LDL⁻ is a lipid hydroperoxide-enriched circulating lipoprotein

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Abstract A subclass of LDL described on the basis of its greater electronegativity and oxidative status is further characterized using a new, highly sensitive single photon counting technique to measure lipid hydroperoxides. We describe in this report that these particles, which we refer to as LDL⁻, are enriched in lipid peroxides and other peroxidation products as compared to the bulk of the unmodified, normal LDL (nLDL) recovered from human plasma. This chemiluminescence-based, single photon counting technique has unique advantages in that analyses are performed on whole LDL, thus avoiding artifactual lipid peroxidation during lipid extraction. Evidence for increased amounts of lipid hydroperoxides in LDL⁻ versus nLDL are in agreement with other analytical methods such as measurement of conjugated dienes as well as cholesterol oxidation products. LDL⁻ also has lower proportions of polyunsaturated fatty acids than nLDL. Analysis of the amino acid composition of apoB-100 and fatty acid composition of total LDL lipids also revealed major differences between nLDL and LDL⁻ consistent with an oxidative modification of the latter. Thus, LDL⁻ has significantly lower proportions of the oxidizable amino acids histidine and lysine, and marked differences in other neutral and acidic amino acids. The deficit in specific amino acids is in agreement with a reduced TNBS reactivity and increased relative electrophoretic mobility of LDL⁻. We postulate that LDL⁻ is a major carrier of lipid hydroperoxides associated with plasma LDL and may arise from oxidative events in the vasculature and/or by ingestion of peroxide-enriched meals.—**Sevanian, A., G. Bittolo-Bon, G. Cazzolato, H. Hodis, J. Hwang, A. Zamburlini, M. Maiorino, and F. Ursini.** LDL⁻ is a lipid hydroperoxide-enriched circulating lipoprotein. *J. Lipid Res.* 1997. **38:** 419–428.

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There is mounting evidence that oxidatively modified LDL plays an important role in atherogenesis in that several processes underlying the formation and progression of an atheromatous lesion appear to be initiated by oxidized LDL (1). LDL can be oxidized in vitro by a variety of catalysts or pro-oxidants, including free (2) and bound (3) transition metals, metalloproteins (4), enzymes (5, 6), and by a number of vascular cells (7–9) utilizing metabolically generated oxidant species. Peroxidation under these conditions is usually extensive resulting in not only accumulation of peroxides but also substantial peroxide decomposition to aldehydes and complete depletion of vitamin E. A more gradual oxidation of LDL lipids, as in the case of autoxidation, produces a minimally modified particle via propagation from pre-existing peroxides with marginal consumption of vitamin E (10). There is evidence that LDL peroxidation occurs in vivo, where formation and accumulation in the vascular intima has been described (1). Furthermore, a form of LDL containing lipid peroxidation products has been isolated from the circulating blood (11). As evidence for oxidized LDL in human plasma remains limited, some investigators have suggested that oxidation of LDL may occur when particles become trapped in the proteoglycan matrix of the intima, where in these antioxidant-depleted domains the LDL particles experience progressive oxidation (1). The rate and extent of oxidation is related to the levels of LDL peroxides regardless of the mechanism for catalysis, indicating that the propensity for LDL oxidation is peroxide-dependent (12). The origin of modified LDL

Abbreviations: LDL, low density lipoprotein; ChOx, cholesterol oxides; HPLC, high pressure liquid chromatography; BHT, butylated hydroxytoluene; GC, gas chromatography; TNBS, trinitrobenzene sulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; LOOH, linoleic acid hydroperoxide.

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in the circulation remains speculative as indications of mild oxidation have been reported (11, 13) and contested (14). This controversy is due, in part, to the absence of suitable analytical tools that are sensitive enough to measure peroxidation products in the physiological concentration range. Nevertheless, modified LDL in the circulation possess a number of features distinct from unmodified LDL that impart a more electronegative character to the modified particle (14). However, a detailed analysis of the constituents that account for the more oxidized nature of circulating modified LDL has yet to be performed.

Utilizing ion exchange chromatography, a more electronegative LDL subfraction (LDL^-) has been isolated from human plasma. This lipoprotein has been reported to be enriched in oxidized lipids, contains vitamin E at levels similar to or lower than that found in unmodified or normal LDL (nLDL), shows a lower (15) or similar (14) binding affinity to LDL receptors on human skin fibroblasts and endothelial cells, is a poor ligand for scavenger receptors (14), and is cytotoxic to cultured endothelial cells (14, 16), unlike nLDL which demonstrates no toxicity. Recent evidence also indicates that particles with characteristics similar to LDL^- have an increased content of sialic acid and much higher levels of apoC-III and apoE (14), however, the sialic acid content is also subject to controversy (17). The total LDL fraction from hypercholesterolemic subjects can have sizable amounts of LDL^-, in the range of 2–6% of total LDL in most subjects (13), and in some cases as high as 10 mg/dL. As the content of cholesterol oxidation products may at times comprise over 25% of total lipoprotein cholesterol (16), LDL^- may also be a major carrier of plasma oxidized cholesterol. As LDL^- is different from nLDL in several ways, a distinct difference in origin may also be possible. In the present study, freshly isolated LDL^- was further characterized with the objective of precisely quantifying the levels of lipid hydroperoxides and describing the characteristics of its apoprotein content and lipid fractions. Using a newly developed method for measuring lipid hydroperoxides by means of single photon counting (18) we show that LDL^- represents a lipid hydroperoxide-enriched lipoprotein subpopulation. It is also enriched with other lipid peroxidation products and has a modified apoB-100 composition.

MATERIALS AND METHODS

Isolation of LDL subfractions

Venous blood was obtained from fasting adult human volunteers with total plasma cholesterol levels ranging from 160–210 mg/dL. Blood was collected into Vacutainer test tubes containing ethylenediamine tetraacetic acid (EDTA, from Sigma, St. Louis, MO) and immediately centrifuged at 1500 *g* for 10 min at 4°C. LDL (δ 1.019–1.063 g/mL) was separated by preparative ultracentrifugation of the plasma using a Beckman L8-55 ultracentrifuge equipped with an SW-41 rotor as described previously (16). The isolated LDL was dialyzed against argon-sparged 0.05 M Tris-HCl buffer, pH 7.2, containing 10 μM EDTA at 4°C overnight and cholesterol levels were measured enzymatically using a VP Super System instrument (Abbott, Dallas, TX). The isolated LDL was kept in argon-sparged, EDTA-buffer at 4°C for no more than 24 h before further analysis.

Separation of LDL^- from unmodified LDL was accomplished using anion exchange high pressure liquid chromatography (Perkin Elmer Series 4 HPLC) as described previously (16). The purity of the isolated LDL was checked by means of rocket gel electrophoresis using agarose gels prepared with 10% polyclonal antibodies to apoprotein B-100, apoA-I, and Lp[a] using commercially available antibodies for these apoproteins (Apo-Tek, Organon Teknika/Biotechnology Research Institute, Rockville, MD). The isolated LDL was then injected into the HPLC at an adjusted concentration of 0.5 mg cholesterol/ml buffer. The eluent was monitored at 280 nm and peaks corresponding to defined/normal LDL (nLDL) or LDL^- were collected in 1-mL aliquots and immediately used for further studies described below. Chelex-treated buffers were used during all steps of the isolation procedure. The amount of LDL protein was determined for each peak using the method of Lowry et al. (19) and used for peak area calibration from which the amounts of nLDL and LDL^- were routinely computed. Fractions were collected into tubes containing 50 μM EDTA in 0.01 M Tris-HCl buffer, pH 7.2, and those fractions containing LDL^- were pooled, concentrated, and all salts were removed by centrifugation with Centricon 10,000 molecular weight microconcentrators (Beverly, MA). Lipoprotein electrophoresis was done using a Beckman Paragon Lipo Cell in 50 mM barbital buffer (pH 8.6). Bovine albumin (Sigma) at a final concentration of 20 mg/mL was added to dilute lipoprotein samples to ensure reproducible migration distances.

Determination of amino acid composition in LDL proteins

Amino acid analysis of LDL was performed on aliquots of delipidated fractions where the protein residue was hydrolyzed at 110°C in 6 M HCl for 24 h in evacuated sealed borosilicate tubes. The hydrolysates were evaporated, washed in distilled water, evaporated again to dryness, derivatized with fluoroaldehyde reagent

(OPA, Pierce Chemical Co., Rockford, IL) and then analyzed by HPLC using a 4 × 150 mm Bio-Sil ODS.5S column as described previously (20). The effluent was monitored with a Perkin-Elmer LS-5 fluorometer (excitation 360, emission. 455 nm). Amino acid standards were obtained from Sigma or Pierce and used to identify amino acids derived from LDL protein using external standard calibration and peak identification using HPLC proprietary software. Free amino groups on LDL subfractions were estimated using trinitrobenzenesulfonic acid (TNBS) according to Habeeb (21). LDL was mixed with 1 mL of 4% $NaHCO_3$ (pH 8.4) and 50 μL of 0.1% TNBS and then heated for 1 h at 37°C. The concentration of amino groups was determined by reference to a valine standard.

Measurement of LDL conjugated diene content

Conjugated dienes were measured in lipid extracts of LDL by means of second derivative UV spectroscopy as described previously (22). Samples of LDL containing 500 μg protein/mL were extracted with 6 mL chloroform–methanol 2:1; the organic phase was collected and saved; the aqueous phase was re-extracted with another 3 mL chloroform–methanol 2:1; and the organic phases were pooled. After evaporation of the solvent under a stream of nitrogen at room temperature, the lipid residue was redissolved in absolute ethanol and the absorbance was monitored over the frequency range of 220–300 nm against an ethanol blank. The amounts of conjugated dienes were estimated from the sum of the absorbance minima at 242 and 233 nm. Linoleic acid hydroperoxide (LOOH), prepared as described previously (23), was used to develop a calibration curve. All scans were taken with a Beckman DU650 spectrophotometer. Conjugated diene content was estimated from the sum of the conjugated diene absorbances using the LOOH calibration standard.

Chemiluminescence measurements

For determinations of LDL peroxides using photon emission counting, all LDL samples were prepared in Chelex-treated normal saline containing 25 mM Tris-HCl buffer, pH 7.4. Photon counting was performed using an SEAS luminometer containing a thermally isolated and cooled photocathode which has been previously described (18). The reaction mixture contained 30 μM luminol, 0.3% (w/v) Triton X-100, 0.12 mL methanol, and 4 μM hemin in 0.1 M CAPS buffer, pH 10, in a final volume of 1.2 mL. The reaction was started by loading the LDL sample into the loop of a 6-way valve containing a computer-controlled syringe that aspirates 1 mL of the reaction mixture in front of the photomultiplier and then re-injects the mixture through the loop containing the sample into the vial in 2 s. Samples were continuously mixed by a built-in minivortex in the dark. For internal calibration, 20 μL of LDL was injected 6 to 8 times in duplicate along with increasing amounts of standard hydroperoxide (1,palmitoyl-2, linoleoylhydroperoxy-phosphatidylcholine, i.e., PLPCOOH) in methanol. Integrated areas of the mono-exponential fitting of the photon emission rate were plotted versus the amount of internal standard added and second-order polynomial fitting of data was used to calculate the absolute value of lipid hydroperoxide by extrapolation through the x-axis intercept.

Measurement of LDL cholesterol oxides

Characterization and quantitation of cholesterol oxides (ChOx) was performed by capillary gas chromatography, as described previously (24). All procedures were performed under nitrogen in subdued light in the presence of 0.01% BHT and 50 μM EDTA. Sample aliquots containing approximately 500 μg/ml LDL protein were extracted with chloroform–methanol 2:1(v/v) containing 0.01% BHT, and 100 μl of 5α-cholestane (10 μg/ml) was added to each sample as an internal standard. The lipid extract was dried under nitrogen and the residue was redissolved in 1.0 ml toluene–ethyl acetate 1:1(v/v). The neutral lipid fraction (triglycerides, cholesterol, ChOx) and polar lipid fraction (phospholipids) were isolated by sequential elution through solid phase extraction columns (Bakerbond Diol columns) with toluene–ethyl acetate followed by methanol. The neutral lipid fraction was subjected to mild, two-phase alkaline saponification and methylated with ethereal diazomethane (25). The samples were then transferred into autoinjector vials; lipids were derivatized to o-trimethylsilyl ethers (TMS) using dimethylformamide and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (1:1); and the vials were sealed, purged with argon, heated at 80°C for 20 min, and injected into a gas chromatograph (Shimadzu GC-14A) fitted with a 30 meter DB-1 column (0.32 mm I.D., 0.25 micron film thickness, J & W Scientific, Folsom, CA) and operated with a split ratio of 1:10. Helium was used as a carrier gas at a flow rate of 1 ml/min, the injection temperature was set at 290°C and initial column temperature at 260°C. A programmed temperature ramp of 3°C/min was used until the final temperature of 290°C was achieved. The flame ionization detector temperature was set at 300°C. Peaks of interest were integrated with Axxi-chrom 747 chromatography software. Quantitative analysis of all chromatograms was performed by the internal standard method. Standards used to identify commonly encountered ChOx included: 5-cholestene-3β,7α-diol (7α-OH), 5-cholestene-3β,7β-diol (7β-OH), cholestan-5α,6α-epoxy-3β-ol (α-epox), cholestan-5β,6β-epoxy-3β-ol (β-epox), cholestan-3β,5α,6β-triol (CT), 5-cholesten-

3β-ol-7-one (7-keto), and 5-cholesten-3β,25-diol (25-OH).

Measurement of LDL phospholipids

The polar lipid fraction isolated from LDL as described under Measurement of LDL cholesterol oxides, above, was dissolved in methanol and injected into an HPLC column (Perkin-Elmer Series 4) fitted with a 4.6 × 150 mm normal phase silica column (Alltech, Inc., Deerfield, IL). Separation was achieved using a tertiary solvent system and 0.5-min linear gradients according to the following elution program and solvent proportions.

	Acetonitrile	Water	Methanol
0.01–5.0 min	86	7	7
5.5–35.5 min	80	12	8
36.0–53.0 min	67	20	13

The eluent was monitored in series using a Perkin-Elmer LC-95 UV detector set at 204 nm and a Shimadzu SPD-6AV UV/VIS detector set at 234 nm. The UV chromatograms were displayed using Axxiom 747 chromatography software. Authentic lipids consisting of phosphatidylinositol (PI) from bovine liver, phosphatidylserine (PS) from bovine brain, phosphatidylethanolamine (PE) from bovine liver, phosphatidylcholine (PC) from soybean, sphingomyelin (SM) from bovine liver, and lysophosphatidylcholine (LPC) from bovine liver, were obtained from Avanti Polar Lipids (Pelham, AL) and used as calibration standards. The amount of conjugated dienes in PC (the major LDL phospholipid) was determined by second derivative UV spectroscopy of the lipid extract from the isolated PC peak (collected using a programmed fraction collector).

Measurement of LDL fatty acid composition

Total lipid extracts of LDL samples were hydrolyzed by means acidic methanolysis and dissolved in a solution of 25 μL of DMF + 25 μL of BSTFA (Supelco, Bellefonte, PA), heated for 10 min at 80°C under argon in sealed autoinjector vials. An aliquot of 2 μl of each sample was injected into a Shimadzu Model 14A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a 30 m × 0.32 mm DB225 capillary column (J&W Scientific, Folsom, CA). The column temperature was held at 185°C for 8 min, increased to 220°C at a rate of 2°C/minute, and held at 220°C for 16 min. The injector temperature was set at 190°C and the detector at 230°C. Fatty acid identification and quantitation were determined using Axxiom 747 chromatographics software.

All data are presented as mean and standard errors based on the analysis of 5–11 samples from different individuals. Statistical significance for differences between nLDL and LDL$^-$ was determined using the Student's *t*-test.

RESULTS

The isolation of nLDL and LDL$^-$ from a total of 11 subjects was found to result in 82–94% recovery with respect to the initial amounts of total LDL subjected to dialysis and HPLC based on cholesterol content. Although the amount of LDL$^-$ can be as low as 0.5% and as high as 8% of the total LDL, the mean percentage of the pool of samples in this study was 4.5%. Electrophoretic analysis of total LDL, nLDL, and LDL$^-$ indicated that only apoB-100 was detectable in all samples and there was no indication of Lp[a] or other apoproteins (data not shown). **Figure 1** indicates that LDL$^-$ has a 10–20% greater electrophoretic mobility compared to nLDL. Analysis of samples from eight subjects showed that this increased mobility correlates with a reduction in TNBS reactivity, suggesting that increased electro-

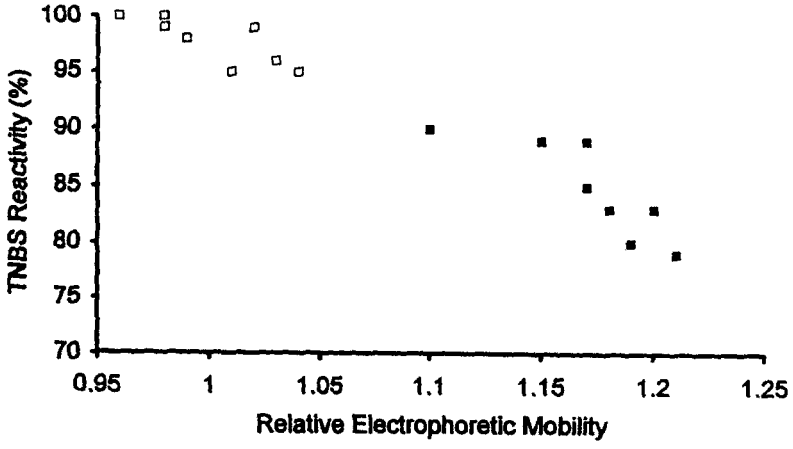

Fig. 1. The relationship between relative electrophoretic mobility and TNBS reactivity for eight independent sample isolates of nLDL (□) and the corresponding LDL$^-$ (■) isolated from each sample. Each plasma LDL sample was subjected to anion exchange HPLC and the nLDL and LDL$^-$ peaks were isolated and analyzed as described under Methods. Freshly prepared total LDL was used to establish electrophoretic mobility for LDL which was arbitrarily set as 1.0. The relative electrophoretic mobility of the isolated nLDL and LDL$^-$ was then calculated and shown along the x-axis. TNBS reactivity is expressed as a percent of the valine standard which was prepared at a protein concentration equivalent to the amount of nLDL and LDL$^-$ protein analyzed.

TABLE 1. Fatty acid composition of nLDL and LDL⁻ total lipids

Fatty Acid	nLDL	LDL⁻
16:0	34.8 ± 1.80	35.3 ± 0.81
18:0	18.4 ± 3.93	21.1 ± 4.11
18:1	15.4 ± 0.94	17.0 ± 1.11
18:2	20.8 ± 2.89	18.2 ± 2.63
20:3	1.33 ± 0.24	0.90 ± 0.21[a]
20:4	5.37 ± 0.37	1.33 ± 0.13[c]
22:6	0.93 ± 0.14	0.13 ± 0.03[b]
Total PUFA	28.5 ± 2.62	20.5 ± 2.97[a]

Values are expressed as mean and standard error for the percent composition of fatty acids from 5 independent measurements using samples obtained from different subjects. Major fatty acids of LDL are shown and those comprising less than 0.5% of fatty acids are omitted.
[a] $P < 0.05$.
[b] $P < 0.005$.
[c] $P < 0.001$.

negativity is related to a reduction in free amino groups in LDL⁻ (Fig. 1). Characterization of LDL⁻ by means of nondenaturing PAGE has also shown it to be more electronegative with indications of greater size heterogeneity than nLDL and greater mass due likely to aggregation (16, 26).

To further exclude the possibility that LDL⁻ was formed during the isolation procedures used, we compared the effects of various antioxidants, including addition of BHT and desferal to the plasma prior to the isolation steps described. No differences in LDL⁻ content were found as compared to the conventional method described. Moreover, when we did not use Chelex-treated buffers or sparge solvents with argon, the isolated nLDL and LDL⁻ contained up to 10 times more peroxides but the content of LDL⁻ did not increase. Finally, rechromatography of nLDL diluted 10-fold gave only one peak corresponding to nLDL (data not shown).

The composition of fatty acids obtained from the total lipid extracts of nLDL and LDL⁻ is presented in **Table 1.** Results were analyzed using the paired *t*-test as

TABLE 2. Composition of cholesterol oxides in nLDL and LDL⁻

ChOx	nLDL	LDL⁻
7α-OH	0.08 ± 0.02	0.46 ± 0.09[a]
7β-OH	0.06 ± 0.02	0.55 ± 0.17[a]
7-Keto	0.47 ± 0.11	1.94 ± 0.29[c]
α-Epox	0.28 ± 0.10	0.66 ± 0.19[b]
β-Epox	0.33 ± 0.17	0.69 ± 0.21
CT	0.03 ± 0.02	0.19 ± 0.07[a]
25-OH	0.02 ± 0.009	0.17 ± 0.05[a]

Values are expressed as mean and standard error for the ChOx expressed as a percent of total cholesterol from 11 independent measurements using samples obtained from different subjects.
[a] $P < 0.001$.
[b] $P < 0.05$.
[c] $P < 0.01$.

TABLE 3. Composition of phospholipids from nLDL and LDL⁻

Phospholipid	nLDL	LDL⁻
PI	3.03 ± 0.98	2.07 ± 0.87
PE	6.42 ± 1.66	1.02 ± 0.63[a]
PC	78.87 ± 1.23	65.90 ± 7.90[b]
LPC	2.00 ± 1.70	3.50 ± 2.09
SM	12.60 ± 1.57	21.50 ± 7.50[b]

Values are expressed as mean and standard error for the percent composition of phospholipids from five independent measurements using samples obtained from different subjects.
[a] $P < 0.001$.
[b] $P < 0.01$.

LDL⁻ and nLDL were compared from the same samples. No differences were found in the proportions of saturated (palmitic and stearic), monounsaturated (oleic), and diunsaturated (linoleic) fatty acids. Marginally lower proportions of trienoic fatty acids (8,11,14-eicosatrienoic acid, 20:3) were found in LDL⁻ and all polyunsaturated fatty acids (PUFA) measured in LDL⁻, and notably (arachidonic acid, 20:4), were significantly lower than in nLDL.

Analysis of the conjugated diene levels in the lipid extracts of freshly isolated LDL and LDL⁻ subfractions showed that the amounts measured were much greater than that found by chemiluminescence detection of lipid peroxides **(Fig. 2).** Nevertheless, there was agreement between these two methods of measurement in terms of differences between LDL⁻ and nLDL contents. LDL⁻ had more than 6-fold higher levels of lipid peroxides than nLDL and 4-fold higher levels of conjugated dienes. In all cases measurements are based on LDL cholesterol content. Accordingly, peroxide values measured via chemiluminescence were 13-fold lower than that measured by means of conjugated diene content for nLDL and 8-fold lower for LDL⁻. Reasons for a discrepancy between amounts of peroxidation products measured via conjugated dienes versus direct measurements of peroxides will be discussed later. In any case, it is clear that LDL⁻ is greatly enriched in lipid peroxidation products, and specifically, lipid hydroperoxides. Results for hydroperoxide measurements by chemiluminescence are in agreement with measurements of total cholesterol oxidation products (ChOx) as shown in Fig. 2 and provided in greater detail in **Table 2** for specific ChOx species. Previous studies showed that total plasma ChOx vary according to the plasma cholesterol levels (16, 24, 27). The results are, therefore, expressed as a percentage of the LDL cholesterol measured by GC together with the ChOx. LDL⁻ was found to contain approximately four times more ChOx than nLDL with the major oxidation product being 7-keto. Indeed, 7-keto was highly representative of the general increase

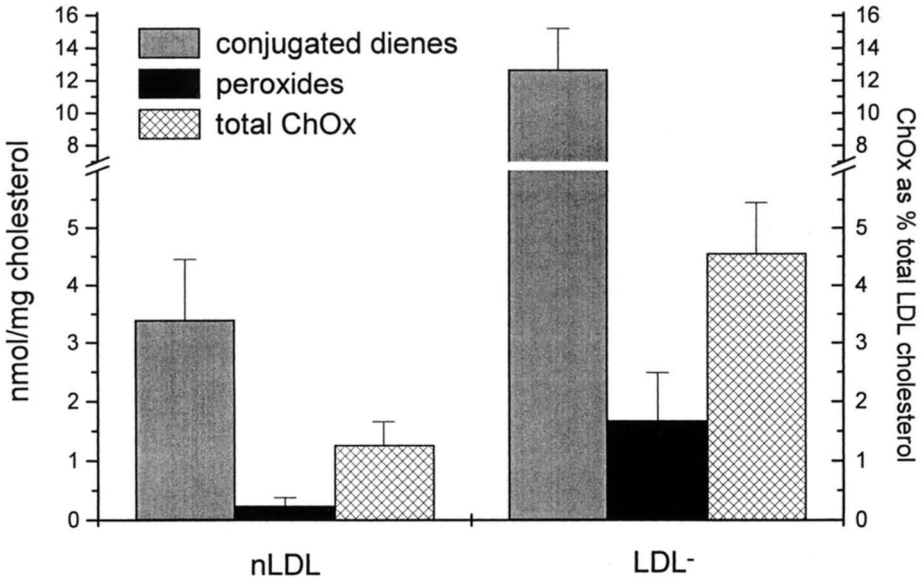

Fig. 2. A comparison of the total lipid conjugated dienes, lipid hydroperoxides, and total cholesterol oxides (ChOx) in nLDL and LDL⁻. Conjugated dienes were measured spectrophotometrically while lipid hydroperoxides were measured by means of chemiluminescence as described under Methods and expressed as nmol/mg LDL cholesterol. ChOx were determined by gas chromatography and expressed as a percent of cholesterol measured in each sample. Results for conjugated dienes and peroxides are based on 5 independent samples whereas ChOx are from 11 samples.

in LDL⁻ ChOx levels although proportionately greater increases in isomeric 7α/β-OH were found.

HPLC analysis of the phospholipid fractions of nLDL and LDL⁻ showed five major phospholipid species of which PC was predominant. The results are shown in **Table 3** and expressed as percent of the total phospholipid content. The proportions of all phospholipids except for SM and LPC were lower in LDL⁻ as compared to nLDL with the largest difference in phospholipid mass accounted for by the PC and PE fractions. As the largest loss occurred for PC, this fraction was collected and analyzed by second derivative UV spectroscopy for total conjugated diene content. Conjugated dienes associated with PC were 5.42 ± 0.42 nmol/mg protein for nLDL and 16.51 ± 3.25 nmol/mg protein for LDL⁻, representing a significantly greater content in the latter lipoprotein fraction.

The amino acid composition of the total LDL protein is shown in **Table 4.** The differences in percent composition for the major amino acids in LDL were most apparent for glycine (+75%), serine (+46%), alanine (+34%), histidine (−52%), lysine (−43%), isoleucine (−37%), and threonine (−35%), where the values in parentheses indicate changes in percent composition for LDL⁻ as compared to nLDL. Amino acids not shown either could not be detected with the method used or were too small to be quantitated. In particular, cysteine could not be reliably measured with the HPLC method used, thus, nothing can be concluded regarding the apparent deficit in this oxidant-sensitive amino acid, however, the lower content of lysine and histidine is suggestive of an oxidative attack on susceptible amino acids. The higher proportion of many of the neutral amino

TABLE 4. Composition of amino acids in LDL apoB protein

Amino Acid	nLDL	LDL⁻
Valine	6.2 ± 0.13	6.7 ± 0.29
Leucine	11.7 ± 0.36	8.9 ± 0.32[d]
Isoleucine	6.7 ± 0.15	4.2 ± 0.26[d]
Serine	8.2 ± 0.24	12.0 ± 0.78[c]
Threonine	6.3 ± 0.16	4.1 ± 0.45[c]
Tyrosine	3.3 ± 0.21	2.9 ± 0.21
Arginine	4.3 ± 0.16	4.3 ± 0.25
Glycine	6.0 ± 0.58	10.5 ± 0.98[d]
Lysine	7.2 ± 0.28	4.1 ± 0.49[d]
Phenylalanine	6.9 ± 0.40	7.4 ± 0.63
Methionine	1.6 ± 0.14	1.4 ± 0.11
Aspartic acid	10.2 ± 0.37	8.7 ± 0.46[a]
Glutamic acid	12.1 ± 0.32	13.3 ± 0.91
Glutamine	3.2 ± 0.11	3.2 ± 0.08
Alanine	6.5 ± 0.24	8.7 ± 0.69[a]
Histidine	2.7 ± 0.26	1.3 ± 0.25[b]

Values are expressed as nmol/100 nmol total amino acids from 5 independent determinations.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.005$.
[d] $P < 0.001$.

acids may reflect the deficit in the specific amino acids indicated.

DISCUSSION

Recent studies on LDL⁻ recovered from human plasma have shown that they represent an oxidatively susceptible population of low density lipoprotein particles that exhibit LDL receptor binding characteristics similar to nLDL (13, 14, 28). Previous studies are in conflict regarding the content of lipid peroxidation products in LDL⁻ and the degree to which the apoprotein is modified in terms of binding to LDL receptors versus scavenger receptors (13–15, 28). The present findings show that LDL⁻ is indeed enriched in lipid peroxides by more than 6-fold as compared to the major "unoxidized" nLDL fraction using a direct and sensitive measurement technique. The elevated peroxide levels in LDL⁻ correspond to the conjugated diene and ChOx levels, all of which are substantially elevated (approximately 3.5-fold) in LDL⁻ as compared to nLDL. The new technique used to measure peroxides also reveals that nLDL, and in some cases LDL⁻, could be considered as "peroxide-free" based on other conventional and less sensitive methods for measuring lipid peroxidation products.

Some of the products measured in LDL are not peroxides as the values for conjugated dienes are considerably greater than those obtained by means of chemiluminescence detection. This may be at the basis of the discrepancy found between measurements of LDL lipid peroxidation determined by conjugated diene levels versus direct analysis of lipid peroxides. Additionally, as the levels of conjugated dienes were estimated from a calibration curve derived from linoleic acid hydroperoxide, peroxide levels interpreted from these results are likely to be overestimated. The conjugated diene absorbance is specific only for the double bonds but not for the peroxidic group, thus, other oxidation products, such as alcohols, or non-peroxidation products, such as 9,11 fatty acids are measured. Comparison to the peroxide levels measured via chemiluminescence suggests that considerable lipid peroxidation likely occurred during the isolation and analysis of the lipoprotein phospholipids. This may be a particular problem for LDL⁻ lipid extracts whose components are more prone to oxidation based on the higher peroxide levels. On the other hand, non-peroxide conjugated dienes may also be associated with PC. For example, the 9,11-fatty acids, particularly of linoleic acid, are thought to be derived from various dietary sources (29) and can be incorporated into phospholipids (30), hence accounting for the high levels of conjugated dienes in the phospholipid fraction of LDL. A point to be stressed from these observations is that a clear advantage exists with the use of chemiluminescence detection of peroxides as it provides a direct measure of peroxides in LDL particles without need for lipid extraction.

The levels of ChOx in LDL⁻ are increased in parallel with other lipid peroxidation products suggesting a common origin. The ChOx are thought to be derived from lipid peroxide attack at the allylic C-7 hydrogen of cholesterol to produce the isomeric 7-α/β cholest-5-en-3β-ol hydroperoxides (7-α/β ChOOx) from which other ChOx are derived (31). The markedly higher amounts of isomeric 7-OH are indicative of a peroxidative attack on cholesterol to yield 7-β ChOOx and in turn these biologically active ChOx (32). The profiles of ChOx are quite consistent among individuals as well as in LDL isolated from animals (16, 27, 33) suggesting that similar mechanisms underlie their accumulation in LDL. Table 2 also indicates that although LDL⁻ is enriched with ChOx relative to nLDL, the bulk of the LDL-associated ChOx (approximately 85%) are found in the nLDL fraction which makes up about 95% of the total LDL as isolated by HPLC. However, this also applies to lipid peroxides as indicated in Fig. 2 and suggests that oxidation processes may have involved the cholesterol or cholesteryl ester fractions, and specifically the cholesterol B-ring to a comparable extent as other lipid peroxidation products. This is expected for lipoproteins rich in cholesterol but, nevertheless, indicates that much higher concentrations of peroxidation products are found in LDL⁻. On the other hand, it is also possible that considerable amounts of ChOx may be derived from dietary sources as discussed below.

Our findings show a substantial difference between LDL⁻ and nLDL amino acid composition, suggesting either apoB-100 oxidation or other undefined modifications of the apoprotein. However, the general composition of apoB amino acids in nLDL and LDL⁻ agrees with that reported by Fong et al. (34) for LDL, both before and after oxidation. Although data for some oxidant sensitive amino acids are incomplete, the general decrease in basic amino acids is consistent with the loss in TNBS reactivity as shown in Fig. 1. Moreover, the relative decrease in the proportions of oxidant-sensitive amino acids (e.g., histidine, lysine, methionine), and increase in unoxidizable amino acids, suggests that LDL⁻ proteins reacted with oxidants. Despite these differences, decreases in total acidic amino acids are not as great as for basic and other oxidant-sensitive amino acids, indicating that the increased negative charge for LDL⁻ may, in part, be explained by amino acid composition. It is unclear why LDL⁻ interacts with the LDL receptor given the 40% decrease in lysine residues as

previous reports show that even a 10% decrease in lysine content can result in major decreases in LDL receptor-mediated uptake (35). One explanation may be that our measurements of apoB represent the proportion of lysine residues among total amino acids, whereas the effects on receptor uptake are based on free lysine residues. TNBS reactivity is consistent with previous results showing a reduced uptake of LDL^- by the LDL receptor, but not a lack of uptake. A reduced reactivity with monoclonal antibodies that recognize domains involved in receptor binding was found (15) and the same results may explain the lack of reactivity with the scavenger receptor. As LDL charge characteristics can be influenced by other components, such as phospholipids, it is also possible that the substantially lower content of PE may account for some of the net electronegativity and decreased TNBS reactivity (36) of LDL^-. Hindering of specific amino groups due to a different folding of the protein, altered size/structure of the particle, or its increased mobility could be unrelated to an oxidant-mediated modification. Demuth et al. (14) isolated particles from human plasma with characteristics similar to LDL^- which have increased sialic acid content and much higher levels of apoC-III and apoE. The increased sialic acid content may contribute to the electronegative character of this modified form of LDL. However, others have found that LDL subclasses with similar characteristics and isolated in a similar manner contain less sialic acid residues (17). Lacking a thorough characterization of glycoprotein composition, the increased electronegative charge of our LDL^- may tentatively be explained by the loss of electropositive amino acids, the total charge of which is much higher than the charge contribution of sialic acid residues removed via desialylation (37).

Modifications in apoB protein can arise from a concerted reaction between lipid hydroperoxides and free amino groups in the protein (38) or by decomposition of hydroperoxides to aldehydic products and their reaction with free amino groups (39). However, the very low levels of Schiff-base fluorescence (data not shown) suggest that reactions between LDL amino groups and lipid peroxide decomposition products are not appreciable in LDL^-. Modifications in LDL^- amino acids based on its enhanced content of lipid peroxides and decreased TNBS reactivity can be reconciled by the finding that many of the lysine residues can form metastable conjugates that are not measured via TNBS fluorescence but degrade to yield free lysine upon acid hydrolysis of apoB (38).

It is reported that LDL phospholipids, and particularly PC, are major targets for lipid peroxidation (39) from which certain biologically active and potentially atherogenic products are derived. Among these are LPC (40, 41) and PC-hydroperoxide decomposition products resembling PAF (42) that stimulate inflammatory cell influx and accumulation during early atherosclerosis. A marginal increase in LPC was also found consistent with its formation after in vitro LDL oxidation (43). Comparison of this data to the results for LDL fatty acid composition (Table 1) suggest that accumulation of lipid peroxidation products in LDL^- may derive from oxidation of LDL–PUFA which are preferred targets during peroxidation of LDL lipids (39).

A dietary origin for plasma ChOx and lipid peroxides may be related to the LDL^- levels. Studies with animals have shown that the diet accounts for the majority of the ChOx found in lipoproteins, although some ChOx appear not to come from the diet (27, 44). Similarly, lipid peroxides can be ingested from peroxide-enriched foods and appear in serum lipoproteins (45). Whether the ingested peroxides persist by the time plasma samples are analyzed remains to be determined. As indicated in Fig. 2, LDL^- is substantially enriched in all lipid peroxidation products as compared to nLDL, however, lipid peroxide levels are proportionately much greater in LDL^- than the other indices measured, suggesting that LDL^- is an enriched carrier of either ingested peroxides or of peroxides accumulating due to the tendency of these particles to undergo lipid peroxidation.

LDL^- may represent a population of old LDL particles that are not sufficiently modified to be cleared from the circulation. Under these circumstances peroxides gradually accumulate either by radical propagation reactions, and/or by deposition of peroxidized lipids from dietary sources or generated by cells. In the latter case, the vessel wall has been shown to generate modified LDL as a component of the LDL oxidized in the interstitial spaces (1). Being minimally modified, LDL^- may escape clearance by phagocytic cells or receptor-mediated uptake and egress back into the circulation in a manner similar to normal LDL (46). Until more definitive findings are available concerning the source of peroxides in human plasma, an oxidative origin linking LDL^- to nLDL must be approached with caution.

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