LDL particle subclasses in hypercholesterolemia: molecular determinants of reduced lipid hydroperoxide stability

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Abstract Hypercholesterolemia is characterized by elevated plasma levels of LDL in which the cholesteryl ester (CE)-rich LDL subclasses of light and intermediate density (LDL1+2 and LDL3, respectively) typically predominate. The molecular mechanisms implicated in oxidation of LDL particle subclasses in hypercholesterolemia are indeterminate. Lipid hydroperoxides (LOOH), primary oxidation products in LDL, are implicated in atherogenesis. LOOH formation was evaluated in light (LDL1+2), intermediate (LDL3), and dense (LDL4+5) LDL subclasses from hypercholesterolemic (HC) subjects (n = 7) during copper-mediated oxidative stress, and compared with that in corresponding subclasses from normolipidemic subjects (n = 7). HC LDL subclasses were distinguished by lower polyunsaturated phospholipid-α-tocopherol ratios ($P < 0.02$), lower contents of phosphatidyl choline (PC)16:0-18:0/18:2 and PC16:0-18:0/20:4+22:6 ($P < 0.002$), and higher surface phospholipid-free cholesterol ratios ($P < 0.04$). The LDL3, LDL4, and LDL5 subclasses in HC subjects displayed low-core polyunsaturated CE-α-tocopherol ratios ($P < 0.05$), despite similar PUFA CE content. These physicochemical differences did not modify the oxidative susceptibility of HC LDL but underlie the marked instability of cholesterol linoleate hydroperoxides in HC LDL1+2, LDL3, and LDL4 subclasses. Elevated concentrations of large, CE-rich, light, and intermediate LDL subclasses (LDL1+2, LDL3) in hypercholesterolemia may therefore act as an abundant proatherogenic source of highly unstable LOOH in the arterial wall.— Chancharme, L., P. Thérond, F. Nigon, S. Zarev, A. Mallet, E. Bruckert, and M. J. Chapman. **LDL particle subclasses in hypercholesterolemia: molecular determinants of reduced lipid hydroperoxide stability. *J. Lipid Res.* 2002.** 43: **453–462.**

Supplementary key words light, intermediate, and dense LDL • oxidative stress • copper-mediated oxidation • surface phospholipid-free cholesterol ratio • polyunsaturated cholesteryl ester-α-tocopherol ratio • lipophilic antioxidants • cholesteryl ester hydroperoxide stability • apoB-100 conformation • atherogenicity

Elevated circulating levels of LDL are the central feature of hypercholesterolemia, a major risk factor for premature development of coronary artery disease (1). The marked atherogenicity of cholesterol-rich LDL reflects their direct role in the initiation and formation of lipid-rich atherosclerotic lesions (2). However, quantitative elevations in plasma LDL do not alone account for the atherogenicity associated with these particles, and it is significant that similar plasma LDL levels can be associated with markedly different degrees of cardiovascular risk (3). Indeed, the atherogenic risk associated with LDL particles is linked to their structure, physicochemical properties, and in vivo metabolism as much as to their circulating concentration (4, 5). Plasma LDL are highly heterogeneous, consisting of multiple subpopulations, although a single copy of apolipoprotein B-100 (apoB-100) predominates in the protein moiety of all LDL subclasses (6). On a physicochemical basis, LDL particles may be grouped into three major density subclasses: light, large LDL (LDL1+2; d = 1.018–1.030 g/ml), intermediate LDL (LDL3; d = 1.030–1.040 g/ml), and small, dense LDL (LDL4+5; 1.040–1.065 g/ml). In primary hypercholesterolemia of type IIA, the elevated plasma concentrations of both light, large LDL (LDL1+2), and LDL of intermediate density (LDL3) frequently predominate relative to those of small, dense LDL (LDL4+5) (7, 8). In this context, it is relevant that available evidence substantiates the elevated atherogenic potential not only of small, dense LDL, but also of large, cholesterol-rich LDL particles.

Extensive epidemiologic and experimental evidence indicates that oxidative modification of cholesterol-rich LDL plays a key role in the development of atheroscle-

Abbreviations: CE, cholesteryl ester; CEOOH, cholesteryl ester hydroperoxide; FC, free cholesterol; HC, hypercholesterolemic; NL, normolipidemic; PC, phosphatidyl choline; PCOOH, phosphatidyl choline hydroperoxide; PL, phospholipid; SM, sphingomyelin.

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rotic lesions (9–12). Hypercholesterolemia is intimately associated with elevated oxidative stress and endothelial dysfunction (13). Indeed, the oxidative susceptibility of LDL in hypercholesterolemic (HC) patients is of considerable relevance to disease progression, as the susceptibility of LDL to oxidation can be closely correlated with the severity of atherosclerosis (14). Retention of LDL in the arterial intima exposes these particles to oxidative stress, involving the action of pro-oxidant enzymes, reactive oxygen species, and transition metal ions (9, 15). The initial phase of oxidation involves the concomitant destruction of the lipophilic antioxidant content of LDL with formation of conjugated dienes and lipid hydroperoxides (LOOH), among which cholesteryl ester (CE) hydroperoxides (CEOOH) predominate (16–18). LOOH formation is a key step in the Cu^{2+}-stimulated oxidation of LDL, and the stability of LOOH species derived from phospholipids (PL) and CE containing PUFA is an integral feature of the oxidative susceptibility of LDL particles (9, 17, 19). Indeed, CEOOH derived from LDL constitute key components of atherosclerotic plaques and may promote plaque fragilization (20, 21). More advanced stages of lipid peroxidation ensue, with formation of several species of oxysterols (22), the loss of PUFAs, and the decomposition of LOOH to aldehydes and to other reactive products (9, 10, 16, 22).

Studies to date in HC subjects have focused on the oxidizability of heterogeneous mixtures of LDL particles consisting of all subclasses, i.e., LDL of d = 1.019–1.065 g/ml, and marked discrepancies are evident (23–27). Such discrepancies may reflect differences not only in the physicochemical properties of the overall mixture of LDL particles between different patient groups, but also in the relative quantitative contribution of each major LDL subclass of distinct oxidative resistance to the total LDL particle population in each patient group (8). The degree to which LDL may resist oxidative stress is determined by multiple factors that are mutually interactive and that are either endogenous or exogenous to the lipoprotein particle. The principal endogenous factors include particle content of antioxidants such as ubiquinol-10 and vitamin E (α-tocopherol), the radical trapping efficacy of these antioxidants, lipid hydroperoxide content, particle size, the number and relative affinities of binding sites for metal cations such as copper and iron, the degree of unsaturation of fatty acids in lipid esters (mainly CE and PL), and the molecular composition and packing of the lipoprotein core and surface constituents (9, 17–19, 28–31).

Insight into the molecular mechanisms of LOOH formation in the major subclasses of HC LDL, i.e., large CE-rich LDL particles in the light (LDL1+2; CE-apoB ratio = 1,900 mol/mol) and intermediate (LDL3; CE-apoB ratio = 1,800 mol/mol) density subclasses is lacking, and may shed new light on their atherogenicity in this population at high cardiovascular risk. Our present aims were therefore *i*) to identify the major lipid targets of LOOH formation during the initial stages of copper-mediated oxidation in the major LDL subclasses (light, intermediate, and dense) from HC subjects, *ii*) to evaluate the stability of defined molecular species of LOOH in these same LDL subclasses, and *iii*) to compare these parameters with those of the corresponding LDL subspecies in normolipidemic (NL) control individuals.

MATERIALS AND METHODS

Patients and blood samples

Patients were recruited and carefully selected from our Outpatient Clinic (Centre for the Detection and Prevention of Atherosclerosis) and displayed moderate primary polygenic type IIA hypercholesterolemia (creatinine, thyroid stimulating hormone, and fasting blood sugar levels, together with hepatic enzyme activities, were in the normal range); they were not receiving any lipid lowering drugs. NL subjects were healthy volunteers. Venous blood was collected in sterile evacuated tubes (Vacutainers) containing K_3EDTA (final concentration 1 mg/ml) after an overnight fast. Plasma lipid and apolipoprotein levels in both groups are summarized in **Table 1**. HC subjects (total plasma cholesterol concentration, 220–320 mg/dl) displayed significantly elevated levels of total cholesterol, LDL-cholesterol (LDL-C), and apoB-100. No significant differences in HDL-C, apoA-I, TG, or lipoprotein [a] levels were detectable between the two groups. None of the subjects included in this study was receiving antioxidant vitamin supplementation or drugs known to affect lipoprotein metabolism; all subjects were non-smokers, and either abstainers or only moderate alcohol consumers. All participants gave informed consent. These studies were approved by the local Institutional Ethics Committee.

After blood collection, plasma was immediately separated by low-speed centrifugation at 4°C, and lipoproteins were protected from degradation as described earlier (19).

Isolation of plasma LDL subfractions

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation as previoulsy described (19). LDL subclasses isolated from HC plasma are referred to as "HC LDL." The five subfractions of LDL obtained corresponded to the following density intervals: LDL-1, 1.018–1.023 g/ml; LDL-2, 1.023–1.030 g/ml; LDL-3, 1.030–1.040 g/ml; LDL-4, 1.040–1.051 g/ml; and LDL-5, 1.051–1.065 g/ml. In view of the low concentrations of lipoproteins in subfractions 1 and 2, they were pooled and termed "LDL1+2" or "light LDL" (d = 1.018–1.030 g/ml); LDL3 corresponds to the "intermediate" subclass, and LDL4 and LDL5 are considered together as the "dense" subclass (d = 1.040–1.065 g/ml) (6). We detected no hydroperoxides in native LDL subspecies by HPLC analysis of lipid extracts, thereby

TABLE 1. Plasma lipid and apolipoprotein levels in hypercholesterolemic (HC) subjects and normolipidemic (NL) controls

	NL (n = 7)	HC (n = 7)
Sex (M/F)	6/1	4/3
Total cholesterol	1.96 ± 0.38	2.76 ± 0.37[a]
TG	0.97 ± 0.53	1.24 ± 0.38
LDL-C	1.21 ± 0.39	1.95 ± 0.33[a]
HDL-C	0.56 ± 0.16	0.57 ± 0.20
ApoB	0.99 ± 0.24	1.38 ± 0.14[b]
ApoA-I	1.59 ± 0.35	1.55 ± 0.24
Lp[a]	0.13 ± 0.11	0.24 ± 0.27

Data are expressed as mean ± SD. Units are mg/dl throughout.
[a] $P < 0.01$ HC versus NL.
[b] $P < 0.05$ HC versus NL.

indicating that LDL oxidation had not occurred to a significant degree during ultracentrifugal fractionation.

Chemical analysis of plasma lipids, apolipoproteins, and lipoprotein subfractions

Analyses of plasma lipids, apolipoproteins, and lipoprotein subfractions were performed by a series of procedures previously described (19).

Determination of antioxidant content

The particle content of α-tocopherol and carotenoids (α and β-carotene, lutein, lycopene, and cryptoxanthin) was determined in each LDL subfraction by reverse phase HPLC as described earlier (32).

Determination of the oxidative susceptibility of LDL subfractions

The susceptibility of LDL subfractions to in vitro copper-mediated oxidation was assessed as previously described (19). The time-course of LDL oxidation was monitored continuously at 234 nm and at 37°C. Oxysterol (7-ketocholesterol and 7-ketocholesterol esters) formation was monitored simultaneously by means of UV-spectroscopy (33). As described earlier (19), three characteristic time points were determined from the absorbance curve at 234 nm that describe the oxidative behavior of LDL. The first time point, the lag time (T_{lag}), was defined as the intersection of the baseline with the tangent of the slope of the absorbance curve during the propagation phase. Second, the maximum time (T_{max}) was defined as the time at the end of the propagation phase when diene production reached its maximal value. The third time is the propagation half-time ($T_{1/2}$) and corresponds to the midpoint of the propagation phase.

Lipid extraction and chromatographic analysis of molecular species of phospholipids and CEs

Lipids were extracted with methanol-hexane (4:10, v/v) from aliquots of LDL subfractions. Native molecular species of sphingomyelin (SM), phosphatidyl choline (PC), CE, and their corresponding hydroperoxides, were identified by HPLC as described by Therond et al. (34), with slight modifications (19). Lipid hydroxides formed upon reduction of LOOH were detected on the HPLC chromatographic elution profile at 205 nm.

Detection of apoB carbonylation

Ovalbumin irradiated by γ-radiolysis in oxygenated aqueous buffer was used as a standard for detection of apoB-100 carbonylation. Oxidized LDL was first derivatized, and carbonylated apoB-100, together with the carbonylated ovalbumin standard, were then electrophoresed in SDS-polyacrylamide gels (4–10% gradient). After migration, protein bands were electroblotted onto nitrocellulose membranes (0.45 μm, Biorad) for 2 h at 150 mA. Immunoassay was performed by incubation of the blots with rabbit anti-dinitrophenylhydrazone (DNP) antibody (150-fold diluted in 1% TTBS-BSA). After washing, a second incubation with the goat anti-rabbit peroxidase-labeled antibodies (300-fold diluted in 1% TTBS-BSA) was performed and the blot incubated with luminol and H_2O_2 solution (Pierce). The bands containing DNP-apoB-100 were visualized by chemoluminescence and exposed to XAR-5 X-ray film (Eastman Kodak); band intensities were integrated by densitometry on a Preference apparatus (Sebia).

Statistical analysis

All results are presented as means ± SD. Comparison of the plasma lipid and apolipoprotein levels between the two groups (NL vs. HC) was performed by the nonparametric Mann-Whitney U test. Comparisons of the different LDL subfractions between the two groups were performed globally across the various LDL fractions by using a multivariate ANOVA with Hotelling-Lawlet trace statistics. Whenever a significant difference was found at the $P < 0.05$ level, each LDL subfraction was subsequently compared between the two groups, using a standard ANOVA, setting the comparison error rate to 0.0125, according to the Bonferroni criterion for multiple comparisons.

Furthermore, lag time values, levels of α-tocopherol and 7-ketocholesterol in LDL particles, and PUFA-α-tocopherol ratio values were compared between LDL subfractions with an ANOVA for repeated measures, allowing testing for both the fraction effect as well as possible fraction-group interaction. Whenever a global difference was found between LDL subfractions, a linear trend was evaluated using the appropriate contrast (GLM procedure, SAS Institute, Cary, NC), tested at the 0.05 level.

RESULTS

Mass distribution of LDL subfractions

The mass distribution profile of LDL subfractions in HC patients and NL subjects is shown as a function of density in **Fig. 1**. The LDL profiles are characterized by a predominance of the intermediate LDL3 subclass in each case, which represented approximately 40% of total lipoprotein mass in both HC and NL subjects. HC type IIA patients displayed an asymmetrical profile dominated by the light (LDL1+2) and intermediate (LDL3) subfractions, the former accounting for a significantly higher proportion (33.7% of total LDL mass) in HC patients as compared with NL subjects (17.5%) ($P < 0.0001$). Such profiles are similar to those reported earlier in patients displaying familial hypercholesterolemia (8). Furthermore, because of the elevated levels of plasma LDL in HC individuals as compared with NL subjects ($P < 0.009$), the former displayed significantly greater amounts of both light (157 ± 65 vs. 59 ± 36 mg/dl; $P < 0.0001$) and intermediate (207 ± 47 vs. 112 ± 48 mg/ml; $P < 0.02$) LDL subclasses.

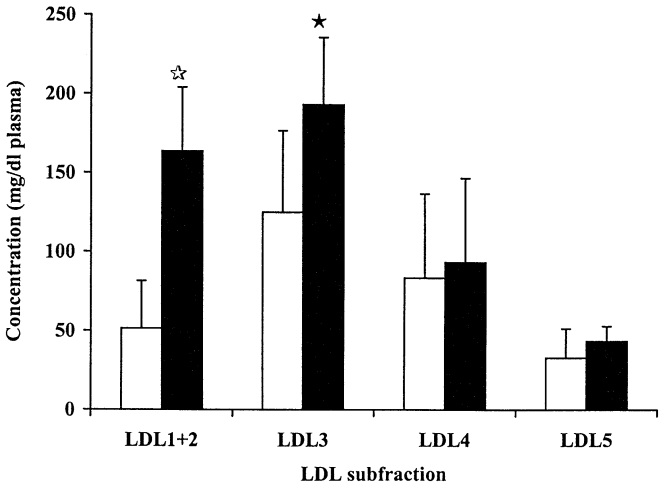

Fig. 1. Mass distribution of LDL subfractions from normolipidemic (NL) subjects (open bar) and hypercholesterolemic (HC) patients (closed bar) (mean ± SD, n = 7 for each group). Closed star: Fraction statistically different from NL subjects; $P < 0.05$, (open star) $P < 0.0001$.

TABLE 2. Particle content of molecular lipid species and antioxidants in LDL subfractions from HC patients and NL controls

	LDL Subfraction								
	LDL1+2 d = 1.018–1.030 g/ml		LDL3 d = 1.030–1.040 g/ml		LDL4 d = 1.040–1.051 g/ml		LDL5 d = 1.051–1.065 g/ml		
	NL	HC	NL	HC	NL	HC	NL	HC	P^e
Component, mol/mol LDL									
PC16:0-18:0/18:2[g]	215 ± 70	133 ± 43	165 ± 34	112 ± 29[a]	148 ± 28	94 ± 30[b]	136 ± 19	74 ± 16[c]	<0.002
PC16:0-18:0/20:4+22:6	67 ± 18	41 ± 11[a]	52 ± 6	37 ± 8[b]	50 ± 10	32 ± 7[b]	48 ± 9	25 ± 4[c]	<0.002
CE18:2	612 ± 90	642 ± 174	523 ± 87	561 ± 78	482 ± 66	457 ± 78	364 ± 89	283 ± 48	ns
CE20:4	52 ± 8	49 ± 15	44 ± 7	41 ± 8	39 ± 7	33 ± 7	31 ± 4	22 ± 6	ns
FC	780 ± 92	683 ± 83	665 ± 26	650 ± 95	555 ± 64	501 ± 129	403 ± 28	337 ± 89	ns
Phospholipid/free cholesterol	1.14 ± 0.19	1.32 ± 0.19[a]	1.13 ± 0.13	1.19 ± 0.16	1.19 ± 0.21	1.38 ± 0.25	1.44 ± 0.40	1.89 ± 0.41[a]	<0.04
Antioxidant content (mol/mol LDL)									
α-tocopherol	11.1 ± 2.0	11.6 ± 3.0	7.5 ± 2.0	14.0 ± 2.6[d]	7.6 ± 2.2	12.6 ± 1.4[d]	6.9 ± 1.1	9.8 ± 2.2	<0.0004
PUFA-α-tocopherol ratio (mol/mol)									
Total PUFAs[f] in CE and PL	84 ± 16	85 ± 51	109 ± 33	54 ± 9[b]	101 ± 35	49 ± 9[b]	87 ± 22	44 ± 15[b]	<0.04
CE containing PUFAs[f]	61 ± 13	68 ± 43	80 ± 26	44 ± 8[b]	73 ± 25	39 ± 8[a]	59 ± 14	33 ± 10[b]	<0.05
PL containing PUFAs[f]	24 ± 6	17 ± 9	29 ± 9	11 ± 3[b]	28 ± 11	10 ± 2[b]	28 ± 10	11 ± 6[b]	<0.02

Molecular weights employed for the calculations are: 3.04 10^6, 2.62 10^6, 2.33 10^6, and 2.00 10^6 Da for LDL1+2, LDL3, LDL4, and LDL5 respectively. The molecular weight of sphingomyelin was taken as 750 Da. α-tocopherol and PUFAs were analyzed by HPLC (see Materials and Methods). [a] $P < 0.01$, [b] $P < 0.005$, [c] $P < 0.0005$, and [d] $P < 0.0001$ statistically different HC versus NL (n = 7 in each population); ns, non significant.

[e] Multivariate ANOVA was performed for all LDL subfractions in HC tested against NL subjects as described in Materials and Methods.

[f] Polyunsaturated fatty acids (linoleic acid, C18:2; arachidonic acid, C20:4; and docosahexaenoic acid, C22:6).

[g] PC16:0-18:0/18:2 denotes a phosphatidylcholine species containing either C16:0 or 18:0 at *sn*-1 position as the saturated fatty acid component, and 18:2 as the polyunsaturated fatty acid at the *sn*-2 position.

Chemical composition of LDL subfractions in normo and in HC subjects

Chemical analysis of LDL subspecies [expressed as percent weight composition of PL, CE, TG, free cholesterol (FC), and protein] failed to reveal any significant difference between corresponding subfractions from HC and NL subjects (data not shown), as described previously (8). Nevertheless, the molar ratio of PL-FC was some 5–31% higher in HC LDL subclasses; moreover, when PL-FC values in HC LDL subclasses were tested as a series by multivariate analyses against those in the corresponding subclasses from NL subjects, these differences were statistically significant ($P < 0.04$). In addition, on an individual subfraction basis, the PL-FC ratio in LDL1+2 and LDL5 from the HC group was significantly different from the corresponding subclasses in NL subjects (**Table 2**, **Fig. 2A**).

Concerning the particle content of carotenoids, we observed no differences between LDL from HC and NL subjects. Nevertheless, significantly higher levels of α-tocopherol per particle were systematically detected in LDL subfractions from HC patients when compared by multivariate analysis to those from NL subjects ($P < 0.0004$), and especially in LDL3 and LDL4 ($P < 0.0005$ in each), in which α-tocopherol content was elevated to 1.8-fold as compared with that in controls. Indeed, maximal levels of α-tocopherol were present in LDL3 and LDL4 (14.0–12.6 mol/mol-LDL, respectively) in HC individuals. LDL subspecies from HC subjects, with the exception of light LDL, can therefore be considered enriched in α-tocopherol as compared with their counterparts in NL controls.

With the exception of LDL1+2, the ratio of PUFA-α-tocopherol content was significantly lower (up to 2-fold) in LDL subclasses from HC subjects, as compared with those in the NL group when tested by multivariate analysis ($P < 0.04$). These data indicate that each molecule of α-tocopherol must protect a greater number of PUFA molecules in intermediate and dense LDL from NL subjects (Table 2, Fig. 2B and 2C). ANOVA for repeated measures also revealed that the ratio of PUFA-α-tocopherol decreased with an increase in density of LDL particles in HC subjects alone ($P < 0.02$). By contrast, peak PUFA-α-tocopherol ratios (101:109 mol/mol) were seen in LDL3 and LDL4 in the NL group. When we focused more precisely on PUFA esterified in CE and in PC, the ratio of polyunsaturated CE-α-tocopherol was found to diminish from LDL1+2 to LDL5, specifically in the HC group ($P < 0.007$; ANOVA for repeated measures). No clear relation, however, was found between LDL particle density and the phospholipid-α-tocopherol ratio in the HC and NL LDL subclasses. Nevertheless, multivariate ANOVA revealed distinct differences ($P < 0.02$) between polyunsaturated PL-α-tocopherol ratios in the HC and NL groups (Fig. 2C).

Esterified PUFA content of PLs and CEs in native LDL subclasses from HC and NL subjects

All LDL subfractions from HC subjects were depleted in PUFA esterified PCs as they contained significantly lower amounts (≈1.6-fold less) of PC16:0-18:0/18:2 ($P < 0.002$) and PC16:0-18:0/20:4+22.6 ($P < 0.002$; multivariate ANOVA) as compared to corresponding LDL subfractions from NL subjects (Table 2). Molar particle contents of these molecular PC species diminished in parallel with increase in density from LDL1+2 to LDL5. We observed no differences in the molecular contents of SM, PC16:0-18:0/-18:1, CE18:2, and CE20:4 between LDL subfractions from NL and HC subjects. Clearly, linoleate-containing PC and

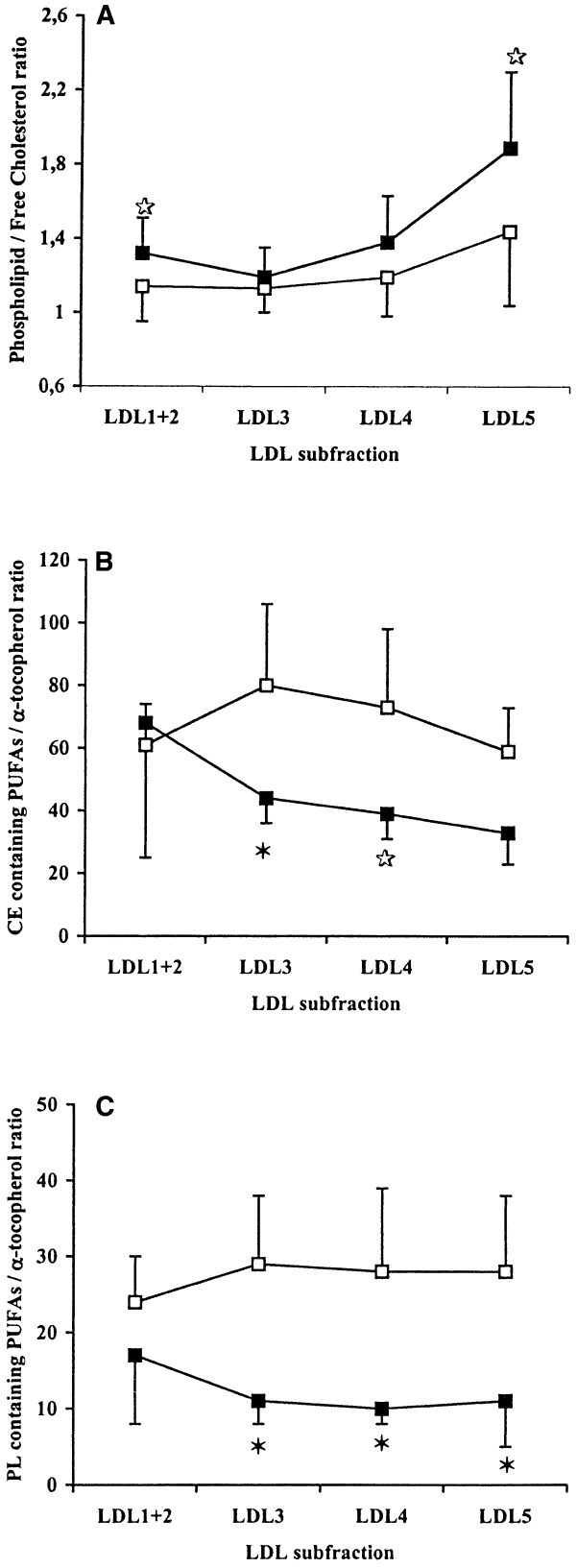

Fig. 2. Chemical features of LDL subspecies from NL subjects (open square) versus HC patients (closed square). Phospholipid-free cholesterol (A), cholesteryl ester (CE) containing PUFAs-α-tocopherol (B), and phospholipid (PL) containing PUFAs-α-tocopherol (C) were calculated (mean ± SD, n = 7 for each group). Open star: Fraction statistically different from NL subjects; $P < 0.01$, (closed star) $P < 0.005$.

TABLE 3. Determination of the lag time during copper-mediated oxidation of LDL subfractions

LDL Subfraction	T_{lag} NL	T_{lag} HC
	min	
LDL1+2	54.1 ± 11.1	47.1 ± 7.8
LDL3	42.5 ± 11.7	40.6 ± 6.9
LDL4	40.5 ± 9.9	35.4 ± 6.2
LDL5	35.6 ± 5.7	31.8 ± 4.5

Aliquots of LDL subfractions (250 μg LDL mass/ml) were oxidized in the presence of 1.6 μM cupric chloride for 3 h at 37°C. The time course of LDL oxidation was monitored by the change in absorbance at 234 nm as described in Materials and Methods. n = 7 in each population (HC and NL).

CE represented the major substrates for lipid peroxidation in all LDL subspecies from both HC and NL subjects quantitatively (Table 2).

Characteristics of copper-mediated oxidation in LDL subfractions

In both NL and HC subjects, values of lag times decreased as the density of the LDL particles increased ($P < 0.0001$), and the overall pattern of oxidative susceptibility increased in the order LDL1+2 > LDL3 > LDL4 > LDL5. However, we observed no differences in the susceptibility to oxidation between corresponding LDL subspecies from HC and NL (**Table 3**), despite slightly shorter lag times in LDL subfractions from HC patients. Furthermore, no differences were detected for $T_{1/2}$ and T_{max} between LDL in NL and HC groups (data not shown).

Oxidative modification of PUFAs in PCs and CEs and LOOH formation

Preformed LOOH content in HC and NL LDL subfractions was below the level of detection of our HPLC-chemoluminescence methodology (<0.1 mmol/mol LDL). During the propagation phase of oxidation ($T_{1/2}$) and at the end of the propagation phase (T_{max}), several marked dissimilarities were documented in the oxidation behavior of LDL subfractions from NL as compared with HC subjects (**Table 4**). Firstly, significantly fewer molecules of PC16:0-18:0/20:4+22:6 were consumed at $T_{1/2}$ in all LDL subspecies from HC patients in comparison with those from NL subjects ($P < 0.008$), and especially in dense LDL4 ($P < 0.005$) and LDL5 ($P < 0.02$). At T_{max}, the same phenomenon was observed for both PC16:0-18:0/18:2 ($P < 0.04$) and PC16:0-18:0/20:4+22:6 ($P < 0.02$). This result presumably reflects the lower content of PUFA esterified PC species in native LDL subfractions seen in the HC group at baseline (Table 2). By contrast, no significant differences were evident in the oxidative consumption of the CE18:2 and CE20:4 esters in corresponding LDL subspecies from the NL and HC group during the propagation phase. Remarkably however, some 40% fewer CE18:2 and CE20:4 were consumed in dense LDL5 from HC patients than in their counterparts in controls. As with the results for PC-PUFA, this finding could be explained by the lower levels of CE-PUFA initially present in LDL5

TABLE 4. Amounts of polyunsaturated fatty acid esterified species of phosphatidylcholine and cholesteryl esters consumed and formation of the derived hydroperoxides during copper mediated oxidation

	LDL Subfraction								
	LDL1+2 d = 1.018–1.030 g/ml		LDL3 d = 1.030–1.040 g/ml		LDL4 d = 1.040–1.051 g/ml		LDL5 d = 1.051–1.065 g/ml		
Component (mol/mol LDL)	NL	HC	NL	HC	NL	HC	NL	HC	P^e
$T_{1/2}$									
PC16:0-18:0/18:2	46 ± 24	45 ± 30	38 ± 13	37 ± 16	36 ± 13	26 ± 14	33 ± 15	20 ± 6	ns
PCOOH18:2	1.8+1.1	2.7 ± 1.9	1.5 ± 0.5	4.0 ± 4.4	1.9 ± 1.2	1.9 ± 1.9	1.5 ± 0.7	1.1 ± 0.6	ns
PC16:0-18:0/20:4+22:6	29 ± 8	20 ± 8	22 ± 6	19 ± 8	23 ± 5	14 ± 5[b]	20 ± 8	11 ± 3[a]	<0.008
PCOOH20:4+22:6	0.3 ± 0.2	0.7 ± 0.5	0.3 ± 0.2	0.8 ± 0.7	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	ns
CE18:2	140 ± 57	145 ± 76	90 ± 28	125 ± 85	114 ± 34	120 ± 49	116 ± 59	71 ± 27	ns
CEOOH18:2	58.1 ± 11.2	23.9 ± 7.4[d]	64.6 ± 16.4	32.3 ± 11.5[b]	45.5 ± 9.1	17.4 ± 10.1[d]	31.3 ± 22.1	12.3 ± 6.6	<0.0008
CE20:4	22 ± 8	23 ± 11	19 ± 6	17 ± 8	18 ± 6	15 ± 5	16 ± 5	10 ± 5	ns
CEOOH20:4	2.3+0.9	0.9 ± 0.7[a]	2.1 ± 0.8	1.3 ± 1.0	1.9 ± 0.8	0.6 ± 0.4[b]	0.8 ± 0.5	0.5 ± 0.5	<0.01
T_{max}									
PC16:0-18:0/18:2	104 ± 41	76 ± 37	74 ± 13	59 ± 18	63 ± 17	48 ± 20	65 ± 17	35 ± 8[b]	<0.04
PCOOH18:2	7.6+6.0	5.8 ± 1.8	6.2 ± 1.3	8.3 ± 6.8	5.4 ± 1.4	8.1 ± 7.0	5.7 ± 3.4	3.4 ± 2.0	ns
PC16:0-18:0/20:4+22:6	52 ± 11	33 ± 11[a]	39 ± 4	30 ± 8	37 ± 8	25 ± 7	37 ± 8	19 ± 4[c]	<0.02
PCOOH20:4+22:6	1.2 ± 0.4	1.9 ± 1.6	1.2 ± 0.5	1.9 ± 1.3	1.1 ± 0.5	1.8 ± 1.3	1.1 ± 0.6	0.9 ± 0.6	ns
CE18:2	397 ± 92	403 ± 108	291 ± 67	331 ± 67	277 ± 55	283 ± 78	244 ± 56	175 ± 60	ns
CEOOH18:2	87.6 ± 28	46.2 ± 17.5[a]	83.6 ± 33.4	46.3 ± 17.7	51.1 ± 15.7	34.6 ± 11.9	43.5 ± 16.1	18.9 ± 9.7[b]	<0.04
CE20:4	46 ± 7	41 ± 15	37 ± 7	34 ± 4	30 ± 8	29 ± 8	26 ± 4	18 ± 6	ns
CEOOH20:4	4.4 ± 2.3	1.7 ± 0.8[a]	3.3 ± 1.9	1.9 ± 0.9	2.8 ± 1.6	0.9 ± 0.4[a]	1.8 ± 0.9	0.7 ± 0.3[a]	<0.03

PUFAs were analyzed by HPLC (see Materials and Methods).

[a] $P < 0.01$, [b] $P < 0.005$, [c] $P < 0.0005$, and [d] $P < 0.0001$ HC statistically different from NL (n = 7 in each population); ns: non significant.

[e] Multivariate analysis of variance was performed for all LDL subfractions in HC tested against NL subjects as described in Materials and Methods.

from HC as compared with NL. Nevertheless, we detected markedly lower quantities of CEOOH18:2 in all HC LDL subspecies at both $T_{1/2}$ ($P < 0.0008$) and T_{max} ($P < 0.04$) in comparison with corresponding subspecies in controls (Table 4). A similar tendency was observed for CEOOH20:4, as significantly lower amounts of CEOOH20:4 were detectable at $T_{1/2}$ ($P < 0.01$) and T_{max} ($P < 0.03$) in LDL subfractions from HC as compared with NL and especially in LDL1+2 and LDL4.

We therefore calculated the ratio of the number of molecules of LOOH formed from a defined molecular PUFA-containing species of PC or CE relative to the number of molecules of the same species that had been transformed to an oxidatively modified form (**Fig. 3**). The first step in lipid peroxidation in LDL involves the formation of monohydroperoxide derivatives, and it is assumed that each oxidized PUFA gives rise to formation of a hydroperoxide before being degraded to secondary oxidation products. The detected amounts of hydroperoxides at a defined time point are therefore a direct reflection of the quantities of PUFA at the initial step of oxidation. Consequently, the calculated ratio provides an index of the stability of the LOOH formed. A high value indicates that most oxidized PUFA species are still present as hydroperoxides. In the present study, we observed no difference in the stability of PCOOH18:2 and PCOOH20:4+22:6 (Fig. 3A and 3B) between the two populations. By contrast, we observed significantly lower values for this ratio for CE18:2 (Fig. 3C) during the propagation phase ($T_{1/2}$, $P < 0.0001$ by multivariate ANOVA) of HC as compared with NL LDL subfractions; a similar pattern was seen in this ratio for CE20:4 (Fig. 3D) in HC as compared with NL LDL subfractions, with the exception of LDL5, for which the ratio was similar in the two groups. These results indicate that CEOOH18:2 formed in each LDL subfraction from HC patients displayed a lower stability as compared with those formed in LDL subspecies from NL subjects. The present data for LDL subfractions in NL subjects are consistent with those reported earlier (19), since we detected a lower stability of CEOOH18:2 formed in dense LDL5 as compared with LDL3 in similar subjects at $T_{1/2}$ (28.3 mol CEOOH18:2 detected for 100 mol of CE18:2 consumed vs. 74.7, respectively; $P < 0.0001$). A similar but nonsignificant, tendency was observed between LDL5 and LDL3 from HC patients (16.9 mol CEOOH18:2 detected for 100 mol of CE18:2 consumed vs. 29.4, respectively). It is important to note that this lower stability was not accompanied by a lower accumulation of conjugated dienes because we did not observe any significant difference between corresponding LDL subfractions from HC and NL subjects (data not shown. We interprete these data to indicate that rates of LOOH formation and stability have little bearing on LDL oxidation, as suggested by the lack of difference in protein carbonyl formation).

Spectrophotometric estimation of 7-ketocholesterol (33) revealed that *i*) formation of this component at T_{lag} was similar in corresponding subfractions from both HC and NL subjects, and *ii*) 7-ketocholesterol formation decreased significantly at the three characteristic time points (T_{lag}, $T_{1/2}$, and T_{max}; $P < 0.0001$) with an increase in density from LDL1+2 to dense LDL5 in both groups (**Table 5**). The higher lability of CEOOH in HC LDL subclasses was, however, reflected in a consistent tendency to higher 7-ketocholesterol levels in LDL3, LDL4, and LDL5, as compared with their counterparts in controls (Table 5).

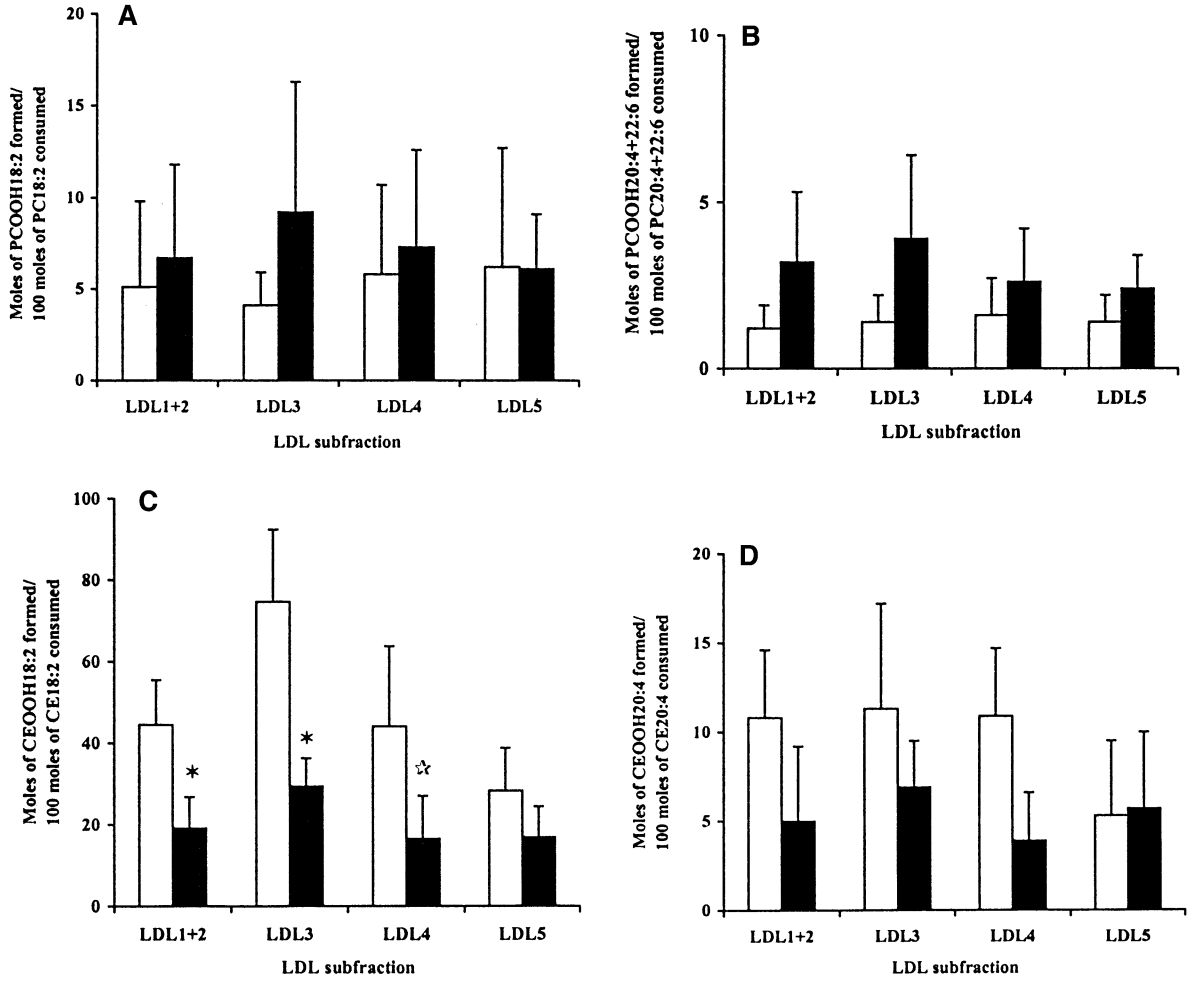

Fig. 3. Ratio of the number of lipid hydroperoxides (LOOH) species formed to the number of native phosphatidyl choline (PC) species lost because of oxidative transformation. Ratios are shown for PC16:0/18:2 (A), PC16:0/20:4+22:6 (B), CE18:2 (C), and CE20:4 (D) at $T_{1/2}$ of oxidation for LDL subfractions from NL subjects (open bar) and HC patients (closed bar). The molar ratio (A–D) is indicated as the number of mol of LOOHs derived from a defined PUFA-containing lipid species to each 100 mol of the same species present in an oxidatively altered form at $T_{1/2}$ (mean ± SD, n = 7 for each group). Open star: Fraction statistically different from NL subjects; $P < 0.01$, (closed star) $P < 0.0005$.

Carbonylation and fragmentation of apoB-100

We focused on the possible reaction occurring between apoB-100 and hydroperoxide-derived alkoxyl and peroxyl lipid radicals, and especially as lipid peroxidation enhances copper-mediated carbonyl formation (35). The breakdown of apoB-100 during the oxidative modification of LDL is linked to oxidative attack on the polypeptide chain, either directly or secondarily to peroxidation of bound LDL lipids (36). As major differences in CEOOH18:2 stability were detected between LDL3 and LDL5, we evaluated protein carbonylation in intermediate and dense LDL subfractions from HC and NL subjects. Patterns of carbonylation and fragmentation were alike, however, not only between corresponding LDL subclasses from HC or NL, but also between LDL3 and LDL5 in each group (data not shown).

TABLE 5. 7-Ketocholesterol content as a function of the oxidative process in LDL subfractions from HC patients and NL controls

	LDL Subfraction								
	LDL1+2 d = 1.018–1.030 g/ml		LDL3 d = 1.030–1.040 g/ml		LDL4 d = 1.040–1.051 g/ml		LDL5 d = 1.051–1.065 g/ml		
7-Ketocholesterol (mol/mol LDL)	NL	HC	NL	HC	NL	HC	NL	HC	P^a
T_{lag}	74.4 ± 14.7	80.3 ± 11.4	64.1 ± 14.3	73.8 ± 20.7	51.1 ± 10.9	65.6 ± 11.5	43.3 ± 8.2	48.1 ± 10.5	ns
$T_{1/2}$	297.4 ± 34.6	283.3 ± 52.2	260.4 ± 31.6	278.4 ± 37.0	203.0 ± 14.9	246.5 ± 36.8	158.2 ± 37.9	162.2 ± 23.8	ns
T_{max}	420.3 ± 37.3	418.6 ± 53.9	363.7 ± 48.9	404.4 ± 37.6	307.1 ± 15.1	341.2 ± 39.7	235.5 ± 27.8	237.9 ± 26.6	ns

ns: non statistically different HC versus NL (n = 7 in each population).
[a] Multivariate ANOVA was performed for all LDL subfractions in HC tested against NL subjects as described in Materials and Methods.

Finally, it is noteworthy that no significant effect of gender could be detected in any parameter analyzed in the NL and HC groups.

DISCUSSION

Molecular analysis of physicochemically defined subclasses of LDL particles at progressive stages of copper-mediated oxidation has revealed a markedly diminished stability of CE-derived hydroperoxides in LDL subspecies from HC patients. This phenomenon was highly significant in CE-rich light LDL1+2 (CE-apoB ratio = 1,900 mol/mol), in the CE-rich intermediate LDL subclass (LDL3; CE-apoB ratio = 1,800 mol/mol), and in CE-poor small, dense LDL4 (CE-apoB ratio = 1,350 mol/mol), and was centered on hydroperoxides of cholesteryl linoleate (Fig. 3), although similar instability was seen in CEOOH20:4, which did not attain significance. This phenomenon was the direct reflection of significant and marked reductions in the contents (up to 2.5-fold) of the CEOOH18:2 and CEOOH20:4 species during the propagation phase of oxidation in HC LDL subclasses as compared with their counterparts in NL subjects. However, such diminished CE hydroperoxide stability was not associated with shorter lag times in HC LDL subclasses in comparison with those in controls (Table 3). Clearly then, these findings emphasize the limitations of the lag phase for copper-mediated oxidation as a unique end point on which to base overall conclusions regarding the oxidative susceptibility, and ultimately potential atherogenicity, of LDL particles. On the contrary, Stocker (37) has recently proposed that measurement of LOOH and peroxides LO(O)H constitutes a reliable index of the overall degree of lipoprotein oxidation in the vessel wall. This suggestion derives from the finding that oxidation in the presence of vitamin E favors accumulation of LO(O)H in both lipoproteins in vitro (21, 38) and in atherosclerotic plaque lipids (39); indeed, under such conditions, LO(O)Hs become a major class of oxidized lipids (39–41).

LDL subclasses from HC subjects were distinguished by several features of immediate relevance to LOOH formation. Firstly, α-tocopherol levels were elevated in native HC LDL subclasses relative to those in NL subfractions (up to 1.8-fold; $P < 0.0004$ by multivariate ANOVA), mainly in LDL3 and LDL4 ($P < 0.0005$ in each) but not in LDL1+2. As a consequence, the molar ratio of PUFAs-vitamin E per LDL particle was dramatically reduced (up to 2-fold) in LDL3, LDL4, and LDL5 subclasses from HC patients as compared with their counterparts in NL subjects ($P < 0.04$ by multivatiate ANOVA; $P < 0.005$ by ANOVA); again, such differences were not observed for light LDL1+2. This compositional feature was evident in CE species containing PUFAs, and specifically in LDL subclasses LDL3, LDL4, and LDL5 ($P < 0.01$), but was especially pronounced in polyunsaturated phospholipid species in the same HC LDL subclasses (Table 2), thereby reflecting their diminished absolute content of PC16:0-18:0/18:2 and PC16:0-18:0/20:4+22:6. We hypothesize, therefore, that the low PUFA-vitamin E ratio characteristic of all HC LDL subfractions (with the exception of LDL1+2) may favor radical-initiated oxidation of lipid esters containing PUFA by the process of tocopherol-mediated lipid peroxidation (37, 38); indeed, regeneration of vitamin E from α-tocopheroxyl radical could not occur in our in vitro system and therefore vitamin E may play a pro-oxidant role.

All HC LDL subspecies were further distinguished by a consistent and significant higher mean molar ratio of PL-FC in their surface monolayer as compared with control LDL (Table 2) ($P < 0.04$ by multivariate ANOVA); this feature was especially pronounced in HC LDL subclasses LDL1+2 and LDL5 ($P < 0.01$), indicating a greater degree of surface fluidity in HC LDL subclasses. The PL-FC ratio is equally relevant to LDL oxidability, as LDL FC content is negatively correlated with this parameter (42). Indeed, FC molecules can diminish lipid fluidity in LDL particles, thereby retarding lipid peroxidation by limiting the diffusion of oxygen-free radicals (42). The direct chain-breaking action of FC may be equally relevant (43).

Clearly then, the stability of cholesteryl linoleate-derived hydroperoxides was consistently lower in HC LDL subclasses, and especially in LDL1+2, LDL3, and LDL4 versus controls (Fig. 2). In absolute terms however, the amounts of CEOOH detected at $T_{1/2}$ were consistently superior to those detected in the corresponding form of PCs (i.e, 18:2 or 20:4) in all LDL subfractions from both HC and NL populations (Table 4). Indeed, quantitatively, CEOOH18:2 was the most abundant hydroperoxide at both $T_{1/2}$ and T_{max} in all LDL subspecies in both populations. The low values of the hydroperoxide stability ratio in PCs (<10:1) and the contrasting high values in CEs (up to 75:1) (Fig. 2) typically found in both HC and NL populations presumably reflects their surface and/or core accessibility, rendering their hydroperoxides potentially susceptible to reaction with apoB-100.

Lipid hydroperoxides may directly reduce apoB-100, (44); indeed, PCOOH display a higher reactivity than CEOOH due to greater accessibility of protein methionine residues. This hypothesis can be rejected, however, because we failed to detect any difference between HC and NL LDL subclasses in the amounts of PC and CE hydroxides by analysis of the HPLC chromatographic elution profile at 205 nm (data not shown), and the lower stability of hydroperoxides was only seen in CE species but not in PC. As we failed to detect differences in the amount of carbonyl apoB by Western blot analysis in LDL subfractions from HC and NL subjects, and as the detection of lipid hydroxides from reduction of LOOH showed no marked difference between HC and NL, we can equally reject the hypothesis that the diminished stability of CEOOH in HC LDL was due to a greater conversion of CEOOH into alkoxyl and peroxyl radicals by copper or a greater reduction by apoB-100 (44).

One potential explanation for the lower stability of CEOOH in HC LDL subfractions could arise from a direct reaction of CEOOH with apoB-100. This hypothesis is supported by the observation of Kato et al. (45), who showed

that during oxidation by copper ion, large amounts of oxidized esterified fatty acids could covalently react with apoB-100. To confirm this point, delipidated apoB-100 from the oxidized LDL of NL subjects was analyzed in preliminary studies for the presence of bound lipids; gas-liquid chromatography revealed a peak corresponding to the retention time of cholesterol, thereby indicating that CEOOH reacted with amino acids in apoB-100 (data not shown).

It is established that the conformation of apoB-100 is distinct between LDL particles of the light, intermediate, and dense subclasses (46, 47). Such differences are clearly accompanied by distinct features of lipid surface and core composition in LDL subclasses from HC subjects. Surface lipid features specific to LDL subclasses in HC subjects include a lower polyunsaturated phospholipid-α-tocopherol ratio reflecting lower contents of PC16:0-18:0/18:2 and PC16:0-18:0/20:4+22:6, and a higher PL-FC ratio (especially in LDL1+2 and LDL5). Among core lipid features specific to HC LDL subclasses, the diminished polyunsaturated CE-α-tocopherol ratio is prominent, especially in LDL3, LDL4, and LDL5. Modifications in apoB-100-lipid interactions may therefore exist in both the surface and core of LDL subclasses from HC subjects.

It is critical to consider the relevance of our findings to the potential atherogenicity of LDL subfractions in HC individuals at high cardiovascular risk. The LDL profile in the present HC subjects was dominated by elevated levels of large, CE-rich light (LDL1+2), and intermediate (LDL3) LDL subclasses, and it is these particles that predominate in NL subjects with coronary artery disease (48, 49). Furthermore, such an LDL profile is distinct and results in large part from the decreased fractional catabolic rate of circulating LDL particles; their increased residence time in plasma extends their exposure to CETP, for which they represent a preferential target for CE transfer from HDL (7). Core CE content thus increases with consequent expansion in size and molecular weight. As a direct consequence of unique compositional core and surface features of HC LDL, oxidative stress favors enhanced reactivity of CE hydroperoxides preferentially in the intermediate, light, and dense LDL subclasses (CEOOH18:2; LDL3 > LDL1+2 > LDL4 > LDL5). Large, CE-rich LDL particles (LDL1+2 and LDL3) in HC subjects may therefore exert enhanced atherogenicity in the environment of the arterial wall, by virtue of three mechanisms; *i*) a bulk effect resulting from their presence in elevated numbers in HC plasma (1.4-fold elevated as compared with controls), *ii*) acting as a source of LOOH derived from both oxidized cholesteryl arachidonate and linoleate, which are not only cytotoxic and highly chemically reactive (50), but also equally exert multiple biological actions via specific cellular receptors (20), and *iii*) their elevated content of cholesterol molecules per particle (4). Clearly, future therapeutic approaches must be aimed not only to reduce LDL particle numbers in HC patients, but also to protect their lipid components from oxidative modification, and thereby reduce the propensity of their complement of PUFA-containing lipid esters to form labile hydroperoxide derivatives with potential pro-atherogenic activity at the arterial wall.

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