Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation

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Abstract Oxidative modification of low density lipoprotein (LDL) has been suggested to play a casual role in human atherosclerosis, and prevention of LDL oxidation may be an effective strategy to prevent or slow the progression of this disease. It is important, therefore, to identify the factors that determine LDL's susceptibility to oxidation. We have analyzed 62 human LDL samples for content of antioxidants, preformed lipid hydroperoxides, and cholesterol. To investigate their oxidative susceptibility, the LDL samples were exposed to either a metal ion-dependent (Cu^{2+}) or -independent (aqueous peroxyl radicals) oxidizing system; the length of the lag phase of inhibited lipid peroxidation was measured, as well as the rate of lipid peroxidation during the lag and ensuing propagation phases. The susceptibility of LDL to metal ion-dependent oxidation was not related to its susceptibility to metal ion-independent oxidation. A strong predictor of an increased susceptibility of LDL to metal ion-dependent oxidation was a decreased vitamin E-to-cholesterol ratio, in contrast to the vitamin E-to-protein ratio. Elevated levels of performed lipid hydroperoxides in LDL and an increased cholesterol content were also associated with an increased susceptibility of the lipoprotein to Cu^{2+}-induced oxidation. Remarkably, a strong predictor of an increased susceptibility of LDL to metal ion-independent oxidation was an increased, rather than decreased, vitamin E content relative to protein. An increased cholesterol content also was associated with an increased oxidative susceptibility of LDL to aqueous peroxyl radicals, while preformed lipid hydroperoxides showed no significant correlation. Ubiquinol-10, β-carotene, and lycopene, whether quantitated relative to cholesterol or protein, did not show significant protective effects against both metal ion-dependent and -independent oxidation of LDL. Our data suggest that a high lipid content of LDL, relative to its protein content, renders the lipoprotein more susceptible to oxidative modification, while vitamin E may have either a protective or promoting effect on LDL oxidation, depending on the oxidative stress conditions. Other known antioxidants in LDL do not appear to play a significant role in protecting LDL against oxidative modification. **—Frei, B., and J. M. Gaziano.** Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *J. Lipid Res.* 1993. **34:** 2135–2145.

Supplementary key words atherosclerosis • modified lipoprotein • lipid peroxidation • vitamin E • ubiquinol-10 • β-carotene • lycopene

Several lines of evidence from both in vitro and in vivo studies suggest that oxidation of low density lipoprotein (LDL) critically contributes to human atherosclerosis (1, 2). Oxidatively modified LDL is taken up readily by macrophages leading to formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions (3). Other properties of modified LDL that may increase its atherogenicity include, but are not limited to, induction of monocyte chemotactic protein-1 in endothelial and smooth muscle cells, stimulation of leukocyte adhesion to the vascular endothelium, cytotoxicity, and inhibition of nitric oxide-mediated vasodilation (1, 4–7). The array of atherogenic properties of oxidized LDL make the prevention of its formation an attractive strategy to prevent and possibly treat coronary heart disease in humans. Therefore, it is important to understand the mechanisms of LDL oxidation, and to identify the components of LDL that determine the susceptibility of this lipoprotein to oxidative modification.

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; apoB, apolipoprotein B; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; PBS, 10 mM phosphate-buffered saline, pH 7.4; R_{lag}, R_p, rate of lipid peroxidation in LDL during the lag and uninhibited propagation phases, respectively; T_{lag}, length of the lag phase of inhibited lipid peroxidation in LDL.

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The mechanism of LDL oxidation in vitro involves lipid peroxidation and covalent modification of apolipoprotein B (apoB) by lipid hydroperoxide breakdown products (8, 9). All three major cell types present in the arterial wall, i.e., endothelial cells, smooth muscle cells, and monocyte-macrophages, can oxidize LDL in vitro (10–12). Although there probably are major differences in the mechanisms by which these cell types oxidize LDL in vitro, catalytic amounts of redox-active transition metal ions appear to be strictly required (10, 11). Indeed, redox-active transition metal ions in the absence of cells can also oxidatively modify LDL in vitro to a form that is taken up rapidly by macrophages (10, 11). Metal ion-dependent oxidation of LDL in vitro seems to be initiated by breakdown of preformed lipid hydroperoxides, resulting in formation of lipid alkoxy and peroxy radicals that can start lipid radical chain reactions (13–15). Whether metal ions are also required for LDL oxidation in vivo in the arterial wall remains to be established.

The factors determining the susceptibility of human LDL to oxidative modification have received increased attention recently (13–25). From the current knowledge of the mechanism of LDL oxidation in vitro as explained above, one would predict that preformed lipid hydroperoxides are an important factor in metal ion-dependent LDL oxidation (13–15). Furthermore, antioxidants that can prevent lipid peroxidation should be critically important in preventing LDL oxidation (13, 16–24). Human LDL contains a number of antioxidants that can inhibit lipid peroxidation, viz. α-tocopherol (the biologically and chemically most active form of vitamin E), γ-tocopherol, ubiquinol-10, β-carotene, lycopene, and some other carotenoids and oxycarotenoids (13, 16, 19). α-Tocopherol is by far the most abundant antioxidant in LDL, and most clinical studies aimed at increasing LDL's resistance to oxidation have employed supplementation with vitamin E (20, 22–24). Although such supplementation did increase LDL's resistance to oxidative modification as expected, numerous studies have shown that the oxidative susceptibility of LDL from non-vitamin E-supplemented donors is not related to the lipoprotein's α-tocopherol content (13, 17, 18, 20, 21, 25). These findings suggest that vitamin E does contribute to the protection of LDL against oxidation, but in unsupplemented LDL other factors appear to be of greater importance.

The study reported here is a detailed investigation of the contribution of selected components of human LDL to the susceptibility of this lipoprotein to oxidative modification, including endogenous antioxidants, preformed lipid hydroperoxides, and free and esterified cholesterol. While most other studies only investigated Cu^{2+}-induced oxidation of LDL (16, 20–23, 25), we chose to use both metal ion-dependent and -independent oxidizing conditions as it is currently not clear which of these conditions is more relevant to LDL oxidation in vivo. We not only observed striking differences between these two types of oxidative stress, but also discovered that appropriate quantitation of endogenous vitamin E levels in LDL determines whether these are significantly correlated with LDL's oxidative susceptibility.

MATERIALS AND METHODS

Materials

$CuCl_2$, diethylenetriaminepentaacetic acid (DTPA), KBr, Sephadex G-25-300, d,l-α-tocopherol, coenzyme Q_{10}, lycopene, β-carotene, lithium perchlorate, cholesterol, cholesteryl oleate, cholesteryl linoleate, cholesteryl arachidonate, and Lowry protein assay kit (procedure P5656) were purchased from Sigma (St. Louis, MO). 2,2'-Azobis-(2-amidinopropane) hydrochloride (AAPH) was obtained from Kodak (Rochester, NY), and sodium heparin Vacutainers (286 USP units per 15 ml of blood) were from Becton-Dickinson (Rutherford, NJ). Chelex 100 resin (100–200 mesh) was purchased from Bio-Rad (Richmond, CA), and 15(S)-hydroperoxyeicosatetraenoic acid was from Cayman Chemical (Ann Arbor, MI). All reagents used for LDL preparations and incubations were made up in chelex-treated, doubly-distilled deionized water in order to minimize contamination with trace amounts of metal ions and prevent fortuitous oxidation of LDL. $CuCl_2$ was prepared in water adjusted to pH 3.0, and was diluted from a 10 mM stock solution prior to use in experiments.

Human subjects

The 16 subjects who participated in this study were 12 healthy males and 4 healthy females aged 33.0 ± 9.4 years (mean ± standard deviation), without prior history of coronary heart disease, cancer, active liver disease, cirrhosis, atrophic gastritis, alcoholism, pancreatic disease, bleeding disorder, insulin-requiring diabetes, small bowel disease or resection, or renal disease. The subjects agreed to refrain from use of vitamin E supplements.

Each subject donated four blood samples over a 15-week period, except for two subjects who donated three samples over 8 weeks. The subjects were treated with β-carotene supplements for the first 7 weeks of a 19-week study period (samples were drawn after 4, 7, 12, and 19 weeks) to examine the effects on the oxidative susceptibility of isolated LDL. We found no protective effect of β-carotene supplementation on LDL oxidation, which is consistent with two published studies also showing no effect of in vivo β-carotene supplementation on in vitro LDL oxidizability (24) or only a very slight decrease (22). β-Carotene treatment, therefore, was not taken into account for the purposes of the present study. The results of our β-carotene supplementation study will be reported in detail elsewhere.

Isolation of LDL

LDL was prepared from plasma by single vertical spin discontinuous density gradient ultracentrifugation as described by Chung et al. (26). Blood was collected into sodium heparin Vacutainers between 7:30 and 9:30 AM from the study subjects after an over-night fast, and plasma was prepared from blood by centrifugation at 4°C for 10 min at 1,000 *g*. To remove vitamin C and uric acid, residual amounts of which in the LDL preparations would have interfered with determination of the lipid peroxidation lag phase (16, 19, 27), plasma was treated by gel filtration (see below). The density of the gel-filtered plasma was adjusted to 1.21 g/ml by addition of solid KBr, and LDL isolated using a near vertical tube 90 rotor in an L8-80M ultracentrifuge (Beckman Instruments, Palo Alto, CA). Centrifugation was performed at 7°C and 80,000 rpm (443,000 mean g) for 45 min. The LDL fraction was collected into a syringe by inserting the needle directly into the orange-colored (carotenoids) LDL band and applying gentle suction. Chelex resin was added to the LDL preparation to bind adventitious metal ions, followed by filtration through a 0.2 μm syringe filter to remove the resin. Protein content was measured by the method of Lowry et al. (28) with sodium dodecylsulfate added routinely to the assay buffer to facilitate dissolution of the lipoprotein. Bovine serum albumin was used as standard. The LDL preparations were free of detectable amounts of vitamin C and uric acid as assessed by high-performance liquid chromatography (HPLC) with electrochemical detection (27, 29), as well as other lipoprotein classes as assessed by agarose gel electrophoresis with Sudan Black B staining (27).

Treatment of plasma by gel filtration

Plasma was subjected to gel filtration in order to remove vitamin C and uric acid. For each Sephadex G-25-300 column, 10 ml of a suspension containing 6.25 g of filtered, moist resin in 5 ml of 10 mM phosphate-buffered saline, pH 7.4 (PBS) was transferred into a polypropylene Econo-Column (Bio-Rad). The column was centrifuged for 5 min at 600 *g* and 4°C. To equilibrate the resin, 2.5 ml of PBS was added and the column spun again for 15 min. This step was repeated once. Subsequently, 820 μl of plasma was slowly applied to the dry resin, and the column was spun for 15 min, collecting the eluate. LDL was prepared from these filtered plasma samples.

Determination of the length of the lag phase and the rate of lipid peroxidation

Freshly isolated LDL was incubated at a concentration of 0.1 mg of protein/ml in PBS with either 1.25 μM $CuCl_2$ or 4 mM AAPH and 0.1 mM of the metal chelator DTPA. AAPH is a water-soluble azo compound that thermally decomposes and thereby generates peroxyl radicals at a known and constant rate (29). Incubations were carried out at 37°C with stirring in a thermostatted 6-cell holder in a spectrophotometer (U-2000, Hitachi, Japan). The reference cell contained 1.25 μM $CuCl_2$ or 4 mM AAPH and 0.1 mM DTPA in PBS. Lipid peroxidation was measured as diene conjugation at a wavelength of 234 nm as described by Esterbauer et al. (30). Absorbance was recorded at 10-min intervals. Tangents were drawn to the segments of the absorbance curve corresponding to the lag and propagation phases of lipid peroxidation, and the length of the lag phase was determined as the intercept of the two tangents (**Figs. 1A and B**). The coefficients of variation for the length of the lag phase in Cu^{2+}- and AAPH-exposed LDL were 7.5% and 5.1%, respectively. The rates of lipid peroxidation during the lag and propagation phases were calculated from the slopes of the corresponding tangents to the absorbance curve, using a molar extinction coefficient for conjugated dienes of $\epsilon_{234\ nm}$ = 2.95 × 10^4 M^{-1} cm^{-1} (30). The rate of lipid peroxidation

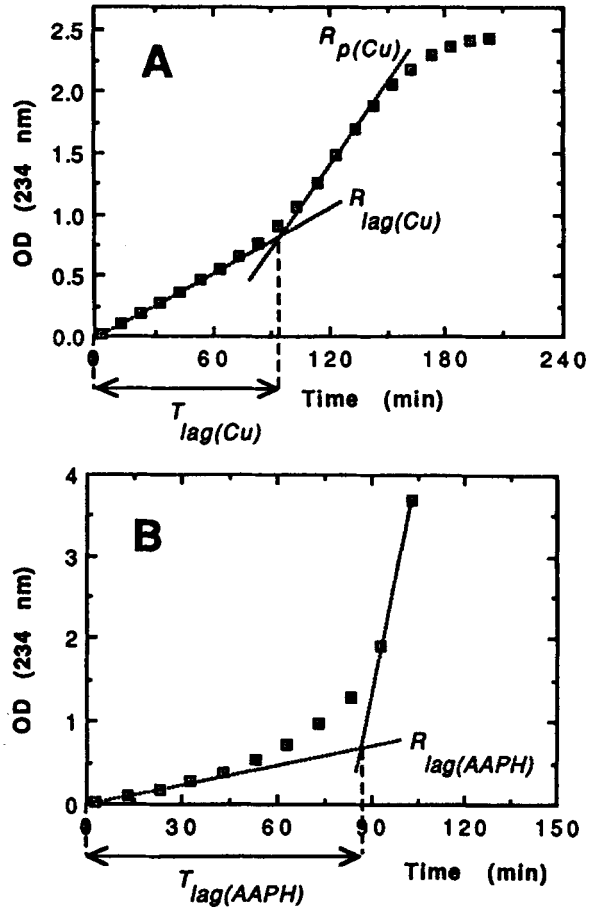

Fig. 1. Determination of the length of the lag phase of inhibited lipid peroxidation (T_{lag}) and the rate of lipid peroxidation during the lag phase (R_{lag}) and the propagation phase (R_p) in LDL exposed to Cu^{2+} (A) or AAPH (B). LDL was isolated and incubated as described under Methods, and lipid peroxidation was measured as diene conjugation at 234 nm. T_{lag}, R_{lag}, and R_p were determined as shown in the figure. One representative example of 62 LDL samples is shown for each type of oxidizing condition.

during the propagation phase in LDL incubated with AAPH (Fig. 1B) and in about half the LDL samples incubated with Cu^{2+} could not be determined because the absorbance at 234 nm reached maximal measurable values before the propagation phase came to an end.

The initial rate of diene conjugation in our LDL preparations exposed to Cu^{2+} (Fig. 1A) was somewhat higher than the rates observed by others (13, 15, 16, 23). This difference may be due to different methods used to prepare LDL, and/or different incubation conditions. For example, we incubated our LDL samples at 0.1 mg of protein/ml and 37°C with 1.25 μM Cu^{2+} under constant stirring, while other investigators incubated at 0.25 mg LDL mass/ml (equal to 0.05 mg of protein/ml) with 1.6 or 3.0 μM Cu^{2+}, at room temperature, and presumably without stirring (13, 16, 20).

Quantitation of preformed lipid hydroperoxides and free and esterified cholesterol

Preformed lipid hydroperoxides were quantitated by HPLC with chemiluminescence detection as described (31). This assay is very sensitive (detection limit 10 pmol/mg LDL protein, i.e., 0.005 molecules of lipid hydroperoxides/LDL particle) and measures the hydroperoxy group itself, rather than indirect indices of lipid peroxidation such as thiobarbituric acid reactive substances (TBARS). Briefly, a 200-μl sample containing 0.1 mg of LDL protein in PBS was extracted with 200 μl methanol and 2.5 ml hexane. Two ml of the hexane phase was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 240 μl ethanol. A 100-μl aliquot of this solution was chromatographed on an LC18 column (Supelco, Bellefonte, PA) using methanol–*tert*-butanol 1:1 (v/v) as mobile phase. The eluate was analyzed at 210 nm (Hewlett-Packard 1050 Series Variable Wavelength Detector, Sunnyvale, CA) to detect free and esterified cholesterol, and then mixed in a mixing T (No. 0653-0024, Applied Biosystems, Foster City, CA) with a reaction solution containing isoluminol and microperoxidase (pumped at a flow rate of 1.5 ml/min) followed by chemiluminescence detection (Chemi Lumi Detector/S-3400, Soma Optics, Japan) to monitor lipid hydroperoxides. Quantitation of free cholesterol, cholesteryl oleate, cholesteryl linoleate, and cholesteryl arachidonate was done using calibration curves constructed with authentic commercial standards, and cholesteryl ester hydroperoxides were quantitated as described previously using 15(S)-hydroperoxyeicosatetraenoic acid as standard (31).

Quantitation of vitamin E, lycopene, β-carotene, and ubiquinol-10

A 50-μl aliquot of the organic extract of LDL in ethanol (prepared as described above) was analyzed by reversed-phase HPLC using an LC-8 column (Supelco), and 1% water in methanol containing 20 mM lithium perchlorate as mobile phase (19). The eluate was analyzed by electrochemical detection at an applied potential of +0.6 V in an LC 4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN). Lycopene, β-carotene, and ubiquinol-10 eluted as single peaks with retention times of 6.6, 8.3, and 11.4 min, respectively; γ- and α-tocopherol eluted with retention times of 4.1 and 4.3 min, respectively, and were quantitated together as vitamin E using d,l-α-tocopherol as a standard. Calibration of the HPLC system was done daily using fresh solutions of authentic antioxidant standards dissolved in hexane (lycopene, β-carotene) or ethanol (α-tocopherol, ubiquinol-10). The concentrations of the standard solutions were determined spectrophotometrically using the following molar extinction coefficients: α-tocopherol, $\epsilon_{292\,nm} = 3.27 \times 10^3\ M^{-1}\ cm^{-1}$; lycopene, $\epsilon_{472\,nm} = 1.85 \times 10^5\ M^{-1}\ cm^{-1}$; β-carotene, $\epsilon_{465\,nm} = 1.29 \times 10^5\ M^{-1}\ cm^{-1}$; and ubiquinol-10, $\epsilon_{290\,nm} = 4.01 \times 10^3\ M^{-1}\ cm^{-1}$.

Statistical analysis of the data

As mentioned above, β-carotene supplementation of the 16 test subjects did not result in statistically significant protection of LDL against oxidation. All the other parameters investigated in this study also were not affected by β-carotene supplementation, except for lycopene levels in LDL which decreased slightly. Thus, treatment with β-carotene was not considered for the purposes of the analyses reported in this paper. Each sample was treated as an individual data point due to the variability of the covariates for each time point during the 19-week study period.

For each covariate normality was determined by examining the frequency distributions, the normal probability plots, and the Shapiro-Wilk test for normality. Pearson correlation coefficients were used for normal comparisons, and Spearman correlation coefficients for comparisons with grossly non-normal covariates. The only covariate falling into the latter category were preformed lipid hydroperoxides. Therefore, values given in the Results section are Pearson correlation coefficients, unless otherwise indicated.

RESULTS

The 62 human LDL samples studied in this investigation were analyzed immediately after isolation from plasma for the endogenous antioxidants α- and γ-tocopherol (henceforth together referred to as vitamin E), ubiquinol-10, β-carotene, and lycopene, preformed cholesteryl ester hydroperoxides (henceforth referred to as lipid hydroperoxides), and free and esterified cholesterol (cholesteryl oleate, linoleate, and arachidonate) (**Table 1**). The LDL samples (0.1 mg protein/ml in PBS, 37°C) were subjected to either metal ion-dependent oxidation by in-

TABLE 1. Levels of antioxidants, free and esterified cholesterol, and preformed lipid hydroperoxides in human LDL, and length of the lag phase of inhibited lipid peroxidation (T_{lag}) and rate of lipid peroxidation during the lag phase (R_{lag}) and the propagation phase (R_p) in LDL exposed to Cu^{2+} or AAPH

	Mean ± SD	Range	n
Vitamin E (nmol/mg p)	15.5 ± 2.9	7.7–24.1	62
Ubiquinol-10 (nmol/mg p)	0.65 ± 0.28	0.11–1.35	62
β-Carotene (nmol/mg p)	2.74 ± 1.13, 0.85 ± 0.49[a]	0.19–5.08	62
Lycopene (nmol/mg p)	0.53 ± 0.20	0.20–1.10	62
Free cholesterol (nmol/mg p)	817 ± 103	598–992	61
Cholesteryl oleate (nmol/mg p)	598 ± 89	428–836	61
Cholesteryl linoleate (nmol/mg p)	1775 ± 236	1295–2130	61
Cholesteryl arachidonate (nmol/mg p)	433 ± 114	213–710	61
Cholesteryl ester hydroperoxides (pmol/mg p)	243 ± 212	13–886	56
Cholesteryl ester hydroperoxides (ppm)	75 ± 61	4.9–256	56
$T_{lag(Cu)}$ (min)	140 ± 39	76–242	61
$T_{lag(AAPH)}$ (min)	78 ± 10	60–102	61
$R_{lag(Cu)}$ (nmol dienes/min/mg p)	2.46 ± 0.41	1.71–3.64	57
$R_{p(Cu)}$ (nmol dienes/min/mg p)	6.46 ± 1.47	4.10–9.46	33
$R_{lag(AAPH)}$ (nmol dienes/min/mg p)	2.75 ± 0.48	1.83–4.17	57

n, number of LDL samples analyzed; p, protein; SD, standard deviation; ppm, parts per million (cholesteryl ester hydroperoxides per total cholesteryl esters).
[a]During and after the β-carotene supplementation period, respectively (see Methods).

cubation with 1.25 μM $CuCl_2$, or metal ion-independent oxidation by exposure to 4 mM of the aqueous radical initiator AAPH in the presence of 0.1 mM of the metal chelator DTPA. Lipid peroxidation was assessed as diene conjugation at 234 nm, and the length of the lag phase of inhibited lipid peroxidation (T_{lag}) preceding the propagation phase of uninhibited lipid peroxidation was measured (Figs. 1A and B). The rate of lipid peroxidation during the lag and propagation phases (R_{lag} and R_p, respectively) was also determined (Figs. 1A and B). The data are summarized in Table 1. T_{lag}, R_{lag}, and R_p were used as measures of the oxidative susceptibility of LDL.

T_{lag} of LDL exposed to Cu^{2+} ($T_{lag(Cu)}$) was not statistically significantly correlated ($P > 0.05$) with T_{lag} of LDL exposed to AAPH ($T_{lag(AAPH)}$) (**Table 2**), indicating that the mechanisms of metal ion-dependent and -independent LDL oxidation are different. In order to identify the factors in LDL contributing to this difference, we investigated the correlations of $T_{lag(Cu)}$ and $T_{lag(AAPH)}$ with antioxidants, preformed lipid hydroperoxides, and cholesterol in LDL. $T_{lag(Cu)}$ was not correlated with vitamin E levels expressed relative to LDL protein (**Fig. 2A,** Table 2). However, when vitamin E was expressed per mol total cholesterol in LDL (sum of free and esterified cholesterol, henceforth referred to simply as cholesterol) rather than per mg protein, a significant correlation with $T_{lag(Cu)}$ was observed (Fig. 2B, Table 2). These data suggest that vitamin E directly protects LDL's lipids, but not its protein, against Cu^{2+}-induced oxidation. Ubiquinol-10, β-carotene, and lycopene, whether quantitated relative to lipid or protein, did not show significant protection against Cu^{2+}-induced LDL oxidation. For example, the coefficients of correlation between $T_{lag(Cu)}$ and ubiquinol-10 expressed per mg protein or mol cholesterol were $r = 0.13$ and 0.22, respectively ($P > 0.05$).

As metal ion-dependent oxidative modification of LDL has been proposed to be initiated by metal-catalyzed breakdown of preformed lipid hydroperoxides (13–15), we

TABLE 2. Coefficients of correlation (r-values) for the length of the lag phase of inhibited lipid peroxidation (T_{lag}) and rate of lipid peroxidation during the lag phase (R_{lag}) in LDL exposed to Cu^{2+} or AAPH

			Vitamin E			
	$T_{lag(Cu)}$	$T_{lag(AAPH)}$	nmol/mg p	mmol/mol ch	LOOHs	Total Cholesterol
	min	*min*			*ppm*	*nmol/mg p*
$T_{lag(Cu)}$	(1.00)	0.10	0.25	0.42[c]	−0.37[b]	−0.28[a]
$R_{lag(Cu)}$	−0.39[b]	n.d.	0.03	−0.32[a]	0.39[b]	0.46[c]
$T_{lag(AAPH)}$	0.10	(1.00)	−0.42[c]	−0.20	−0.17	−0.33[b]
$R_{lag(AAPH)}$	n.d.	−0.49[d]	0.31[a]	−0.13	0.43[b]	0.61[d]

Values are Pearson coefficients, except for correlations with preformed lipid hydroperoxides (LOOHs), which are Spearman coefficients. Ch, total cholesterol (sum of free and esterified cholesterol); n.d., not determined; p, protein.
Significance levels are: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P < 0.0001$.

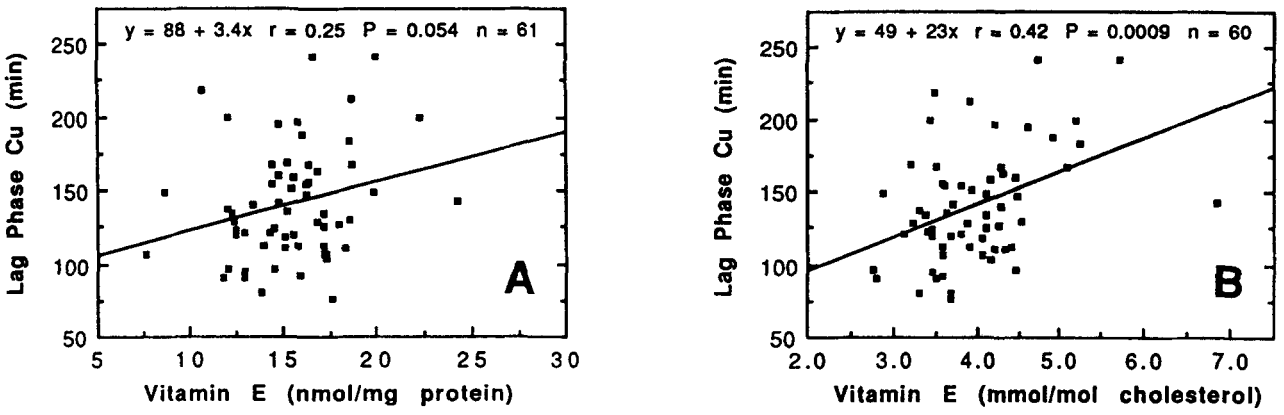

Fig. 2. Correlation between vitamin E expressed relative to protein (A) or cholesterol (B) and the length of the lag phase of inhibited lipid peroxidation in LDL exposed to Cu^{2+}. One LDL sample in panel B had an unusually high vitamin E-to-cholesterol ratio (6.85 mmol/mol), which is more than two standard deviations (2 × 0.72 mmol/mol) higher than the mean value for all LDL samples (3.99 mmol/mol). Omission of the data point for this particular LDL sample resulted in an increase of the *r*-value for the correlation shown in panel B from 0.42 ($P = 0.0009$) to 0.49 ($P < 0.0001$).

investigated whether $T_{lag(Cu)}$ is correlated with lipid hydroperoxide levels present in isolated LDL before incubation with Cu^{2+}. Lipid hydroperoxides were measured by a sensitive and specific HPLC assay with chemiluminescence detection (31), and quantitated on a parts per million (ppm) basis by calculating the ratio of cholesteryl ester hydroperoxides (pmol/mg protein) to total cholesteryl esters (sum of cholesteryl oleate, linoleate, and arachidonate, μmol/mg protein). We found a significant inverse correlation between $T_{lag(Cu)}$ and preformed lipid hydroperoxides (Table 2), i.e., LDL containing smaller amounts of lipid hydroperoxides was less susceptible to Cu^{2+}-induced oxidation. $T_{lag(Cu)}$ was also inversely associated with LDL cholesterol (Table 2), suggesting that a high lipid-to-protein ratio renders LDL more susceptible to Cu^{2+}-induced oxidation.

The rate of lipid peroxidation during the lag phase in Cu^{2+}-exposed LDL ($R_{lag(Cu)}$) was inversely correlated with $T_{lag(Cu)}$ (Table 2), i.e., the faster the rate of lipid peroxidation during the lag phase, the shorter it was. Consistent with this finding, other parameters that were significantly correlated with $T_{lag(Cu)}$ were also significantly correlated, in the opposite direction, with $R_{lag(Cu)}$: there was a significant inverse association between $R_{lag(Cu)}$ and the vitamin E-to-cholesterol ratio (but not the vitamin E-to-protein ratio), and strong direct associations between $R_{lag(Cu)}$ and preformed lipid hydroperoxides and cholesterol (Table 2). The rate of lipid peroxidation during the propagation phase ($R_{p(Cu)}$) was directly correlated with $R_{lag(Cu)}$ ($r = 0.51$, $P < 0.01$). Correspondingly, $R_{p(Cu)}$ was strongly inversely associated with $T_{lag(Cu)}$ ($r = -0.68$, $P < 0.0001$) and directly with LDL cholesterol ($r = 0.63$, $P < 0.0001$). Unlike $R_{lag(Cu)}$, however, $R_{p(Cu)}$ was not significantly correlated with preformed lipid hydroperoxides or the vitamin E-to-cholesterol ratio. These data indicate that the rate of lipid peroxidation in Cu^{2+}-exposed LDL both during and after the lag phase depends on the lipoprotein's lipid content, and that the rate during the lag phase, but not the propagation phase, is also influenced by levels of preformed lipid hydroperoxides and vitamin E.

$T_{lag(AAPH)}$, in contrast to $T_{lag(Cu)}$, was significantly correlated with vitamin E quantitated relative to LDL protein (**Fig. 3A,** Table 2). Most remarkably, however, this correlation was inverse, indicating that the higher the vitamin E content, the less resistant LDL to oxidation induced by aqueous peroxyl radicals. This strong inverse association was not observed when vitamin E levels were expressed relative to cholesterol (Fig. 3B, Table 2). Ubiquinol-10, β-carotene, and lycopene, irrespective of whether expressed relative to lipid or protein in LDL, were not significantly correlated with $T_{lag(AAPH)}$. These findings indicate that the antioxidants in LDL do not protect the lipoprotein's lipids against aqueous peroxyl radical-induced oxidation, and that vitamin E may actually promote peroxidative damage to LDL under the experimental conditions used.

Unlike $T_{lag(Cu)}$, $T_{lag(AAPH)}$ was not associated with preformed lipid hydroperoxides (Table 2), suggesting that these do not contribute significantly to initiation of lipid peroxidation in LDL by aqueous peroxyl radicals. Similar to $T_{lag(Cu)}$, however, $T_{lag(AAPH)}$ was inversely correlated with LDL cholesterol (Table 2). Thus, a high cholesterol content was associated with increased susceptibility of LDL to both metal ion-dependent and -independent oxidation.

In order to address the question of why the vitamin E-to-protein ratio, but not the vitamin E-to-cholesterol ratio, was inversely correlated with $T_{lag(AAPH)}$ (see Fig. 3A), we plotted LDL cholesterol against vitamin E (in nmol/mg protein). A significant positive correlation was observed ($r = 0.37$, $P < 0.01$). Thus, vitamin E levels in LDL appear to reflect cholesterol levels, which themselves

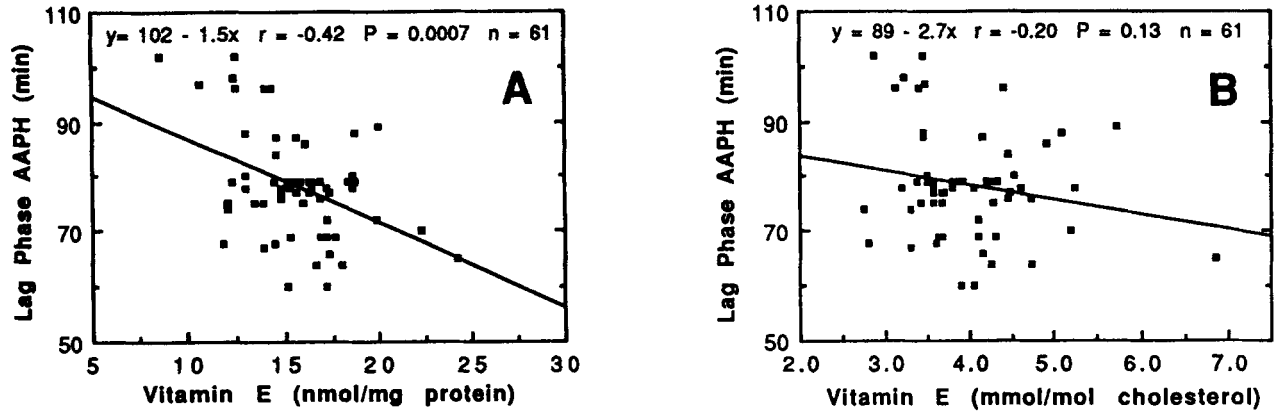

Fig. 3. Correlation between vitamin E expressed relative to protein (A) or cholesterol (B) and the length of the lag phase of inhibited lipid peroxidation in LDL exposed to AAPH. Omission of the data point for the LDL sample with the vitamin E-to-cholesterol ratio of 6.85 mmol/mol (see legend to Fig. 2) resulted in a decrease of the *r*-value for the correlation shown in panel B from −0.20 (P = 0.13) to −0.13 (P = 0.33).

are inversely associated with $T_{lag(AAPH)}$. This may explain in part why vitamin E content relative to protein, but not cholesterol, is inversely associated with $T_{lag(AAPH)}$.

Similar to the correlation between R_{lag} and T_{lag} for Cu^{2+}-exposed LDL, the rate of AAPH-induced lipid peroxidation during the lag phase ($R_{lag(AAPH)}$) was inversely associated with $T_{lag(AAPH)}$ (Table 2). Consistently, other parameters that were correlated with $T_{lag(AAPH)}$ were also correlated, in the opposite direction, with $R_{lag(AAPH)}$ (Table 2), except for preformed lipid hydroperoxides, which were significantly correlated with $R_{lag(AAPH)}$ but not $T_{lag(AAPH)}$. There was a particularly strong direct association between $R_{lag(AAPH)}$ and LDL cholesterol (Table 2), indicating that the lipid content is of major importance for the rate of lipid peroxidation during the lag phase in LDL exposed to aqueous peroxyl radicals.

DISCUSSION

The aim of this study was to identify components of human LDL that contribute to the susceptibility of this lipoprotein to oxidative modification. We measured antioxidants and preformed lipid hydroperoxides by sensitive and specific methods, and expressed antioxidant levels relative to LDL protein or cholesterol content, the latter defined as the sum of free and esterified cholesterol. The levels of antioxidants and lipids in our LDL samples (see Table 1) were similar to published values (13), except for β-carotene levels, which were higher due to supplementation. Lipid hydroperoxide levels in our LDL preparations were two orders of magnitude lower than those reported by some other investigators (15, 32). On the other hand, Bowry, Stanley, and Stocker (33) estimated that plasma LDL contains about 0.0004 molecules of cholesteryl ester hydroperoxides/LDL particle; using a molecular weight for apoB of 513,000 (13), our mean value for cholesteryl ester hydroperoxides of 243 pmol/mg LDL protein (Table 1) corresponds to 0.125 molecules/LDL particle, i.e., about 300-fold higher than the value reported by Bowry and colleagues (33). These data, therefore, suggest that our LDL preparations have been oxidized ex vivo, but that the degree of oxidation is very low relative to that of LDL used by some other investigators (15, 32).

The most important findings of the present study are summarized in Table 2. We found that the susceptibility of LDL to metal ion-dependent oxidation was not related to its susceptibility to metal ion-independent oxidation, indicating that LDL is oxidized by two different mechanisms. In order to identify the factors in LDL contributing to this difference in susceptibility to oxidation by different types of oxidative stress, we investigated the effects of antioxidants, preformed lipid hydroperoxides, and cholesterol content on LDL oxidizability.

We found no statistically significant correlation between the susceptibility of LDL to Cu^{2+}-induced oxidation and its vitamin E content expressed relative to protein. This result is in agreement with numerous previous studies showing that the α-tocopherol-to-protein ratio of LDL from donors not receiving vitamin E supplements cannot be used to predict LDL's resistance to oxidation induced by either metal ions (13, 17, 20, 21), mouse peritoneal macrophages (17), or γ-irradiation (18). For example, Esterbauer et al. (13) reported a nonsignificant correlation coefficient of r = 0.20 between the susceptibility to Cu^{2+}-induced oxidation and α-tocopherol content (in mol/mol apoB) of LDL prepared from 78 non-vitamin E-supplemented donors; our nonsignificant *r*-value of 0.25 for 61 LDL samples is similar.

However, it may not be appropriate to quantitate vitamin E levels relative to protein. First, vitamin E can be presumed to primarily protect the lipids in LDL, not

apoB, against oxidative attack. Second, lipid peroxidation is an important early step in LDL oxidation (1, 8–10, 13), and its measurement, rather than protein oxidation, was used as an indicator of LDL's susceptibility to oxidation in this and numerous other studies (13, 17, 18, 20–25). When we expressed vitamin E levels relative to cholesterol, it became obvious that this antioxidant does play an important role in preventing Cu^{2+}-induced lipid peroxidation in LDL. In fact, among the components of LDL investigated in this study, an increased vitamin E-to-cholesterol ratio was the strongest predictor of an increased resistance of the lipoprotein to metal ion-dependent oxidation. In addition to vitamin E, water-soluble antioxidants in the interstitial fluid of the arterial subendothelial space may also protect LDL against oxidative modification in vivo. For example, vitamin C and uric acid very effectively protect LDL against Cu^{2+}-induced oxidation (16, 27).

Interestingly, despite the high statistical significance ($P = 0.0009$) of the correlation between the lag phase of lipid peroxidation and the vitamin E content relative to lipid in LDL exposed to Cu^{2+}, the coefficient of correlation was rather low ($r = 0.42$). This r-value of 0.42 corresponds to an r^2-value of 0.18, indicating that about 20% of the lag phase of lipid peroxidation in Cu^{2+}-incubated LDL is determined by the vitamin E-to-cholesterol ratio. Preformed lipid hydroperoxides in LDL may explain another 15% of $T_{lag(Cu)}$ ($r = -0.37$, i.e., $r^2 = 0.14$). However, approximately 65% of $T_{lag(Cu)}$ appear to be determined by unidentified factors in LDL. Similarly, in LDL exposed to aqueous peroxyl radicals only about 20% and 10% of the lag phase of lipid peroxidation are determined by the vitamin E-to-protein ratio and the cholesterol content, respectively, while approximately 70% of $T_{lag(AAPH)}$ remain unexplained. Thus, the parameters investigated in the present study can only partly explain the variations of the susceptibility of LDL to oxidation.

In addition to vitamin E, none of the other known antioxidants in LDL measured by us, i.e., ubiquinol-10, lycopene, and β-carotene, showed significant protection against metal ion-dependent or -independent oxidation of the lipoprotein. This is not surprising as these antioxidants are present in much smaller quantities in LDL than vitamin E, and thus contribute little to the lipoprotein's total antioxidant capacity. Based on the measurements presented in Table 1, we calculate that on an average each particle of LDL contains 7.9 molecules of vitamin E, and only 1.5 molecules of all the other antioxidants combined, in good agreement with published data (13, 33). We had previously argued that ubiquinol-10 plays a significant role in protecting LDL against lipid peroxidation, as ubiquinol-10 forms the first line of antioxidant defense and is consumed before α-tocopherol in LDL exposed to oxidative stress (19, 34). Ubiquinol-10 may play a pivotal role under milder oxidative stress conditions than used in this study, preventing formation of minimally modified LDL. This form of LDL has been shown to possess a number of potentially atherogenic properties, such as stimulation of expression of monocyte adhesion molecules and monocyte and granulocyte colony stimulating factors by vascular endothelial cells (4, 35).

As mentioned above, increased levels of preformed lipid hydroperoxides in LDL were associated with an increased susceptibility of LDL to metal ion-dependent oxidation, which is consistent with the concept that in vitro metal-catalyzed breakdown of preformed lipid hydroperoxides initiates lipid peroxidation in LDL (13–15). However, it should be noted that the lipid hydroperoxides in our LDL preparations, although present only in small amounts, appear to have been formed mostly ex vivo (see above), i.e., appear to represent an artifact. Therefore, the observed correlation between preformed lipid hydroperoxides and the susceptibility to Cu^{2+}-induced oxidation of isolated LDL may be of little relevance to the situation in vivo, where lipid hydroperoxide levels are much lower (31, 33).

Consistent with the lack of a correlation between the susceptibility of LDL to metal ion-dependent and -independent oxidation, we found that an increased vitamin E content relative to LDL protein is associated with a decreased resistance of LDL to metal ion-independent oxidation. This association may be partly explained by the fact that vitamin E levels are positively correlated with, i.e., reflect, cholesterol levels in LDL, which are positively correlated with LDL's oxidative susceptibility. However, the correlation between vitamin E and cholesterol levels was moderate, and T_{lag} of AAPH-exposed LDL was more strongly associated with vitamin E than cholesterol (see Table 2). Therefore, additional factors may contribute to vitamin E's promoting effect on aqueous peroxyl radical-induced LDL oxidation. Most interestingly, two recent papers have concluded, on both experimental and theoretical grounds, that α-tocopherol may exert prooxidant activity in LDL exposed to a low flux of aqueous peroxyl radicals, as under these experimental conditions α-tocopherol may act as a chain-transfer agent rather than as a radical trap, and may also be involved in the transfer of radicals from the aqueous phase into the lipoprotein (36, 37). It is important to note that this effect is strictly concentration-dependent: at low concentrations of LDL and AAPH, as were used in the present study, a prooxidant effect of vitamin E in LDL is observed (36), whereas at high concentrations of LDL and AAPH vitamin E exerts antioxidant activity in LDL (36, 38, 39). However, in vivo the rate of radical production is likely to be very low, and vitamin E's prooxidant effect in LDL may prevail over its antioxidant effect, once ubiquinol-10 and vitamin C have been exhausted (36, 37).

In light of these data, therefore, it appears pivotal to clarify the mechanism of LDL oxidation in vivo, as sup-

plementation with vitamin E may actually promote atherosclerosis if LDL oxidation in vivo occurs by a metal ion-independent mechanism. The protective effects of vitamin E supplementation against experimental atherosclerosis in primates and rabbits (40, 41), and against coronary heart disease observed in epidemiologic studies (42–44) may suggest that LDL oxidation in vivo occurs by a metal ion-dependent mechanism. This notion is also supported by epidemiologic studies suggesting a direct association between serum copper and ferritin levels and risk of cardiovascular disease (45, 46), although these studies need to be confirmed. In addition, a clinical study has shown that the susceptibility of isolated LDL to Cu^{2+}-induced oxidation is significantly correlated with global coronary atherosclerosis in patients with a history of myocardial infarction (47).

Finally, we found that an increased cholesterol content of LDL was associated with an increased oxidative susceptibility of the lipoprotein to both Cu^{2+} and aqueous peroxyl radicals. This is most probably because LDL's cholesterol content represents more than 70% of total lipids in LDL (in nmol/mg protein) (13), and lipids are the substrates for lipid peroxidation. Since not cholesterol itself but esterified polyunsaturated fatty acyl side chains of lipids are the true substrates for lipid peroxidation, it is likely that the levels of these polyunsaturated fatty acids are more strongly associated with the oxidative susceptibility of LDL than the cholesterol content. Reaven et al. (48) have shown that the content of linoleic acid, the most abundant polyunsaturated fatty acid in LDL, is strongly correlated with the susceptibility of the lipoprotein to Cu^{2+}-induced oxidation. It is also interesting to note that our finding of an increased oxidizability of LDL with an increased lipid-to-protein ratio appears to be in conflict with the observations that large, buoyant LDL subfractions are less susceptible to Cu^{2+}-induced oxidation than small, dense LDL subfractions (25, 49, 50). Although we are currently unable to explain this discrepancy, it is noteworthy that in our LDL preparations there was a very strong positive correlation between free and esterified cholesterol ($r = 0.86$, $P < 0.0001$), whereas Tribble et al. (25) reported a significant inverse correlation ($P = 0.0002$) between these two parameters in LDL subfractions, and observed a positive association between free cholesterol and the resistance of LDL to Cu^{2+}-induced oxidation ($r = 0.46$, $P < 0.001$). In contrast, in our study we observed a significant inverse correlation between free cholesterol and the resistance of LDL to Cu^{2+}-induced oxidation ($r = -0.26$, $P < 0.05$).

In summary, the findings reported here show that the LDL particles most resistant to metal ion-dependent oxidation are characterized by a high vitamin E-to-cholesterol ratio, a low cholesterol-to-protein ratio, and low levels of preformed lipid hydroperoxides. The LDL particles most resistant to metal ion-independent oxidation also have a low cholesterol content, but contain small amounts of vitamin E relative to protein.

Note added in proof: In a recent publication (Bowry, V. W., and R. Stoker. 1993. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J. Am. Chem. Soc.* **115:** 6029–6044) it was shown that vitamin E may also act as a prooxidant in LDL incubated with low concentrations of transition metal ions. Therefore, the differences observed by us between oxidative susceptibility of LDL exposed to Cu^{2+} and AAPH may only partially reflect fundamental differences in the mechanisms of metal ion-dependent and -independent oxidation, but may also be due to the different rates of radical production by the concentrations of Cu^{2+} and AAPH used.

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