioxidants, but α-tocopherol was found in the highest concentration. Esterbauer et al. (1991, 1992) demonstrated that the amount of α-tocopherol carried by LDL was directly related to the time required to initiate rapid autoxidation of LDL PUFA. Steps 7 and 8 depict the inhibition of PUFA autoxidation by α-tocopherol.

Using steps 1–6, eq 1 was derived for the formation of conjugated diene hydroperoxide (PUFAO_2H), R_p, after α-tocopherol had been depleted: k_p is the rate constant for propagation, R_i is the rate of initiation, k_t is the rate constant for termination. The derivation assumes that the radical

$$\text{d[conj diene]}/\text{d}t = R_p = k_p[\text{PUFA}]R_i^{0.5}/(2k_t)^{0.5} \quad (1)$$

$$R_i = 2ek_d[\text{ABAP}] = (n_i/t_{lag})[\alpha\text{-tocopherol}] \quad (2)$$

$$t_{lag} = (n_i/R_i)[\alpha\text{-tocopherol}] \quad (3)$$

species are present in low, steady-state concentrations and that the chains are relatively long. In addition, it was assumed that the LDL did not exchange radical species during the course of the oxidation. The low solubility of the lipids in aqueous solutions supports this assumption. The relationship between the lag time, t_{lag}, and α-tocopherol concentration was described by Boozer et al. (1955) and Niki et al., (1984), where e is the efficiency of chain initiation, k_d is the rate of decomposition of ABAP, and n_i is the stoichiometric number of radicals scavenged per molecule of inhibitor. Niki et al. (1984) have shown that each α-tocopherol stops two radical chains.

This report describes how fatty acid composition, particularly the PUFA content, and α-tocopherol concentration affect the oxidation of native LDL. This study demonstrates (1) that the rate of PUFA oxidation is directly related to the concentration of PUFA as suggested by the study of Bonanome et al. (1992) and (2) that the relationship between lag time and α-tocopherol concentration depends upon the method of initiation. One consequence of initiator-dependent t_{lag} is that without knowing how oxidation was initiated *in vivo* it would be difficult to predict how fatty acid substitution would affect *in vivo* oxidation.

EXPERIMENTAL METHODS

Chemicals and Reagents. All of the reagents used in the autoxidation study were of the highest commercial grade available. Azobis(2-amidinopropane) dihydrochloride was from Polyscience, Inc. Potassium phosphate (Ultrex), NaCl, and NaOH were from J. T. Baker. Milli-Q water was used in the preparation of solutions.

Source of LDL. Native LDL was obtained from cynomolgus monkeys fed diets as reported before (Rudel et al., 1991). The diets contained a caloric distribution of fat, 35%, carbohydrate, 48%, and protein, 17%. Sufficient cholesterol, 0.4 mg/calorie, was added to induce high serum cholesterol levels. The fats used in these studies were lard which contains a high percentage of saturated fatty acids, safflower oil enriched in oleic acid, safflower oil enriched in linoleic acid, an ω-6 fatty acid, and menhaden oil which was enriched in ω-3 fatty acids. All of the animals were supplemented with vitamins including α-tocopherol, administered as DL-α-tocopherol acetate, and ascorbic acid included in the diet mixture at 10 g and 450 g per 10 kg of diet, respectively. Tenox 20A, active ingredient *tert*-butylhydroquinone, was included in all of the diets at a level of 0.2 g/kg of fat to retard autoxidation on storage. The animals were maintained in accordance with the guide lines of the Institutional Animal Care and Use Committee.

Blood samples were collected in 3 mM EDTA and stored on ice. The protease inhibitor cocktail of Edelstein and Scanu (Edelstein & Scanu, 1986) was added to prevent protein breakdown during isolation. The lipoproteins were separated by centrifugation, and the $d < 1.225$ g/mL fraction applied to an agarose column and LDLs were isolated by gravity filtration (Rudel et al., 1986). To retard oxidation 0.3 mM EDTA was added to the eluting buffer and the buffer was continuously sparged with nitrogen gas. All operations and sample storage were carried out at 4 °C. Samples were stored under argon.

Autoxidation. The autoxidation of LDL was studied at 37 °C in air-saturated solutions containing 10 μg/mL of apoprotein B in 25 mM phosphate buffer pH 7.4 containing 0.16 M in NaCl (PBS). Copper ion catalyzed autoxidation was carried out by adding sufficient copper sulfate to give a 3 μM solution. For studies employing copper, EDTA was removed by dialysis for 48 h at 4 °C against PBS that was continuously sparged with nitrogen gas. Autoxidation initiated by copper ions was compared to initiation by 666 μM ABAP. The oxidation of PUFA was monitored at 236 nm on a Cary Model 219 UV/vis spectrometer. The concentration of conjugated diene was calculated assuming an ϵ at 236 nm of 26 000 M^{-1} cm^{-1}. All analyses were performed in triplicate. The maximum rate of oxidation was determined from the slope of a plot of [conjugated diene] versus time. The intercept of this line with the time axis is called the lag time, t_{lag}.

Analyses. α-Tocopherol was quantified by the Lipid Laboratory of the Wake Forest University Comprehensive Cancer Center on a fee-for-service basis employing the HPLC method of De Leenheer et al. (1979). Fatty acid analyses

Table 1: Mole Percent PUFA of the Fatty Acids in LDL and in the Cholesteryl Ester and Phospholipid Fractions of LDL[a]

diet	cholesteryl ester[b]	phospholipids[c]	total PUFA[d]
Sat	19.2 ± 5.0†	5.6 ± 2.9†	24.9 ± 2.5†
Mono	13.9 ± 5.6†	5.0 ± 2.1†	18.9 ± 5.6†
ω-6FA	48.9 ± 9.2‡	8.1 ± 2.3†,‡	57.0 ± 9.4‡
ω-3FA	16.0 ± 4.5†	4.2 ± 2.4†,§	20.7 ± 4.0†

[a] Numbers with unlike superscripts are significantly different at the following levels. [b] $\rho = 0.001$. [c] $\rho = 0.03$. [d] $\rho = 0.001$.

Table 2: Fraction of the Different Types of PUFA (± SD) Found in LDL Reported as Mole Percent of the Total PUFA[a]

diet	X:2[b]	X:3[c]	20:4[d]	20:5, 22:5, 22:6[e]
Sat	87.2 ± 2.6†	3.7 ± 0.8†	8.9 ± 1.4†	0.2 ± 0.5†
Mono	87.3 ± 4.1†	4.3 ± 3.6†	5.2 ± 2.2‡	1.0 ± 2.0†
ω-6FA	97.6 ± 1.2‡	0.9 ± 0.8‡	1.3 ± 1.4§	0.3 ± 0.4†
ω-3FA	51.2 ± 9.5§	4.1 ± 2.4†	8.0 ± 2.7†	36.7 ± 8.5‡

[a] Numbers with unlike superscripts are significantly different at the following levels. [b] $\rho = 0.002$. [c] $\rho = 0.02$. [d] $\rho = 0.01$. [e] $\rho = 0.001$.

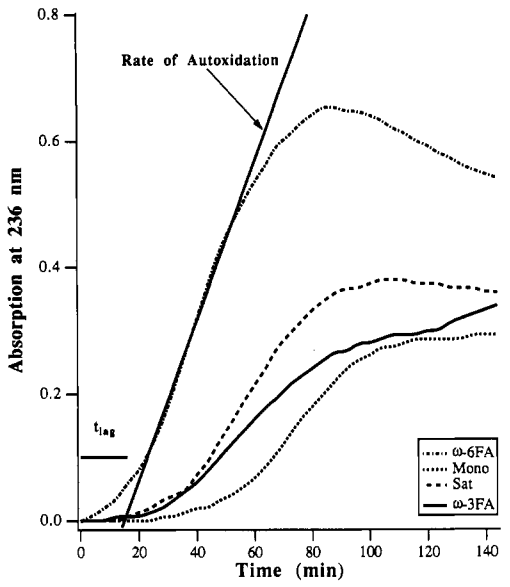

FIGURE 1: Typical plot of the absorption at 236 nm versus time for the copper ion catalyzed oxidation of LDLs from each of the four different fatty acid enrichments.

were carried out after saponification and methylation of the fatty acids (Metcalfe & Schmitz, 1961). Phospholipid and cholesterol ester fractions were separated and quantified by TLC (Rudel et al., 1977). Apoprotein B was measured by ELISA (Koritnik & Rudel, 1983). Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical Co., No. 4503) as the standard. ELISA values were related to Lowry determinations using column purified LDL containing >95% apoprotein B. To detect differences in the means, the results were analyzed by analysis-of-variance using the *F*-test for multiple comparison.

RESULTS

The distribution of PUFA in each of the LDLs was determined by GLC analysis, and the results are summarized in Tables 1 and 2. Table 1 shows the mole percent distribution (±SD) of LDL PUFA among the different diet classes. Table 2 shows the percent distribution (±SD) of the different PUFA types, e.g. dienes, trienes, pentaenes, and hexaenes, in each of the diet classes. The distribution of LDL phospholipids,

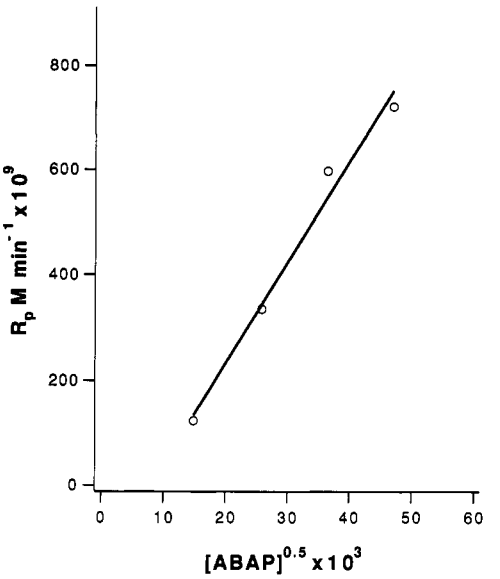

FIGURE 2: A plot of R_p versus $[ABAP]^{0.5}$ for one of the ω-6FA-enriched LDLs demonstrating the linear relationship anticipated from eq 1.

Table 3: Summary of the Rates of Oxidation of LDL Isolated from Nonhuman Primates Fed Diets Enriched in Different Fatty Acids[a]

		rate	
diet	*N*	copper catalyzed	ABAP catalyzed
high Sat	8	204 ± 105†	205 ± 31†
high Mono	8	202 ± 236†	196 ± 88†
high ω-6FA	9	827 ± 530‡	906 ± 393‡
high ω-3FA	9	153 ± 56†	67 ± 13†

[a] The rates of oxidation are given in picomoles conjugated diene per minute ± SD. *N* is the number of different samples. Numbers with unlike superscripts are significantly different at $\rho < 0.006$.

cholesteryl esters, cholesterol, triglyceride, and protein was similar to that reported previously (Rudel et al., 1991): 21.4 ± 1.9, 47.7 ± 4.2, 10.0 ± 1.2, 0.7 ± 1.2 and 20.2 ± 2.7 weight percent, respectively.

The rate of autoxidation and t_{lag} were obtained from plots of conjugated diene formation versus time for LDL treated with either copper ions or ABAP. A typical plot for the oxidation profile is shown in Figure 1. LDL enriched in each of the different fatty acid classes were found to give distinctly different oxidation profiles.

To assess whether the oxidation of LDL followed the equation for radical chain autoxidation, eq 1, the concentration of ABAP was systematically varied. Figure 2 shows a typical response demonstrating that the rate of LDL oxidation depends upon $R_i^{0.5}$ as anticipated from eq 1. The decomposition of ABAP was monitored by following the first-order loss of the azo chromophore at 370 nm as a function of time at 37 ± 0.1 °C giving a k_d of 7.26×10^{-5} min^{-1} (data not shown) which is similar to the value of 7.8×10^{-5} min^{-1} reported by Niki et al. (1986).

Table 3 summarizes the rates of autoxidation (±SD) of LDL initiated by ABAP or by copper. Numbers with unlike superscripts are significantly different at $\rho < 0.006$. Both initiators gave similar rates of autoxidation at the concentrations employed in this study. For both initiators the rate of oxidation had the same order, ω-6FA > Sat ≈ Mono ≈ ω-3FA.

Table 4 shows the average t_{lag} (±SD) and amounts of α-tocopherol in the different LDLs. Unlike superscripts designate numbers that are significantly different at $\rho < 0.05$.

Table 4: Summary of Lag Times, t_{lag}, Moles of α-Tocopherol/Mole of ApoB, and Moles of PUFA/Mole of α-tocopherol[a]

diet	N	copper-catalyzed lag	ABAP-catalyzed lag	mole α-tocopherol/mole ApoB	mole PUFA/mole α-tocopherol
high Sat	8	103.3 ± 35.3†	11.0 ± 3.4†,§	4.1 ± 0.8†	152.4 ± 38.2†
high Mono	8	224.1 ± 144.1‡	18.3 ± 12.1†,‡	5.2 ± 4.1†	131.6 ± 53.8†,¶
high ω-6FA	9	38.0 ± 13.1†,¶	26.9 ± 12.3‡	5.5 ± 1.8‡	243.0 ± 65.1‡
high ω-3FA	9	17.4 ± 7.7§,¶	7.5 ± 6.7§	3.5 ± 1.0†	101.8 ± 33.2§,¶

[a] The lag times are given in min ± SD. N is the number of different samples. Numbers for t_{lag} with unlike superscripts are significantly different at $p < 0.05$.

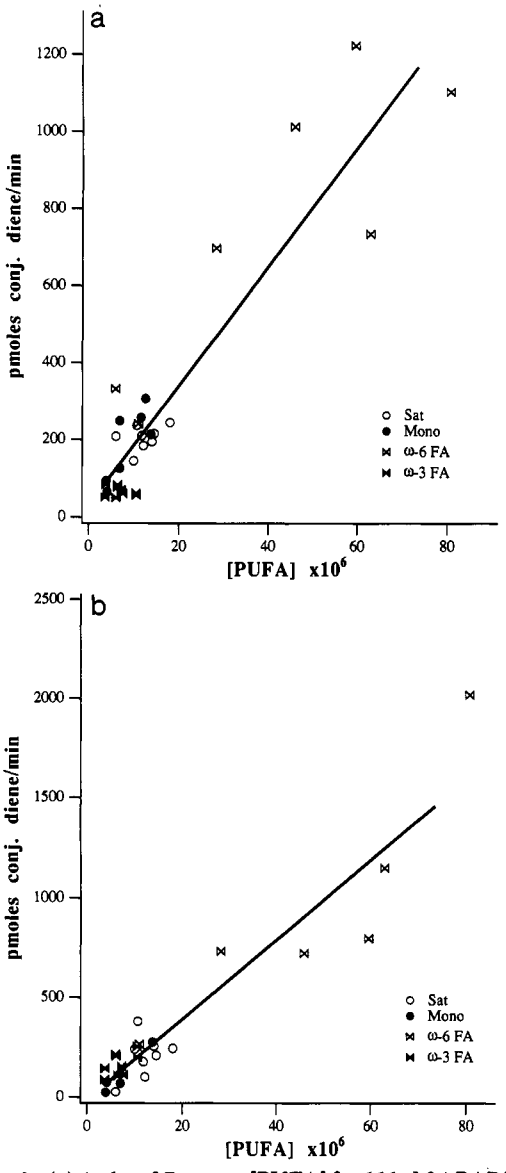

FIGURE 3: (a) A plot of R_p versus [PUFA] for 666 μM ABAP initiated oxidation of LDL. All of the LDLs from the four fatty acid enrichments are included in this plot. See text for details. (b) A plot of R_p versus [PUFA] for 3 μM copper ion initiated oxidation of LDL. All four fatty acid enrichments are included in this plot. See text for details.

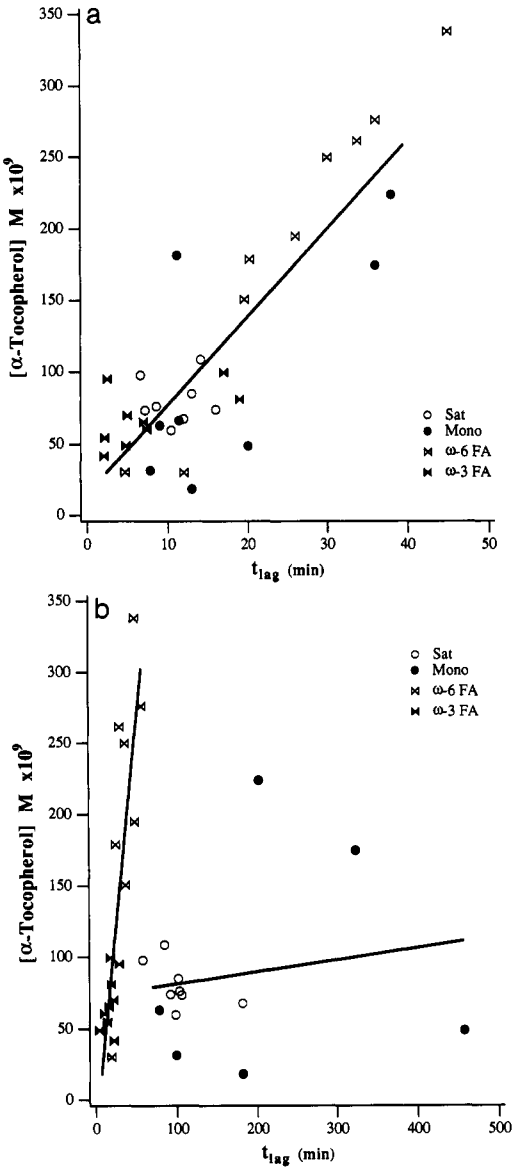

FIGURE 4: (a) A plot of [α-tocopherol] versus t_{lag} for 666 μM ABAP initiated LDL oxidation. All of the LDLs used in this study are included in this plot. See text for details. (b) A plot of [α-tocopherol] versus t_{lag} for 3 μM copper ion initiated LDL oxidation. All of the LDLs used in this study are included in the plot. Note that the LDLs enriched with ω-3FA and ω-6FA show a correlated between [α-tocopherol] and t_{lag}, while the SAT and MONO do not demonstrate such a correlation. See text for details.

The magnitude of t_{lag} for the different fatty acid enrichments can be roughly summarized as follow: for copper oxidation, Mono > Sat > ω-6FA ≈ ω-3FA, while for ABAP-initiated oxidation, ω-6FA ≈ Mono ≈ Sat ≈ ω-3FA. α-Tocopherol levels are similar among the different diet group.

Plots of the rate of oxidation in picomoles per minute versus [PUFA], expressed as a molar concentration, Figure 3, show that [PUFA] and the rate of oxidation are directly related as anticipated for a free-radical chain mechanism described by eq 1. Figure 3a demonstrates this relationship for ABAP-initiated oxidation with $r = 0.92$. A similar relationship is found for copper catalysis, $r = 0.94$ (Figure 3b). The slope of the lines in parts a and b of Figure 3, $(2.26 \pm 0.15) \times 10^7$ and $(1.74 \pm 0.14) \times 10^7$ pmol min^{-1} M^{-1}, respectively, are similar at the concentrations of copper and ABAP chosen for these studies.

A plot of [α-tocopherol] versus t_{lag} for ABAP-initiated oxidation in Figure 4a shows t_{lag} depends upon [α-tocopherol],

r = 0.86, as anticipated from eq 3. When the LDL concentration was changed t_{lag} was changed in direct proportion to the change in LDL (data not shown). Both R_i and R_p, and the kinetic chain length, R_i/R_p (= 34 ± 4), showed little variation at LDL concentrations between 15 and 73 nM (data not shown).

A plot of [α-tocopherol] versus t_{lag} for copper-initiated oxidation did not show a significant correlation. However, when ω-6FA- and ω-3FA-enriched LDL are examined together they showed a correlation of r = 0.83 (Figure 4b). The slopes of the lines for ω-6FA- and ω-3FA-enriched LDLs shown in Figure 4b are equal within experimental error to those found with ABAP, (6.2 ± 0.6) × 10^{-9} and (5.6 ± 0.9) × 10^{-9} M min^{-1}, respectively. No correlation between [α-tocopherol] and t_{lag} was detected when LDLs enriched in Mono FA or Sat FA were analyzed together. Since Tenox 20A was included in the dietary lipids to inhibit autoxidation, it might have been incorporated into LDL and contribute to the lag time. However, analysis of the LDL samples by HPLC did not show detectable levels of Tenox 20A.

DISCUSSION

LDL was isolated from nonhuman primates that had been fed diets that were identical with respect to caloric intake, but differed in the type of fatty acid that was the major constituent of the dietary lipids. The fatty acid composition of the different LDLs reflected the fatty acid composition of the diets. Table 2 shows that linoleic acid was the predominant PUFA in all of the LDLs. The amount of PUFA carried per LDL particle was much greater for the ω-6FA-enriched LDLs than for other enrichments: ω-6FA > Sat ≈ ω-3FA ≈ Mono, respectively. This distribution was unusual because LDLs from animals fed diets enriched in ω-3 PUFAs carry much less total PUFA than the LDLs isolated from animals fed a diet enriched in ω-6 PUFAs.

Figure 1 shows that the LDLs having different fatty acid compositions display different oxidation profiles. Comparing Tables 2 and 3 indicates that the average rate of autoxidation, R_p, for a particular enrichment generally increases as the amount of PUFA in the particle increases for both ABAP and copper-catalyzed oxidations. The dependence of the rate of propagation, R_p, on the concentration of PUFA is more precisely demonstrated in Figure 3 which shows that R_p increases linearly as the PUFA concentration increases. Substituting approximately 40% of the linoleic acid with PUFA having a higher degree of unsaturation did not alter the dependence of the rate of propagation on PUFA concentration, suggesting that concentration was the more important factor affecting the rate of propagation.

To determine if differences in the lipid environment affect oxidation, the data shown in Figure 3 were replotted as $R_p/R_i^{0.5}$ versus [PUFA], Figure 5, so that the slope is independent of the rate of initiation and equal to $k_p/(2k_t)^{0.5}$, a term called the oxidizability of the system. If the fatty acid composition substantially affected diffusion in the different LDLs, both k_p and k_t would be similarly affected and the half-order dependence of k_t in $k_p/(2k_t)^{0.5}$ would give a nonlinear plot. The measured values of slopes are 40.5 ± 3.5 (r = 0.90) and 49.7 ± 7.9 (r = 0.76) $M^{0.5}$ $min^{-0.5}$ for ABAP and copper initiation, respectively, suggesting that the oxidizability of the different LDLs is similar. The combined, cumulative errors in the measurement of the rate of oxidation and the amounts of PUFA and α-tocopherol preclude the detection of small changes in oxidizability.

Besides showing that the different LDLs autoxidize at different rates, Figure 1 also shows that the lag times differ

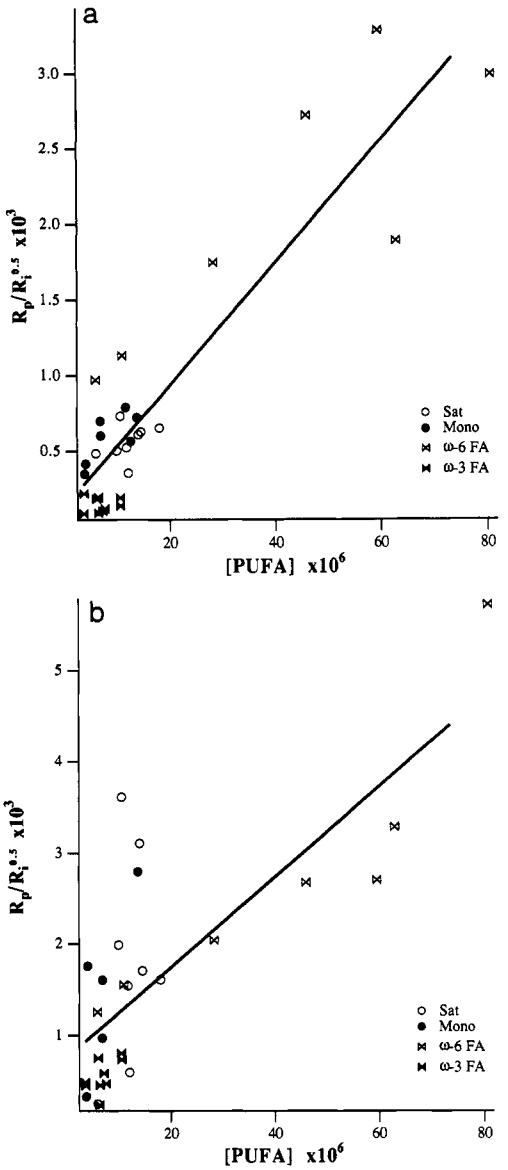

FIGURE 5: (a) A plot of $R_p/R_i^{0.5}$ versus [PUFA] for all of the LDLs in this study. Oxidation was initiated with 666 μM ABAP. R_i was calculated from t_{lag} and [α-tocopherol] assuming n_i = 2. See text for details. (b) A plot of $R_p/R_i^{0.5}$ versus [PUFA] for all of the LDLs in this study. Oxidation was initiated with 3 μM copper ions. R_i was calculated from t_{lag} and [α-tocopherol] assuming n_i = 2. See text for details.

somewhat among the different fatty acid enrichments. Mean lag times for the different LDLs confirm that there were statistically significant differences between several of the enrichments. When ABAP was used to initiate autoxidation, a significant correlation was found between lag time and the amount of α-tocopherol carried by the individual LDLs (Figure 4a). This latter correlation is probably more important for understanding how antioxidants moderate LDL oxidation than the means of the lag times of the various fatty acid enrichments. In contrast to ABAP-initiated autoxidation, copper-catalyzed autoxidation did not yield a correlation between lag time and α-tocopherol concentration (Figure 4b). However, careful examination of Figure 4b suggests that the lag times for oxidation of ω-6FA- and ω-3FA-enriched LDLs were correlated with α-tocopherol concentration while the lag times of LDLs enriched with Sat and Mono fatty acids showed no such correlation. Esterbauer et al. (1991, 1992) have reported a correlation between the lag time and α-tocopherol concentration for copper-catalyzed oxidation of human LDL. The

LDLs used in the Esterbauer study contained on an average 49% PUFA, mostly linoleic acid, making them comparable to the ω-6FA-enriched particles used in this study.

Although the fatty acid composition of LDL would not be expected to directly affect the lag time, the composition may influence how much α-tocopherol is carried by individual LDL particles and the rates of diffusion within the particle. Preliminary analysis show that the amount of α-tocopherol carried by LDL was directly proportional to the amount of PUFA per particle, $r = 0.97$, $\rho < 0.001$ (data not shown). Therefore, as a group LDLs enriched in ω-6FA contain slightly more α-tocopherol per particle than do the other enrichments, but these LDLs also had the largest PUFA to α-tocopherol ratio, Table 4, indicating that each α-tocopherol had to protect more PUFA as compared to LDL having other fatty acid enrichments.

The results presented in Figure 5b also suggest that the lag times of copper-oxidized Sat and Mono-enriched LDLs were not correlated with the α-tocopherol concentration. In this figure the data points for Sat- and Mono-enriched LDLs are not correlated with the concentration of PUFA as they were in Figure 3b. Figure 5b is similar to Figure 3b except that R_p is divided by the rate of initiation to the one-half power, $R_i^{0.5}$, that was calculated from eq 3. Figure 5b shows that the rate of initiation, R_i, calculated from the lag time and α-tocopherol concentration, does not correctly reflect R_i in particles enriched in Sat and Mono fatty acids.

The implication that eq 3 might not yield a correct R_i suggested that the rate of radical formation by ABAP should be more closely examined. The magnitude of the rate of initiation calculated from eq 3 gives an average value of $(1.82 \pm 1.40) \times 10^{-8}$ M min^{-1} for all of the LDL oxidations initiated with ABAP. Using the measured value of $k_d = 1.21 \times 10^{-6}$ s^{-1}, eq 2 gave 4.83×10^{-8} M min^{-1} as the maximum rate of radical formation. The calculated autoxidation efficiency, e ($= R_i/2k_d$[ABAP]), was therefore 37%, a value that is similar to the efficiencies reported for the initiation of radical reactions in emulsions and in liposomes (Niki et al., 1986; Castle & Perkins, 1986).

Several reports have suggested that α-tocopherol may act like a chain-transfer agent in isolated, purified LDL (Bowry et al., 1992; Ingold et al., 1993; Bowry & Stocker, 1993). Support for the suggestion of chain transfer rely on kinetic arguments which suggest that the α-tocopheroxyl radical has a long lifetime in LDL (Ingold et al., 1993). If α-tocopherol were to act exclusively as a chain-transfer agent, the reaction profile would not show a lag period. The effectiveness of α-tocopherol in breaking chains as opposed to transferring them would be reflected in the rate of oxidation during the lag period. Our results suggest that α-tocopherol was an effective antioxidant under the conditions used in this study, but they do not rule out the possible participation of α-tocopherol in chain-transfer reactions during the lag phase.

The lag times measured for copper-catalyzed oxidation of Sat- and Mono-enriched LDLs are significantly larger than those for ω-6FA- or ω-3FA-enriched LDLs. These differences suggest that the initiating radicals are being generated at different rates. The change in rate combined with the apparent absence of α-tocopherol-related inhibition suggests a change in mechanism for the generation of initiating radicals. Although there is no explanation for these results, it is clear that the lipid oxidation process catalyzed by copper is sensitive to factors of lipid composition that are not related to the PUFA content. The sensitivity of Sat- and Mono-enriched LDL to α-tocopherol inhibition when ABAP is used rules out an explanation which invokes differences in lipid viscosity to explain the unusual behavior of Sat and Mono LDLs to copper-catalyzed oxidation.

These studies do not provide direct evidence for a correlation between LDL oxidation and atherosclerosis, but they suggest that the sensitivity of LDL to oxidation depends upon the type of initiator, the fatty acid composition of the particle, and the amounts of PUFA and α-tocopherol carried by the particle. As a group the LDLs enriched in ω-6FA oxidize faster than LDLs having other fatty acid enrichments, although they may be more resistant to the initiation of oxidation. In this study, the resistance to oxidation was also found to depend upon whether copper or an azo initiator was used.

It is not clear how these results relate to the development of atherosclerosis since the radical species initiating *in vivo* oxidation have not been identified. The development of atherosclerosis in nonhuman primates has been studied by feeding diets enriched in some of the different fatty acids employed this study. The results have consistently shown that animals fed Sat-enriched diets developed more diet-induced atherosclerosis than animals fed an ω-6FA-enriched diet (Rudel et al., 1991). With respect to the oxidation hypothesis for the development of atherosclerosis, our findings suggest that the antioxidant effects of the extra α-tocopherol carried by the ω-6FA-enriched LDL may be more effective in preventing oxidation than the increased levels of ω-6FA are in promoting oxidation. The role of arachidonic acid also needs to be considered when assessing how lipid oxidation might facilitate the development of atherosclerosis. Many of the products generated by free-radical autoxidation of arachidonic acid are strong inflammatory agents. Arachidonic acid levels are the lowest in the LDLs of ω-6FA fatty acid fed animals and, therefore, smaller amounts of eicosanoid products would be formed by autoxidation as compared to the other LDLs.

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