Is the Oxidation of High-Density Lipoprotein Lipids Different Than the Oxidation of Low-Density Lipoprotein Lipids?[†,‡]

Michael J. Thomas,*[,§] Quiri Chen,[§] Manal Zabalawi,[§] Rachel Anderson,[||] Martha Wilson,[⊥] Richard Weinberg,[||] Mary G. Sorci-Thomas,[⊥] and Lawrence L. Rudel[§,⊥]

Departments of Biochemistry, Internal Medicine (Gastroenterology), and Pathology/Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

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ABSTRACT: This article gives detailed insight into the kinetics of high-density lipoprotein (HDL) oxidation catalyzed by azobis(2-amidinopropane)·dihydrochloride (ABAP) or by copper. ABAP initialized oxidation of human HDL 3−4 times faster than non-human primate HDL with a similar composition. The oxidizability of non-human primate HDL was 1000 times lower than the oxidizability calculated from rate constants derived from liposome oxidation, suggesting that there is a slow step in HDL oxidation not present in liposomes. Saturable binding of copper to HDL was a significant feature of copper-catalyzed oxidation. Binding constants (K_m) for non-human primate HDL were 2−3-fold lower than those for human HDL. Copper-catalyzed oxidation of non-human primate HDL was slower than that of human HDL, but human HDL_2 and HDL_3 oxidized at about the same rate. Overall, the kinetics describing the oxidation of HDL were mechanistically similar to those reported for LDL, suggesting that HDL lipids were as easily oxidized as LDL lipids and that HDL will be easily oxidized in vivo when exposed to agents that oxidize LDL.

High plasma concentrations of high-density lipoprotein (HDL)[1] are associated with a reduced risk of atherosclerosis (*1*, *2*). Reverse cholesterol transport from peripheral tissues back to the liver is generally assumed to be the major antiatherogenic role of HDL (*3*−*5*). Most of the lipids deposited in the artery wall of humans come from low-density lipoproteins (LDLs), and several studies have shown that the severity of the atherosclerotic lesion may be directly related to the amount of lipid oxidation products in a plaque (*6*−*8*). Chisolm, Steinberg, and others have proposed that the peroxidation of LDL lipids may play an important role in the development of atherosclerosis (*9*−*11*). Several reports have suggested that HDLs retard atherosclerosis by slowing the oxidation of LDL or by removing the products of lipid oxidation (*12*−*20*), while other studies have suggested that HDL may be more easily oxidized than LDL (*21*−*25*).

Several mechanistic studies are beginning to clarify how oxidized HDL might contribute to atherosclerosis. Mild oxidation of the methionine residues in HDL, a process that can be repaired (*26*), did not affect cholesterol clearance (*27*, *28*), while the tyrosylation of apoprotein A-I in HDL enhanced cholesterol clearance in mouse macrophage foam cells and human skin fibroblasts (*29*−*31*). The increased hydrophobicity of tyrosylated apoprotein A-I is believed to facilitate the binding to SR-B1. However, other studies suggest that more extensive HDL oxidation reduces the efficiency of cholesterol clearance from the artery wall (*32*−*34*).

Although HDL transports lipids back to the liver for excretion into bile, there is rapid exchange of both phospholipids and cholesteryl esters among the lipoprotein classes in plasma before removal by the liver. There is ample evidence showing that plasma LDL levels are maintained by facilitated transport of lipids from HDL to LDL. Therefore, exchange of oxidized lipids between HDLs and LDLs trapped in a plaque ensures that these oxidized lipids enter the plasma pool. Conversely, when HDL lipids are oxidized these lipids may diffuse into the plasma lipoprotein pool and be carried by LDL into the artery wall and atherosclerotic plaque.

In vivo mechanisms that explain the initiating events in lipoprotein oxidation have not been established, although several attractive hypotheses have been proposed. Most, if not all, of these mechanisms involve the actions of oxidants or oxidizing enzymes (*35*−*41*). However, quantitatively assessing the kinetics of how lipoprotein lipids oxidize requires a constant rate of initiation that is best achieved in an in vitro system that uses the thermal decomposition of ABAP (*42*). The use of pro-oxidant enzymes for controlled kinetic studies of lipid oxidation is of limited applicability in quantitative studies because the enzymes are not readily obtained in pure form or they generate other products that

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* To whom correspondence should be addressed: Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157-1016. Telephone: (336) 716-2313. Fax: (336) 716-7671. E-mail: mthomas@wfubmc.edu.

[§] Department of Biochemistry.

[||] Department of Internal Medicine (Gastroenterology).

[⊥] Department of Pathology/Comparative Medicine.

[1] Abbreviations: ABAP, azobis(amidinopropane)·2HCl; AP•, amidinopropane radical; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PUFA, polyunsaturated fatty acid; R_i, rate of initiation; R_p, rate of oxidation; t_{lag}, lag time.

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interfere with the analysis. We studied the kinetic of copper-catalyzed oxidation because it is routinely used to prepare in vitro-oxidized lipoproteins for biological studies.

Because both HDL and LDL rapidly exchange lipids in vivo, their total fatty acid compositions are quite similar (*43*, *44*). Moving through the same in vivo compartments exposes HDL to many of the same oxidant stresses that cause LDL autoxidation, and several studies have suggested that HDL may oxidize more readily than LDL (*24*, *25*). To firmly establish the sensitivity of HDL lipids to free radical autoxidation, we studied the rate of HDL lipid oxidation under conditions that were used to assess lipid oxidation in LDL (*45*), and we contrast copper-catalyzed lipid oxidation with oxidation initiated by the water-soluble free radical initiator azobis(2-amidinopropane)·HCl (ABAP).

MATERIALS AND METHODS

Reagents. All the chemicals that were used were of the highest grade available commercially. Azobis(2-amidinopropane)·dihydrochloride was from Polyscience, Inc. Copper sulfate was from Fisher Scientific. Diethylenetriaminepentaacetic acid disodium salt from Aldrich was recrystallized twice from water before being used. Potassium phosphate (Ultrex), NaCl, and NaOH were from J. T. Baker. Milli-Q water was used throughout.

Source of HDL. Native HDL was either from human volunteers or from St. Kitts vervits that had been fed diets with the following caloric distribution: 17% protein, 48% carbohydrate, and 35% fat. Cholesterol (0.4 mg/cal) was added to induce hypercholesterolemia and atherosclerosis in the non-human primates. The non-human primate diets were constituted as previously reported (*46*). The animals were maintained according to the guidelines of the Institutional Animal Care and Use Committee. Blood was drawn from human volunteers according to guidelines approved by the Clinical Research Practices Committee.

Preparation of HDL. *(1) Non-Human Primate HDL*. Blood was collected in 3 mM EDTA and stored on ice. The lipoprotein fraction was separated by centrifugation; the $d < 1.225$ g/mL fraction was applied to an agarose column, and individual lipoproteins were separated (*46*, *47*) at 4 °C. The eluting buffer contained 3 mM EDTA and was continuously sparged with nitrogen gas. Samples were briefly stored at 4 °C under argon. Animals used in this study were maintained on a diet rich in saturated fats or linoleic acid (*45*).

(2) Human HDL. Blood was collected in EDTA and stored on ice. Lipoprotein fractions were separated by sequential density gradient centrifugation. HDL_2 was isolated at 1.125 g/mL and HDL_3 at 1.210 g/mL. The HDL_3 fractions were recentrifuged at 1.210 g/mL to remove residual albumin. Electrospray mass spectroscopy carried out on a Quattro II triple-quadrupole mass spectrometer showed that the samples were not contaminated with albumin. Samples were briefly stored at 4 °C under argon.

Autoxidation. Autoxidation was studied at 37 °C in quartz cuvettes containing 3 mL of air-saturated 25 mM phosphate buffer (pH 7.2) containing 0.1 M NaCl (*46*). Some experiments also included 2 mM $CaCl_2$. The concentration of the other components depended on the nature of the experiment as follows: 58.8 μg/mL for human HDL protein, 20 μg/mL for non-human primate HDL protein, 0.54−10.8 μM for Cu^{2+}, and 267−2668 μM for ABAP. When LDL oxidation was compared with HDL oxidation, the LDL concentration was 3.3 μg/mL and the HDL concentration was adjusted to give equivalent amounts of cholesterol ester. Excess EDTA was removed by dialysis for 48 h at 4 °C against 25 mM PBS and 10 μM diethylenetriaminepentaacetic acid (DTPA) with continuous nitrogen sparging. The small amount of DTPA introduced with HDL did not affect the reaction initiated by ABAP. Copper concentrations were adjusted to compensate for the small amount of Cu^{2+} chelated by DTPA. After equilibration of the sample at 37 °C, the initiator was added to start autoxidation. Conjugated diene formation was assessed at 236 nm with a Hewlett-Packard model 8452 diode array spectrometer. Concentrations were calculated using an ϵ_{236} of 26 000 M^{-1} cm^{-1}. The rates of oxidation (R_p) and lag times (t_{lag}) were determined as previously described for LDL (*46*).

Protein was assessed (*48*) using bovine serum albumin (Sigma Chemical Co., catalog no. 4503) as the standard. The concentration of HDL was calculated assuming two apoprotein molecules (MW = 28 083 g/L) per HDL particle for non-human primate HDL and human HDL-2 and three apoprotein molecules for HDL-3.

Analyses. Fatty acids were analyzed by the method of Metcalfe and Schmitz using pentadecanoic acid as the internal standard (*49*). Cholesterol esters were analyzed using GC and the enzymic Cholesterol/HP kit from Boehringer Manheim. Statistical significance among groups was assessed using ANOVA. A Student's *t* test assuming unequal variances was used to assess the significance of the results between groups.

RESULTS

Initiation of HDL Lipid Oxidation by Azobis(2-amidinopropane)·2HCl. Figure 1a shows a set of typical HDL oxidation profiles where the appearance of the conjugated diene at 236 nm is plotted versus time for several different ABAP concentrations. Each curve was differentiated to find the region of the curve with the maximum rate of change. The tangent to the curve at this point was defined as the maximum rate of oxidation (R_p). To demonstrate that termination was bimolecular, R_p was plotted versus the square root of the ABAP concentration ($[ABAP]^{0.5}$). Figure 1b shows a representative plot for HDL_3. All of the plots were linear, suggesting that free radical chain oxidation was terminated in a bimolecular step.

HDL and LDL from the same non-human primate were oxidized under similar conditions where each sample contained equal amounts of lipoprotein cholesteryl ester. The total fatty acid distributions are shown in Table 2. The mean rates of oxidation were not significantly different ($p = 0.58$, $N = 11$). There was no statistical difference among the diet groups, although the linoleic acid-enriched diet gave an overall higher rate of oxidation. For the linoleic acid rich diet, HDL oxidized at a rate of 118 ± 31 pmol of conjugated diene/min while LDL oxidized at a rate of 99 ± 10 pmol of conjugated diene/min. The saturated fat diet gave an HDL rate of oxidation of 80 ± 35 pmol of conjugated diene/min and an LDL oxidation rate of 82 ± 34 pmol of conjugated diene/min. Correlation, covariance, and regression analysis

Table 1: Summary of HDL Oxidation Parameters

HDL source	relative oxidizability ($M^{-1/2}$ $s^{-1/2}$)	calculated oxidizability ($M^{-1/2}$ $s^{-1/2}$)	V_{max} (pmol/min)	$k_5k_i/2k_6$ (k_i for copper) (s^{-1})	K_m (μM)
human HDL_2	$(1.2 \pm 0.7) \times 10^{-4}$	$(2.0 \pm 1.1) \times 10^{-4}$	$(3.0 \pm 0.7) \times 10^3$	3.2 ± 0.8	1.8 ± 0.3
human HDL_3	$(1.0 \pm 0.2) \times 10^{-4}$	$(1.5 \pm 0.3) \times 10^{-4}$	$(2.7 \pm 0.6) \times 10^3$	3.8 ± 0.8	2.1 ± 0.4
non-human primate HDL	$(0.4 \pm 0.7) \times 10^{-4}$	$(0.5 \pm 0.1) \times 10^{-4}$	$(1.1 \pm 0.5) \times 10^3$	1.8 ± 0.8	0.8 ± 0.2

Table 2: Fatty Acid Distribution in Cholesteryl Esters and Phospholipids from Non-Human Primate HDL and LDL[a]

lipid/lipoprotein	16:0	16:1	18:0	18:1	18:2	20:4	other
			Saturated Fat Diet				
PL/HDL	31.8 ± 1.8	0.5 ± 0.1	12.0 ± 0.6	14.5 ± 1.0	26.3 ± 2.1	1.2 ± 1.2	13.7
PL/LDL	36.0 ± 1.0	0.5 ± 0.1	10.6 ± 0.7	13.5 ± 0.6	26.5 ± 2.5	3.6 ± 0.5	9.3
CE/HDL	14.2 ± 1.0	3.0 ± 0.7	2.8 ± 0.7	45.6 ± 5.5	26.3 ± 5.8	0.9 ± 0.4	7.2
CE/LDL	17.6 ± 1.4	3.1 ± 0.8	3.4 ± 0.8	44.3 ± 5.3	23.7 ± 5.3	0.7 ± 0.2	7.2
			Linoleic Acid Rich Diet				
PL/HDL	25.7 ± 2.4	0.3 ± 0.1	16.0 ± 1.8	5.8 ± 0.8	38.8 ± 3.7	0.2 ± 0.1	
PL/LDL	28.6 ± 2.1	0.3 ± 0.1	14.0 ± 1.3	4.6 ± 0.4	39.7 ± 2.4	2.8 ± 0.6	
CE/HDL	6.7 ± 1.3	0.9 ± 0.2	0.8 ± 0.2	18.5 ± 2.2	65.7 ± 3.7	0.4 ± 0.2	
CE/LDL	7.4 ± 1.1	1.0 ± 0.3	0.8 ± 0.3	19.3 ± 1.9	65.0 ± 2.9	0.5 ± 0.2	

[a] Animals used in this study were fed diets enriched either in saturated fats such as the standard American diet or in linoleic acid. Fatty acids that comprise more than 3% of the total in at least one lipoprotein are shown. Abbreviations: CE, cholesteryl ester; other, other fatty acids; PL, phospholipid; 16:0, myristic acid; 16:1, myristoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid.

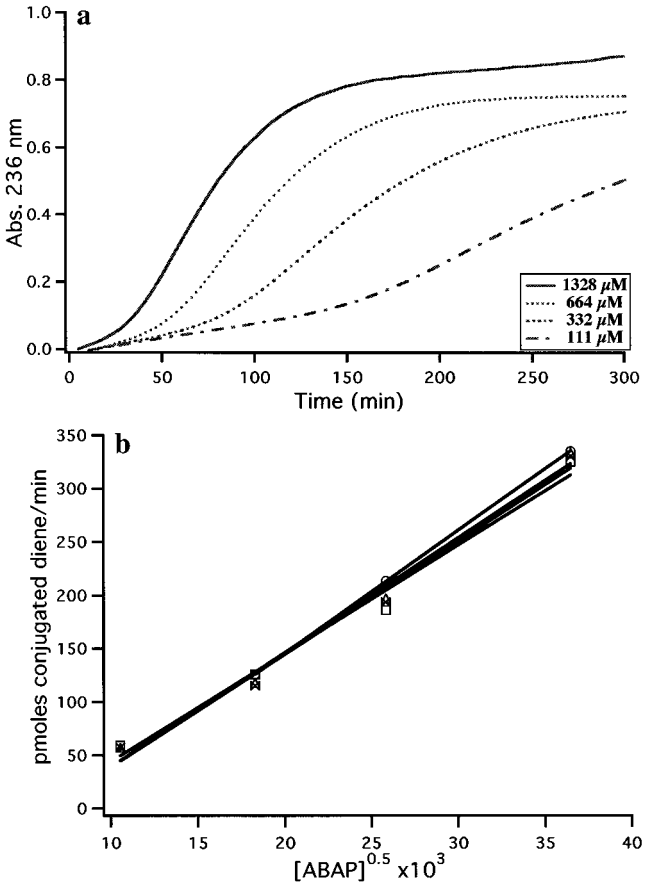

FIGURE 1: (a) Conjugated diene absorption measured at 236 nm plotted vs time for the ABAP-initiated oxidation of HDL_2. The ABAP concentrations are listed in the inset. (b) A plot of the maximum rate of HDL_3 oxidation in picomoles of conjugated diene per minute plotted vs the square root of the ABAP concentration for four different subjects. The solid lines have been fitted by least-squares methods to the equation $R_p = m[ABAP]^{0.5} + b$. Experimental details are found in Materials and Methods.

suggested that there was no systematic variation in the lag time and rates of oxidation for HDL and LDL (data not shown).

Initiation of HDL Lipid Oxidation by Copper Ions. Figure 2 shows a typical plot of HDL oxidation at different concentrations of Cu^{2+}. Both the lag time and the maximum rate of copper-catalyzed oxidation approached limiting values as the Cu^{2+} concentration was increased. At the higher copper concentrations, the rate of oxidation approached a maximum value while the lag time approached a minimum value. These results are consistent with saturation of a fixed number of copper-binding sites on HDL. Plotting the reciprocal of the maximum oxidation rate ($1/R_p$) versus the reciprocal of the Cu^{2+} concentration ($[Cu^{2+}]^{-1}$) gave a straight line for all of the HDLs (data not shown). The maximum rate of oxidation and copper binding constants (K_m's) were determined by nonlinear regression analysis. Typical plots for non-human primate oxidation are shown in Figure 2b, and the results are listed in Table 1.

From the K_m's and the solution concentration of HDL, the number of copper atoms bound at half-maximum velocity was estimated. For human HDL_2 and HDL_3, the number of binding sites was significantly different with a p of <0.001 (1.7 ± 0.3 mol of Cu/mol of HDL and 7.4 ± 1.1 mol of Cu/mol of HDL, respectively). The number of binding sites on non-human primate HDL preparations was more difficult to assess because the individual HDL subspecies HDL_2, HDL_3, and HDL_4 were not isolated. We assumed an average of 2.5 apo A-I molecules per HDL particle. The average amount of copper ions bound per HDL based on this assumption was 2.9 ± 0.8 mol of Cu/mol of HDL.

DISCUSSION

HDL and LDL undergo oxidation by similar mechanisms, with the lipids of both lipoproteins displaying similar sensitivities to free radical chain autoxidation. Therefore, HDL lipids were as easily oxidized as the LDL lipids. In vivo, enzymes associated with HDL may help prevent the oxidation of both HDL and LDL. These enzymes include esterases such as calcium-dependent paraoxonase, a phospholipase A2, i.e., platelet-activating factor acetylhydrolase, or lecithin cholesterol acyltransferase (*12*, *50*−*59*). However,

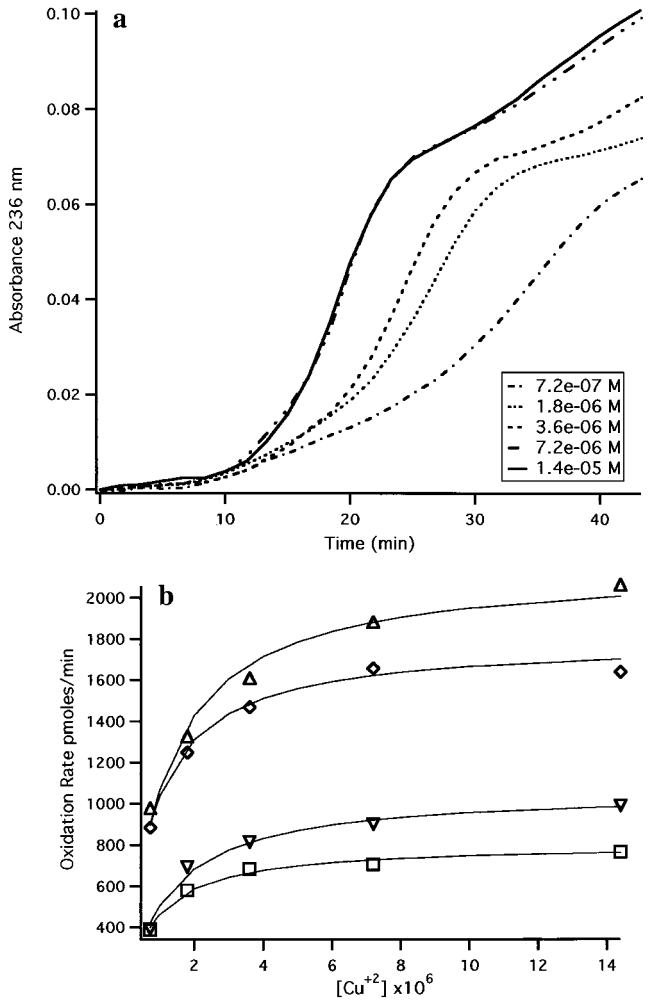

FIGURE 2: (a) Conjugated diene absorption measured at 236 nm plotted vs time for the copper-catalyzed oxidation of non-human primate HDL. The copper concentrations are listed in the inset. (b) A plot of the maximum oxidation rate in picomoles per minute vs the molar copper concentration for four different animals. The solid lines have been fitted by nonlinear regression analysis to the equation $R_p = A[Cu^{2+}]/(K_m + [Cu^{2+}])$. Experimental details are found in Materials and Methods.

lipid hydroperoxides are reported to inhibit lecithin cholesterol acyltransferase (*60*).

In vivo, HDL may protect lipoproteins from oxidation by reverse cholesterol transport where cholesterol and its derivatives are carried from peripheral tissues to the liver for reprocessing. This transport process may carry oxidized lipids (*17*, *19*, *61*) to the liver where they are catabolized. Oxidized HDL will exchange oxidized lipids with other lipoproteins in addition to delivering oxidized lipids to the liver for catabolism, and these lipids may then be carried back to the artery wall. HDL that has undergone mild oxidation was reported to remove cholesterol from cells more rapidly than unoxidized HDL (*29*−*31*). However, HDL lipids are as easily oxidized as LDL lipids, and oxidized HDL may be less efficient in removing cholesterol from peripheral tissues. There is still some controversy regarding how oxidized HDL affects cholesterol transport (*29*−*34*), but it is clear that HDL lipids are not naturally resistant to oxidation.

Initiation by the Azo Initiator. The linearity of these plots showed that free radical chain oxidation was terminated in a bimolecular step. The small volume of the HDL particle suggests that only one free radical chain will be present at any given time as described by Bowry and Stocker (*62*). Therefore, it is likely that the hydroperoxyl radical formed by ABAP decomposition, the amidinopropane hydroperoxyl radical AP-$O_2^\bullet$, participated in both the initiation and termination of lipoprotein oxidation (*45*). Scheme 1 outlines the steps in ABAP-catalyzed oxidation. The overall rate of oxidation derived from this scheme is given by eq I and the rate of initiation by eq II.

Scheme 1

$$\text{ABAP} \rightarrow 2\text{AP}^\bullet \quad (1)$$

$$\text{AP}^\bullet + \text{O}_2 \rightarrow \text{AP-O}_2^\bullet \quad (2)$$

$$\text{AP-O}_2^\bullet + \text{PUFA} \rightarrow \text{AP-O}_2\text{H} + \text{PUFA}^\bullet \quad (3)$$

$$\text{PUFA}^\bullet + \text{O}_2 \rightarrow \text{PUFA-O}_2^\bullet \quad (4)$$

$$\text{PUFA-O}_2^\bullet + \text{PUFA} \rightarrow \text{PUFA-O}_2\text{H} + \text{PUFA}^\bullet \quad (5)$$

$$\text{AP-O}_2^\bullet + \text{PUFA-O}_2^\bullet \rightarrow \text{nonradical products} \quad (6)$$

$$R_p = k_5[\text{PUFA}]R_i^{0.5}/(2k_6)^{0.5} \quad \text{(I)}$$

$$R_i = 2ak_1[\text{ABAP}] \quad \text{(II)}$$

The ease with which a system undergoes autoxidation is defined by its oxidizability, the ratio of the rate constant for free radical chain propagation (step 5) and the square root of the rate constant for free radical chain termination (step 6) [$=k_5/(2k_6)^{0.5}$]. Because the rate of radical generation (R_i) has been established, the product of oxidizability and HDL oxidizable lipid concentration [[PUFA]$k_5/(2k_6)^{0.5}$, called relative oxidizability] was calculated from the slope of the plots such as those shown in Figure 1b. The results are collected in Table 1. Different HDLs had different mean, relative oxidizabilities. Dividing by the estimated PUFA concentrations estimated from the mole fraction of PUFA (0.43, 0.47, and 0.52 for HDL_2, HDL_3, and non-human primate HDL, respectively) gave oxidizabilities for each HDL class collected in Table 1. There was no significant difference in the relative oxidizability of HDL_2 and HDL_3 ($p = 0.5$), but non-human primate HDL was less oxidizable than human HDL_2 and HDL_3 ($p < 0.001$). The oxidizability of non-human primate HDL was similar to that of non-human primate LDL [$(7.9 \pm 2.0) \times 10^{-5}$ $M^{-1/2}$ $s^{-1/2}$ (*45*)]. The slopes of the lines in Figure 1b were proportional to the amount of PUFA oxidized, suggesting that the rate was proportional to the concentration of PUFA (data not shown) with a correlation coefficient of 0.98.

Like LDL oxidizability, HDL oxidizability was lower [$(3.9 \pm 0.7) \times 10^{-5}$ to $(1.0 \pm 0.2) \times 10^{-4}$ $M^{-1/2}$ $s^{-1/2}$] than that calculated from the values of propagation k_5 and termination k_6 reported for micelle and liposome oxidation, 0.05 $M^{-1/2}$ $s^{-1/2}$ (*63*−*65*). The rate for radical termination is always faster than that for propagation, suggesting that the propagation step was unusually slow. A slow propagation step implies that the viscosity was greater than previously estimated (*45*) or that there is an unreactive radical "reservoir" participating in the free radical chain. For example, if α-tocopherol participated in this process, formation of the α-tocopheroxyl

radical with subsequent reinitiation of free radical chain oxidation would reduce the oxidizability by roughly the order of magnitude reported here (*62*). An alternative explanation questions one of the assumptions used for our kinetic analysis. That assumption is that AP-$O_2^{\bullet}$ readily penetrates HDL. The overall rate of termination could be reduced if the AP-$O_2^{\bullet}$ does not penetrate HDL but oxidizes one of the PUFA surface phospholipids. The transfer of the radical from the surface to the core now depends on the surface lipid coming into contact with the oxidized core lipids. Such a step could be rate-limiting and may reduce the rate of oxidation by several orders of magnitude.

The ABAP-initiated oxidation of both HDL and LDL appears to proceed by the same kinetic mechanism. These kinetic similarities appear to translate into similar levels of lipid oxidation. There was not a significant difference between LDL and HDL oxidation when samples were analyzed at similar total cholesterol concentrations. These results suggest that the environment for lipoprotein lipid oxidation was similar for both HDL and LDL. Because HDL is reported to carry a calcium-dependent paraoxonase that might inhibit lipid oxidation, 2 mM $CaCl_2$ was added to both LDL and HDL preparations (*51*, *53*, *57*). The added calcium did not change the rate of oxidation or the lag time when compared to controls that did not contain $CaCl_2$, suggesting that an active paraoxonase was not associated with these HDLs.

Initiation by Copper Ions. Previous studies of copper-catalyzed LDL oxidation have suggested that the binding of Cu^{2+} to LDL could be saturated (*45*, *66*). The same phenomenon was observed for HDL oxidation. Analysis of the maximum rate of oxidation versus copper concentration gives a complex constant, $(k_5k_i/2k_6)$[HDL][PUFA]υ (*45*), that is similar to V_{max}. This term includes the concentrations of HDL and PUFA, the solution volume (υ), and a ratio of rate constants where k_5 is the rate constant for propagation, k_i is the rate constant for radical generation by copper, and k_6 is the rate constant for termination. The mean values of this constant were 2993 ± 735 pmol/min for HDL_2 ($N = 7$), 2688 ± 576 pmol/min for HDL_3 ($N = 7$), and 1094 ± 476 pmol/min for non-human primate HDL ($N = 9$). The value of this constant for the non-human primates was significantly smaller than that of human HDL ($p < 0.001$). After dividing these numbers by the HDL concentration, estimated PUFA concentration, and volume, we found rate constant ratios $(k_5k_i/2k_t)$ of 3.2 ± 0.8, 3.8 ± 0.8, and 1.8 ± 0.8 s^{-1} for HDL_2, HDL_3, and non-human primate HDL, respectively. These results are summarized in Table 1. Because oxidizability $[k_5/(2k_6)^{0.5}]$ is independent of the initiator, these results suggest that copper-initiated human HDL oxidation was roughly twice as fast as the oxidation of lipids carried by non-human primate HDL.

From the K_m values, the number of catalytically active binding sites can be estimated. There were more copper binding sites on non-human primate LDL, approximately 50 (*45*), than on HDL which has approximately 3. The large difference in the number of binding sites suggests that copper atoms bound to HDL were more effective in promoting oxidation than the larger number of copper ions that bind to LDL. When HDL or LDL samples isolated from different individuals are compared, it is obvious that there is considerable variation in K_m among the samples. Because K_m will be related to the rate at which copper initiates oxidation, this variation in K_m makes quantitative comparison between HDL or LDL samples impossible unless the concentrations of reactants are adjusted to reflect differences in K_m.

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