Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1183–1187, February 1994
Medical Sciences

Oxidative susceptibility of low density lipoprotein subfractions is related to their ubiquinol-10 and α-tocopherol content

(parinaric acid/atherosclerosis/antioxidants/conjugated dienes)

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Contributed by Bruce N. Ames, October 1, 1993

ABSTRACT **The conjugated polyene fatty acid parinaric acid (PnA) undergoes a stoichiometric loss in fluorescence upon oxidation and can be used to directly monitor peroxidative stress within lipid environments. We evaluated the course of potentially atherogenic oxidative changes in low density lipoproteins (LDL) by monitoring the oxidation of PnA following its incorporation into buoyant (ρ = 1.026–1.032 g/ml) and dense (ρ = 1.040–1.054 g/ml) LDL subfractions. Copper-induced oxidation of LDL-associated PnA exhibited an initial lag phase followed by an increased rate of loss until depletion. Increased PnA oxidation occurred immediately after the antioxidants ubiquinol-10 and α-tocopherol were consumed but before there were marked elevations in conjugated dienes. Despite differences in sensitivity to early oxidation events, PnA oxidation and conjugated diene lag times were correlated (r = 0.582; P = 0.03), and both indicated a greater susceptibility of dense than buoyant LDL in accordance with previous reports. The greater susceptibility of PnA in dense LDL was attributed to reduced levels of ubiquinol-10 and α-tocopherol, which were ≈50% lower than in buoyant LDL (mol of antioxidant/mol of LDL) and together accounted for 80% of the variation in PnA oxidation lag times. These results suggest that PnA is a useful probe of LDL oxidative susceptibility and may be superior to conjugated dienes for monitoring the initial stages of LDL lipid peroxidation. Differences in oxidative susceptibility among LDL density subfractions are detected by the PnA assay and are due in large part to differences in their antioxidant content.**

Free-radical-mediated oxidation induces potentially atherogenic changes in low density lipoproteins (LDL) (1). Chief among these is an ability to stimulate cholesterol engorgement by macrophages (2, 3), which are progenitors of lipid-laden foam cells found in early atherosclerotic lesions. In addition, oxidized-LDL influences the chemotactic behavior of monocytes and monocyte-derived macrophages (4, 5), promotes endothelial cell damage (6, 7), and induces potent mediators of cell proliferation, monocyte adherence, and platelet aggregation (8–12), all of which may contribute to lesion formation and growth.

LDL oxidation is believed to proceed through initial oxidation of the fatty acyl components. The resulting lipid-derived radicals are intercepted by radical scavenging antioxidants including ubiquinol-10 and α-tocopherol. However, if the oxidant stress is sufficiently great or prolonged, these antioxidants are depleted (13–16) and injury is propagated among LDL lipids with spread to the protein component through reactive lipid breakdown products (6, 17). The atherogenic properties of oxidized-LDL are attributed to this extensively altered particle (1–7, 17), although minimal modifications associated with the initial stages of injury also may be important (10–12).

As evidence has accumulated in support of a role for LDL oxidation in atherogenesis, assays of oxidative susceptibility have been developed to evaluate LDL atherogenic potential. LDL oxidative susceptibility is typically assessed by monitoring the course of oxidation under conditions of controlled oxidant exposure. Time-dependent changes in chemical constituents including antioxidants, lipids, and protein, and in biological properties such as macrophage uptake and cytotoxicity, have been examined. However, many studies have relied primarily on rates of bulk lipid peroxidation (e.g., as indicated by conjugated diene formation) to monitor LDL oxidation. Such measures are characterized by an initial slow or lag phase followed by a period of rapid change. The length of the lag phase provides a convenient index for comparison among LDL samples but is frequently misinterpreted as a period during which no changes are occurring in LDL lipids. Rather, the conjugated diene lag phase masks an active period involving radical initiations, radical chain terminations, and antioxidant depletion (13–16).

The oxidative fluorescence decay of parinaric acid (PnA) has been used to monitor lipid peroxidation processes in bilayer systems (18–21). This polyunsaturated fatty acid is similar in structure to arachidonic acid but contains a conjugated polyene system that accounts for its fluorescent properties and oxidative susceptibility. As a preferred target relative to most endogenous fatty acids, PnA reflects initial peroxidation events not apparent from measures of bulk lipid peroxidation. In the current studies, we investigated whether PnA might be useful for monitoring early peroxidative changes in LDL. The ability of this assay to detect intrinsic differences among LDL preparations was evaluated by performing experiments in buoyant vs. dense LDL subfractions, which have been shown to differ in oxidative susceptibility by standard assays (22–25). Our results show that PnA is an earlier indicator of peroxidative stress than conjugated dienes and may be particularly useful for identifying intrinsic properties that influence the initial stages of LDL oxidation.

MATERIALS AND METHODS

Chemicals. PnA (9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid) was from Molecular Probes. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Aldrich). Ubiquinone-10 and D,L-α-tocopherol standards were from Sigma; ubiquinone-10 (CoQ) was con-

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Abbreviations: LDL, low density lipoprotein(s); PnA, parinaric acid; TBARS, thiobarbituric acid-reactive substance(s).

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verted to ubiquinol-10 ($CoQH_2$) by treatment with sodium borohydride as described (26). All reagents, buffer components, and HPLC solvents were of the highest grade commercially available.

Isolation and Characterization of LDL Subfractions. Subjects were eight healthy normolipidemic adult volunteers (six men and two women) not using vitamin supplements or taking hormones or drugs known to alter plasma lipids. Blood samples were collected by venipuncture into vacutainers containing 1 mg of EDTA per ml and 10 μM Trolox (a water-soluble vitamin E analog), and cells were immediately removed by centrifugation at 2000 × g for 30 min under refrigeration (4°C). LDL subfractions were isolated by two sequential spins at ρ = 1.026 g/ml and ρ = 1.032 g/ml (for buoyant LDL) and ρ = 1.040 g/ml and ρ = 1.054 g/ml (for dense LDL). These density intervals contain predominantly LDL-I and LDL-III, respectively (27), and were selected to obtain preparations containing particles of nonoverlapping size as indicated by gradient gel electrophoresis. The upper densities were obtained by addition of solid NaBr directly to plasma. Samples (6 ml) were centrifuged in a Beckman 40.3 fixed-angle rotor at 40,000 rpm for 20 hr at 10°C. The upper 2 ml was removed, and samples were again brought up to 6 ml and adjusted to the more dense cutoff values using NaBr solutions. Following centrifugation for 20 hr at 10°C, LDL subfractions were recovered in the upper 2 ml. EDTA (1 mg/ml) and Trolox (1 μM) were included in all spin solutions to reduce oxidative changes during isolation.

Lipoprotein particle diameters were determined by nondenaturing 2–16% gradient gel electrophoresis (28) at 8–10°C using 0.09 M Tris/0.08 M-boric acid buffer, pH 8.3, containing 3 mM EDTA. Lipoprotein samples were adjusted to 20% sucrose and 3–10 μl was applied to the gel. Potentials were set at 40 V (15 min) followed by 70 V (24 hr). Gels were stained with Coomassie brilliant blue and scanned at 555 nm using a Transidyne RFT densitometer. Molecular diameters were determined on the basis of migration distance by comparison with standards of known diameter.

Protein concentrations were determined using the Lowry method modified to include sodium dodecyl sulfate (29). Phospholipid phosphorus was analyzed according to the method of Bartlett (30) and was expressed as phosphatidylcholine equivalents. Total cholesterol and triglycerides were measured using standard enzymatic methods on a System 3500 Gilford computer-directed analyzer. Unesterified and esterified cholesterol were determined on a Hewlett-Packard 5830A gas chromatograph (31).

The antioxidants ubiquinol-10 and α-tocopherol were measured in lipoprotein lipid extracts by reversed-phase HPLC with electrochemical detection (32). Isolated LDL was mixed with 1 ml of ethanol and briefly dispersed in a Vortex, and 5 ml of hexane was added. The samples were spun in a Vortex and then centrifuged at 1000 × g, and the upper hexane layer was transferred to a glass tube. An additional 5 ml of hexane was added and the extraction procedure was repeated. Extracts were pooled and dried under a stream of nitrogen, and the resulting residue was dissolved in 0.5 ml of methanol/ethanol, 1:1 (vol/vol). Separations were accomplished using 3-μm particle size Supelcosil (Supelco) LC-8-DB guard (1.5 cm × 4.6 mm) and analytical (15 cm × 4.6 mm) columns. The mobile phase consisted of 20 mM lithium perchlorate in methanol/H_2O, 96:4 (vol/vol), and was delivered at a flow rate of 2 ml/min. Electrochemical detection was performed using an ESA model 5100 Coulochem detector equipped with a 5021 conditioning cell and a 5011 analytical cell. The conditioning cell potential was set at −0.55 V (for reduction). Potentials for the analytical cell were set at +0.01 V and +0.4 V for electrodes 1 and 2, respectively. The signal produced by electrode 2 was used for detection of antioxidants. Antioxidants were quantified by comparison with standards of known amount.

Table 1. Characteristics of buoyant and dense LDL subfractions

	LDL subfraction		
	Buoyant	Dense	P value
Density, g/ml	1.026–1.032	1.040–1.054	
Particle diameter, Å	276.2 ± 6.4	251.9 ± 4.8	0.0001
Protein,*	16.6 ± 2.2	21.3 ± 2.8	0.002
Triglyceride,*	7.8 ± 1.7	4.7 ± 1.9	0.004
Phospholipid,*	24.3 ± 1.4	23.3 ± 1.7	0.02
Unesterified cholesterol,*	10.7 ± 1.9	8.2 ± 0.8	0.005
Cholesteryl esters,*	40.7 ± 3.6	42.4 ± 4.2	0.40

Values represent mean ± SD for eight subjects.
*Expressed as % of total LDL mass, which was derived by summing the mass contributed by protein, triglycerides, phospholipids, unesterified cholesterol, and cholesteryl esters.

LDL Oxidation. LDL subfractions were dialyzed for 24 hr against 0.01 M phosphate-buffered saline (PBS; pH 7.4) to remove EDTA and Trolox prior to oxidation. LDL protein concentrations were adjusted to 100 μg/ml and incubations were carried out at 37°C. Oxidation was initiated by addition of $CuSO_4$.

PnA was added to LDL by injection of an ethanolic solution to a final concentration of 0.85 μM in a total volume of 2 ml of PBS and was allowed to equilibrate (as indicated by signal stability) prior to $CuSO_4$ addition. Fluorescence measurements were performed in a Perkin–Elmer LS5B spectrofluorometer equipped with a thermostated cuvette and a magnetic stirring device. Excitation and emission wavelengths were set at 324 nm (slit width, 3 nm) and 413 nm (slit width, 10 nm), respectively.

Conjugated diene formation was determined by monitoring the increase in absorbance at 234 nm in a Beckman DU-70 spectrophotometer equipped with a six-position automatic sample changer. Initial absorbance was set at zero and was recorded every 5 min for 16 hr. Lag times were defined as the time interval between initiation and the intercept of the tangent of the slope of the absorbance curve (22, 24, 25).

Statistical Analyses. Statistical analyses were performed using the Macintosh STATVIEW II statistical program (Abacus Concepts, Berkeley, CA). Mean differences in composition and oxidation parameters among LDL subfractions were evaluated by analysis of variance. Relationships among oxidation lag times and LDL antioxidants were determined by linear regression. All significance levels are based on two-tailed tests.

RESULTS

LDL Subfraction Characteristics. Characteristics of the LDL subfractions are shown in Table 1. Mean particle diameter was ≈10% greater in buoyant than dense LDL. Buoyant LDL exhibited a higher content of triglycerides and unesterified cholesterol and a lower content of protein when these constituents were expressed as percent of total LDL mass.

Contents of the radical-scavenging antioxidants ubiquinol-10 and α-tocopherol (mol of antioxidant/mol of LDL) were 2- to 3-fold greater in buoyant than dense LDL (0.124 ± 0.070 vs. 0.068 ± 0.060 for ubiquinol-10 and 6.85 ± 0.95 vs. 2.45 ± 0.74 for α-tocopherol), although differences did not attain significance for ubiquinol-10 (P = 0.130) due to its large interindividual variation.

PnA Fluorescence Properties in LDL Subfractions. PnA was minimally fluorescent in the absence of LDL as a result of fluorescence self-quenching in fatty acid micelles, and thus its incorporation into LDL could be monitored by following the appearance of a fluorescence signal. Fluorescence intensity increased rapidly and stabilized within ≈5 min of PnA

addition. When LDL concentrations were held constant at 100 μg of LDL protein per ml, maximal fluorescence increased linearly with PnA concentrations up to ≈2 μM, ultimately achieving saturation followed by a decrease in fluorescence due to collisional quenching. Fluorescence intensity of a fixed concentration of PnA (0.85 μM) increased as LDL concentration increased until sufficient LDL was present to accommodate the probe without quenching. No significant differences in PnA or LDL concentration dependence were noted between LDL subfractions. However, in keeping with the ≈17% greater surface area (calculated from particle diameter), maximal PnA fluorescence intensity occurred at a 15% greater PnA concentration in buoyant (10.9 μM PnA) than dense (9.3 μM PnA) LDL. In subsequent oxidation experiments, we used 0.85 μM PnA and 100 μg of LDL protein per ml for both LDL subfractions (≈5 molecules of PnA per LDL particle). These conditions were such that a linear relationship existed between PnA concentration and fluorescence intensity, and small variations in LDL concentration did not influence PnA fluorescence intensity.

PnA Oxidation as Compared with Conjugated Diene Formation in LDL Subfractions. PnA fluorescence loss exhibited a biphasic response that was characterized by an initial lag phase followed by an increased rate of loss until the fluorophore was depleted. PnA was a more sensitive (earlier) (Fig. 1) indicator of peroxidative stress than conjugated dienes, as illustrated in Fig. 2 for both buoyant and dense LDL. Since conjugated diene formation was measured in separate incubations due to interference by PnA-related absorbance at 234 nm, we evaluated the influence of PnA addition on rates of formation of thiobarbituric acid-reactive substances (TBARS) as an alternative index of LDL lipid peroxidation. At the concentrations used in these studies (0.85 μM), PnA did not alter rates of TBARS formation, which also occurred over a longer time course than PnA depletion (data not shown), suggesting that PnA did not alter the oxidative susceptibility of LDL.

Despite differences in the apparent sensitivity of PnA oxidation and conjugated diene formation, lag time estimates derived from these two assays were correlated ($r = 0.582$; $P = 0.03$) (Fig. 3), and the extent of differences in mean lag time values between buoyant and dense LDL was similar for PnA oxidation (27.7 ± 9.6 min vs. 18.1 ± 8.9 min, $P < 0.05$) and

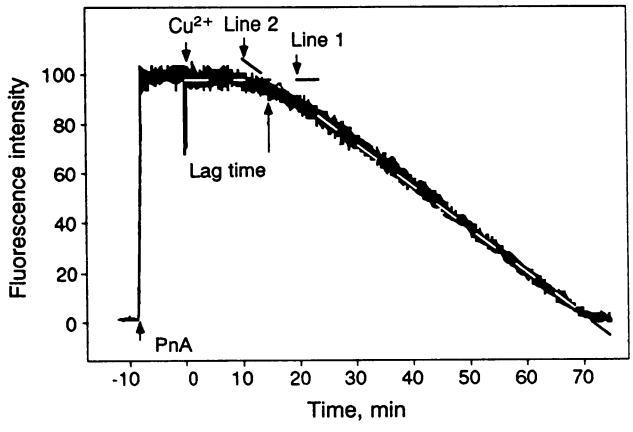

FIG. 1. Time course of Cu^{2+}-induced PnA oxidative fluorescence decay. Shown is a typical fluorescence pattern for PnA in Cu^{2+}-exposed LDL. The current example is of dense LDL; buoyant LDL behaved similarly, albeit with an extended slow phase. Lag times were defined as the time between addition of $CuSO_4$ (time zero) and movement into the rapid phase of PnA oxidation (fluorescence decay) and were derived from the intersect of lines drawn through the initial slow phase (line 1) and the linear portion of the rapid phase (line 2).

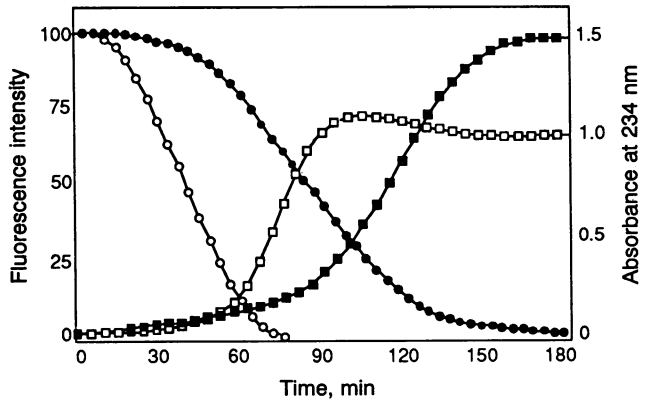

FIG. 2. PnA oxidation and conjugated diene formation in buoyant and dense LDL. PnA fluorescence intensity (circles) and absorbance at 234 nm (conjugated dienes, squares) are shown for buoyant (closed symbols) and dense (open symbols) LDL from a representative subject. Oxidation was initiated by addition of 1.66 μM $CuSO_4$.

conjugated diene formation (190.4 ± 99.7 min vs. 133.0 ± 64.6 min, $P < 0.2$).

PnA Oxidation and Conjugated Diene Formation in Relation to LDL Antioxidant Depletion and Initial Content. PnA oxidation occurred immediately upon depletion of ubiquinol-10 and α-tocopherol. In the example shown in Fig. 4, ubiquinol-10 was depleted within 8 min of Cu^{2+} addition, followed closely by α-tocopherol, which was oxidized within 20 min. Accelerated PnA oxidation began when ≈20% of the α-tocopherol remained. As before, changes in conjugated dienes were not observed until PnA oxidation was nearly complete and thus did not overlap with antioxidant depletion.

In view of the temporal relationship between antioxidant depletion and accelerated PnA loss, and observations of differences in antioxidant content and PnA oxidation lag times between subfractions, we evaluated the extent of correlation between initial antioxidant content and PnA oxidation lag times. As shown in Fig. 5, when buoyant and dense LDL were considered together, PnA oxidation lag times were highly significantly correlated with initial contents of ubiquinol-10 ($r = 0.871$, $P = 0.0001$) and α-tocopherol ($r = 0.583$, $P = 0.01$), which accounted for 80% of the total variation in PnA oxidation lag times ($r^2 = 0.792$; $P = 0.0001$). When LDL subfractions were considered independently, associations between PnA oxidation lag times and antioxi-

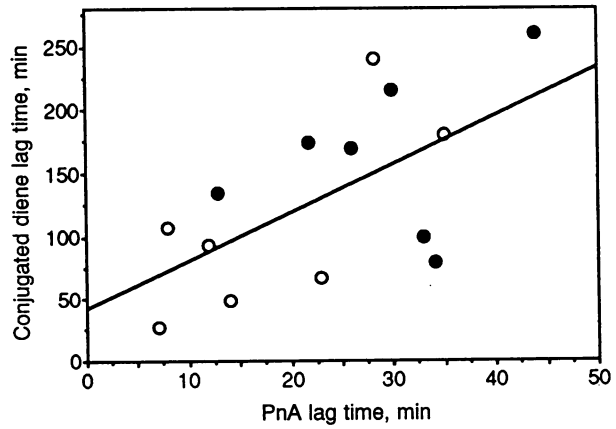

FIG. 3. Relationship of lag times derived from conjugated diene formation and PnA oxidation. Lag times were determined for buoyant (closed circles) and dense (open circles) LDL subfractions. One set of subfractions was eliminated from the current analysis due to the lack of accelerated formation of conjugated dienes within the time frame of the experiment (16 hr).

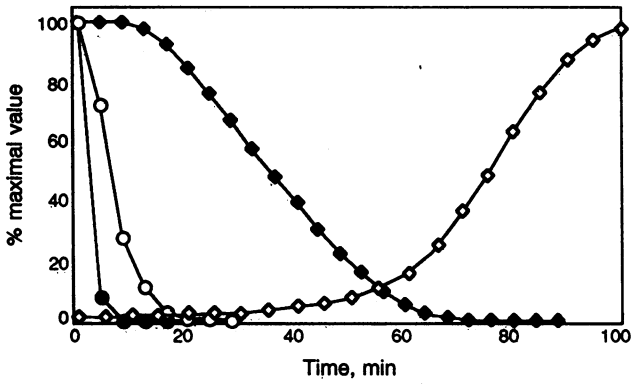

FIG. 4. Sequential changes in antioxidants and indices of lipid peroxidation in Cu^{2+}-exposed LDL. Aliquots were collected immediately prior to and at 4-min intervals after oxidant addition (1.66 μM $CuSO_4$) for assessment of ubiquinol-10 (closed circles), α-tocopherol (open circles), and PnA fluorescence (closed squares). Conjugated dienes (open squares) were measured in a separate incubation. All parameters are expressed as % of their maximal values.

dant content were less impressive but were significant at $P < 0.05$. Variations in ubiquinol-10 and α-tocopherol, in contrast, accounted for <35% of the total variation in conjugated diene lag time estimates ($r^2 = 0.347$; $P = 0.04$), suggesting that other lipoprotein parameters may have had a greater influence on variations and subfraction-dependent differences in this assay.

DISCUSSION

Peroxidative loss of PnA, as indicated by changes in its fluorescent properties, can be used to monitor peroxidative stress within various lipid milieus (18–21). This relatively simple assay has been compared to gas chromatographic analysis of a single fatty acid species over time, but in a continuous manner without the need for sampling and derivitization (18). Although the exceptional lability of the PnA conjugated polyene system and its increased mobility relative to phospholipid fatty acyl components limit direct extrapolation to endogenous lipids (18), PnA fluorescence loss has been shown to parallel the breakdown of other polyunsaturated fatty acids and to respond similarly to modulating factors in the surrounding environment (18–21).

In a recent report of its use in LDL, Laranjinha et al. (33) described PnA oxidation by the radical-initiating agent 2,2'-azobis(2-amidinopropane)dihydrochloride. Under their experimental conditions, however, PnA was merely a probe of oxidation chemistry in the aqueous compartment and was independent of its LDL carrier. In the current studies, we investigated whether PnA could be used as a probe of peroxidative stress within the LDL particle. Cu^{2+}-induced oxidation of LDL-associated PnA exhibited a biphasic response from which a lag time could be derived for comparisons among LDL samples. PnA oxidation lag times were severalfold lower than conjugated diene lag times as predicted from the susceptibility of this probe in bilayer systems (19–22). As indicated by the enhanced lability of dense vs. buoyant LDL-associated PnA, this probe was capable of detecting intrinsic differences in oxidative susceptibility among LDL preparations.

An enhanced oxidative susceptibility of dense LDL has been demonstrated previously based on conjugated diene lag time estimates, rates of formation of TBARS and fluorescent pigments, and changes in anionic electrophoretic mobility (22–25) and is suggested to contribute to the increased risk associated with LDL profiles enriched in small, dense LDL particles (34). de Graaf et al. (22) observed an enrichment of polyunsaturated fatty acids in the most dense of three LDL subfractions and suggested the importance of this factor to its increased susceptibility. We previously showed that unesterified cholesterol content was highly predictive of variations in oxidative susceptibility among six LDL density subfractions, with increased resistance associated with increased content, and suggested a direct protective effect of this constituent through its influence on properties of the surface monolayer (23). Our observation that LDL subfraction differences were reflected by the surface-localized PnA probe suggests that fatty acids of equal chemical sensitivity may be more prone to (Cu^{2+}-induced) oxidation when carried on the surface of dense vs. buoyant LDL particles. This may have been due to differences in the content of the surface-localized antioxidants ubiquinol-10 and α-tocopherol, which were correlated with unesterified cholesterol content (at $P < 0.05$, data not shown).

Previous studies in bilayer systems have shown that addition of radical-scavenging antioxidants extends the PnA oxidation lag phase and that variations in antioxidant content contribute to variations in PnA oxidative susceptibility (20, 21). We observed that ubiquinol-10 and α-tocopherol were depleted immediately prior to PnA oxidation and that their initial contents were highly predictive of PnA oxidation lag times. The association between PnA oxidation lag times and α-tocopherol content was not significant following adjustment for ubiquinol-10 (data not shown), suggesting that the latter, or factors contributing to differences in its LDL content, may have been of greater importance in determining PnA oxidative susceptibility. A key antioxidant role for ubiquinol-10 was suggested previously based on observations that it is rapidly oxidized in plasma and isolated LDL and that lipid hydroperoxides accumulate immediately afterward even when considerable α-tocopherol remains (26, 35).

The potential importance of ubiquinol-10 as a radical-scavenging agent has been challenged, however, due to its low content in nonsupplemented LDL (<1 molecule per LDL particle) (16). Ubiquinol-10 levels may represent a surrogate measure of some other lipoprotein property affecting PnA oxidative susceptibility. Ubiquinol-10 content and the ubiquinol-10/(ubiquinol-10 plus ubiquinone-10) ratio appear to be

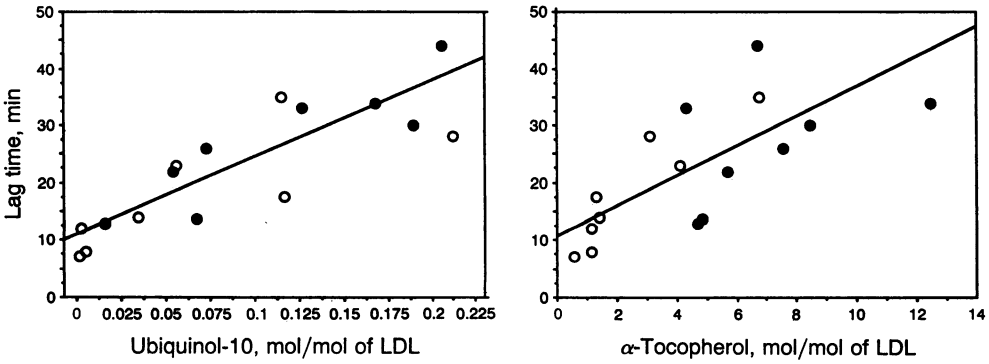

FIG. 5. Oxidative susceptibility of PnA in relation to ubiquinol-10 and α-tocopherol content. PnA oxidation lag times for buoyant (closed circles) and dense (open circles) LDL are expressed relative to initial ubiquinol-10 or α-tocopherol content (mol/mol of LDL). Scatterplots with regression lines are shown for eight subjects.

exquisitely sensitive markers of early oxidative changes in LDL. Bowry *et al.* (36) recently reported an inverse correlation between plasma ubiquinol-10 content and levels of circulating hydroperoxides as measured by a sensitive chemiluminescence assay. The degree of hydroperoxide "seeding" could influence LDL susceptibility to Cu^{2+}-induced oxidation, which is suggested to be dependent on preexisting hydroperoxides (37), and could thus underlie the observed association of PnA oxidation lag times with ubiquinol-10.

LDL ubiquinol-10 content was 2- to 3-fold lower in this study than in previous reports using rapidly isolated LDL (26, 38, 39), suggesting that some autooxidation may have occurred during preparation and/or storage of our samples prior to analysis. To assess the potential importance of antioxidant loss *ex vivo*, we examined the relationship between LDL antioxidant content and time between blood collection and analysis in a parallel study involving 15 sets of LDL subfractions. Loss appeared to be linear for ubiquinol-10 and α-tocopherol. Ubiquinol-10 content was decreased at a rate of ≈0.006 mol/mol of LDL per day in buoyant and dense LDL, whereas α-tocopherol loss occurred at a 2-fold greater rate in dense (0.201 mol/mol of LDL per day) than buoyant (0.110 mol/mol of LDL per day) LDL. Thus, differential loss did not appear to underlie differences in ubiquinol-10 content or associated differences in oxidative behavior among LDL density subfractions but may have been a factor in the reduced α-tocopherol content of dense LDL. Nonetheless, this observation provides additional evidence of the greater oxidative susceptibility of dense LDL, with rate of α-tocopherol autooxidation representing an index of relative oxidative change. Furthermore, results obtained by extrapolation to zero time suggested that the α-tocopherol content did differ by about 30% among buoyant and dense LDL particles while in circulation.

Initial contents of ubiquinol-10 and α-tocopherol accounted for less of the variation in conjugated diene than PnA oxidation lag times. This is not surprising given that conjugated diene lag times reflect the combined effects of a series of chemical events ultimately culminating in accelerated radical propagation among LDL lipids. The composite nature and lack of sensitivity of this index to initial peroxidation events, including those reflected by the PnA assay, limit its sensitivity to individual factors contributing to variations in the initial stages of LDL lipid peroxidation. This may explain the failure of several studies to detect a relationship between conjugated diene lag times and LDL antioxidant content despite its chemical plausability (15, 16, 40).

We thank F. Kuypers, R. Stocker, and B. Frei for helpful discussions, P. Blanche for laboratory assistance, and M. G. Lansberg for help with figure preparation. This research was supported by National Institutes of Health (NIH) Grants HL 27059 and DK 32094 and a grant from the National Dairy Promotion and Research Board administered in cooperation with the National Dairy Council (to R.M.K.) and was conducted at Lawrence Berkeley Laboratory through the U.S. Department of Energy under Contract DE-AC03-76SF00098. D.L.T. and J.J.M.v.d.B. were supported by funds from the Tobacco-Related Disease Research Program of the University of California. P.A.M. was supported by NIH Training Grant ESO 7075. B.N.A. was supported by National Institute on Environmental Health Sciences Center Grant ESO 1896 and NIH Grant CA 39910.

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