Free Radical Research, 2002 Vol. 36 (2), pp. 127–142

Taylor & Francis
healthsciences

New Insight on the Relationship between LDL Composition, Associated Proteins, Oxidative Resistance and Preparation Procedure

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Accepted by Professor A. Azzi

(Received 10 July 2001; In revised form 27 July 2001)

Oxidized low density lipoprotein (LDL) plays an important role in atherogenesis. It is generally thought that LDL is mainly oxidized in the intima of vessel walls, surrounded by hydrophilic antioxidants and proteins such as albumin. The aim of this study was to investigate the possible interrelationships between oxidation resistance of LDL and its protein and lipid moieties. Proteins and to a lesser extent lipids, appeared to be the major determinants in the LDL Cu^{2+}-oxidation resistance, which in turn depend on the ultracentrifugation (UC) procedure used. Comparing high speed/short time (HS/ST, 4 h), high speed/long time (HS/LT, 6–16 h) and low speed/long time (LS/LT, 24 h) conditions of UC, HS with the shortest time (4 h) led to prepare LDL (named LDL·HS-4 h) with higher total protein and triglyceride contents, unchanged total cholesterol, phospholipids and Vitamin E, and higher Cu^{2+}-oxidation resistance. Among proteins, only albumin allows to explain changes. PAF acetyl hydrolase appeared to be unaffected, whereas its pro-oxidant role was established and found only in the absence of albumin. In contrast the pro-oxidant role of caeruloplasmin took place regardless of the albumin content of LDL. The antioxidant effect of albumin (the oxidation lag time was doubled for 20 mol/mol albumin per LDL) is assumed to be due to its capacity at decreasing LDL affinity for Cu^{2+}. Interestingly, the LDL·HS-4 h albumin content mirrored the intrinsic characteristics of LDL in the plasma and was not affected by added free albumin. Moreover, it has been verified that in 121 healthy subjects albumin was the best resistance predictor of the Cu^{2+}-oxidation of LDL·HS-4 h, with a multiple regression equation: lag time (min) $= 62.1 + 0.67(\text{HSA/apoB}) + 0.02(\text{TG/apoB}) - 0.01(\text{TC/apoB})$; $r = 0.54$, $P < 0.0001$. Accounted for by lag time, the oxidation resistance did not correlate with α-tocopherol and ubiquinol contents of LDL. The mean albumin content was about 10 mol/mol, and highly variable (0–58 mol/mol) with subjects. The LDL·HS-4 h may account for the status of LDL in its natural environment more adequately than LDL resulting from other conditions of UC.

Keywords: LDL chemical composition; Albumin-associated LDL; Platelet activating factor-acetylhydrolase; LDL-phospholipase A_2; LDL Cu^{2+}-oxidation resistance; Vitamin E

Abbreviations: IDL, Intermediate low density lipoprotein; LDL, Low density lipoprotein; VLDL, Very low density lipoprotein; TRL, Triglyceride rich lipoprotein; ox-LDL, Oxidized low density lipoprotein; LDL-PLA_2, LDL-associated phospholipase A_2; PAF-AH, Platelet activating factor-acetyl hydrolase; TProt, Total protein; TC, Total cholesterol; CE, Cholesterol esters; TG, Triglycerides; PL, Phospholipids; lysoPCho, Lysophosphatidyl choline; Pcho, Phosphatidyl choline; vit E, Vitamin E; Ub, Ubiquinol; EDTA, Ethylenediamine tetraacetic acid; DTPA, diethylenetriamine pentacetic acid; HS, High speed-isolation procedure; LS, Low speed-isolation procedure; HSA, Human serum albumin; Cp, Caeruloplasmin; CD, Conjugated dienes; NBD-C6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phospho-choline; NBD-X, NBD-hexanoic acid; UC, Ultracentrifugation

INTRODUCTION

In normal subjects, low density lipoprotein (LDL) is specifically distributed within the density interval 1.019–1.063 g/ml. Heterogeneity in physical, chemical, hydrodynamic and immune properties is an inherent characteristic of LDL which is in fact a

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290006466

continuum of different-sized particle subspecies as accounted for by gradient ultracentrifugation (UC)[1] or gradient polyacrylamide gel electrophoresis.[2,3] LDL from most individuals displays a single major peak by UC and two or three bands of bordering sizes by electrophoresis. Regardless of the well-known effect of dietary intake on the LDL particle size profile, individuals can be classified as having mainly large and buoyant LDL (pattern A) or small and dense LDL (pattern B).[2] Many studies confirm that LDL density is inversely related to the particle size.[1,4,5] It is also well established that whereas LDL size decreases, protein content increases, phospholipid content remains practically constant and other lipids decrease.[1] Variations in the ratio of surface-to-core lipids (i.e. phospholipids + cholesterol for the surface and the most part of cholesterol esters + triglycerides for the core) have been related to alterations in the tertiary structure of the protein part (apoprotein B), to the binding to receptors, and to the oxidative resistance of LDL,[3] whereas the organisation of the LDL lipid core has been found to be a major parameter influencing oxidative resistance.[6] These considerations underscore the need to more thoroughly study factors—diet excepted—potentially affecting the composition and the oxidation response of LDL. One of these factors—namely the type of LDL preparation—is of real interest for two particular points: (1) cholesterol and triglyceride contents are already thought to depend on the preparation procedure;[7,8] and (2) LDL properties found by different groups are frequently compared without taking this factor adequately into account.

There is a general agreement on the role played by oxidized LDL (ox-LDL) in atherogenesis.[9,10] The corollary is that antioxidants could prevent the atherogenic process. We have shown that dietary supplementation with red wine phenolic compounds led to a significant Vitamin E enrichment of LDL without a significant increase in LDL resistance to *in vitro* Cu^{2+}-oxidation.[11,12] Even though Vitamin E does contribute to the LDL resistance to oxidation, other factors appear to be of great importance.[13–17] As it is the case for well-known plasma components (Vitamin C, urate, glutathione, etc.), albumin in its native form has been reported to have a LDL antioxidant protective effect.[18–20] By contrast, caeruloplasmin, a protein known for binding the redox-active metal ion Cu^{2+} (which is recognized as an antioxidant property by virtue of copper chelation), has been shown to have a pro-oxidant activity under some conditions,[21,22] whereas there are contradictory results on the role of LDL-associated phospholipase A_2[23–25] (also called PAF-acetylhydrolase—PAF-AH—which is thought to be the same enzyme).[26]

Since the LDL resistance to oxidation depends on intrinsic (lipophilic) and extrinsic (hydrophilic) antioxidants,[25,27] it is interesting to keep in mind that *in vivo* LDL is essentially oxidized in the subendothelial space, surrounded by many types of antioxidants including albumin.[28] Therefore, the albumin-associated LDL form could mimic *in vitro* the *in vivo* LDL environment much better than albumin-free LDL. This form is therefore of crucial interest in assessing oxidation resistance of LDL in the presence of other antioxidants.

Among the characteristics of LDL preparation, the UC time (t_{cent}), its impact on the biological composition—in particular that concerns with proteins—, and the oxidation resistance of LDL are poorly documented. The aim of this study was to investigate the interrelationships between oxidation, associated proteins (albumin, caeruloplasmin and PAF-AH), intrinsic lipids and the preparative UC used for isolation of LDL.

MATERIALS AND METHODS

Subjects

Plasmas were obtained from human normolipidemic subjects, either from 125 healthy men (20–45 years old), participating in a study submitted to the Ethics Committee of University of Montpellier for which they have given informed, written consent, or from patients undergoing plasmapheresis session in the Hematology Department of Lapeyronie Hospital (Montpellier, France) according to a procedure previously described.[29,30] In this last case, the only inclusion rule was that the LDL oxidation parameters from plasmapheresis patients were not statistically different from that of the aforementioned healthy subjects. Healthy subjects were moderate drinkers and nonsmokers, and none was receiving any pharmacological treatment.

For healthy subjects, blood samples were drawn by venepuncture on 1 g/l-EDTA after an overnight fasting. Plasma lipid characteristics were as follows: total cholesterol (TC) = 3.92 ± 0.07 mmol/l, triglycerides (TG) = 0.75 ± 0.02 mmol/l, phospholipids (PL) = 2.25 ± 0.03 mmol/l, apoA-1 = 1.30 ± 0.02 g/l, apoB = 0.74 ± 0.01 g/l. Plasmas from plasmapheresis patients were collected from plasmapheresis bags and added with 0.2 g/l-EDTA.

The influence of the UC protocol upon the LDL composition, albumin association, PAF-AH activity and characteristics of the Cu^{2+}-oxidation resistance were carried out *in vitro* with plasmas from plasmapheresis patients, whereas the main determinants of the LDL oxidation resistance—in the case of choosing the UC protocol keeping albumin

associated to LDL—were investigated in the healthy subjects.

LDL Isolation

LDL was isolated by sequential UC[31] in a TFT 65-13 rotor (Kontron Instruments SA, Montigny Le Bretonneux, France), with density adjustments using potassium bromide. Two isolation procedures were used.

High Speed-isolation Procedure (HS)

Plasma was submitted to sequential UC at the density of 1.0063, 1.0063–1.019 and 1.019–1.063 g/ml for obtaining triglyceride rich lipoproteins (TRL, essentially composed of very low density lipoprotein (VLDL) in the fasting conditions), intermediate low density lipoprotein (IDL) and LDL, respectively. For TRL and IDL, tubes span at 65,000 rpm (300,000 *g*) for 4 h per run. For LDL, the same speed was used, but for either 4 h (LDL·HS-4 h), 6 h (LDL·HS-6 h), 8 h (LDL·HS-8 h) or 16 h (LDL·HS-16 h) per run.

Low Speed-isolation Procedure (LS)

TRL+IDL were first isolated at 40,000 rpm (110,000 *g*) for 20 h per run. LDL was then isolated at 110,000 *g* for 24 h (LDL·LS-24 h) per run.

LDL from fresh plasma was frozen in the presence of 10% sucrose and stored at −80°C until utilization.[32] LDL was thawed at room temperature just before using, extensively dialyzed three times at 4°C (in the dark) for 24 h against a deoxygenated 10 mmol/l phosphate-buffered saline (PBS), pH 7.4, containing 150 mmol/l NaCl and 10 μmol/l diethylenetriamine pentacetic acid (DTPA). LDL was then sterilized through a 0.22 μm filter (Millex-GS, Millipore, Saint Qentin-Yvelines, France). We had previously verified that fresh LDL and LDL stored with 10% sucrose for at least 3 months at −80°C exhibited the same oxidation characteristics.[12] LDL from frozen plasma with 0.6% sucrose[33] was also used. In this last case, LDL was used immediately after isolation. LDL from fresh and frozen plasmas were also verified to exhibit the same oxidation characteristics.

LDL Analysis

Apoprotein B and A-1 concentrations were determined by immunonephelometry using a Turbitimer apparatus (Behring, Rueil-Malmaison, France). LDL concentration was assimilated to that of apoB.[34] The protein content of LDL was assayed using the Markwell's modification of the Lowry's method,[35] with using bovine serum albumin (BSA) as a standard. A coefficient of 0.86 was applied to the protein content in order to adjust for differences between BSA and human apoB.[3] TC, TG and PL were measured by automatized enzymatic methods proposed by Bio-Merieux (Marcy-l'Etoile, France). TC and PL were also measured by chemical procedures.[36,37] A calibration serum (Calimat; Bio-Merieux SA, Marcy l'Etoile, France) was used as a standard. Vitamin E and ubiquinol were simultaneously measured by means of an electrochemical detector after HPLC separation according to a procedure previously described for Vitamin E.[38] After lipid extraction and separation by thin layer chromatography, LDL fatty acids (FA) were transesterified and analyzed according to an usual procedure.[39] The FA composition allowed us to eventually calculate the saturated FA+monounsaturated FA to polyunsaturated FA (SFA+MUFA/PUFA) ratio.

Chromatography

LDL was dialyzed overnight at 4°C against 0.15 mol/l NaCl, adjusted to pH 6.0 with 1N HCl, and then loaded on a Sephacryl-400, 0.5 × 40 cm column, on the one hand, and on a blue Sepharose 6FF, 0.5 × 40 cm column, on the other, after previous equilibration (both gels from Amersham Pharmacia Biotech, Uppsala, Sweden). The column was then washed with 0.15 mol/l NaCl, adjusted to pH 7.5 with 1N NaOH, until the 280 nm absorbance of the eluate was stabilized at the baseline value, and then eluted with the 50 mmol/l Tris buffer solution, pH 8.0, containing NaCl (0.15, 0.5 and 1.0 mol/l, each time until the absorbance of the eluate at 280 nm was stabilized). Fractions were collected and concentrated by ultrafiltration on centrifugal filter devices with YM30 membranes (Amicon, Millipore, Saint Quentin en Yvelines, France) and dialysed overnight against 50 mmol/l Tris buffer solution, pH 8.0.

Electrophoresis

Aliquots (2–3 μl) of LDL were submitted to agarose gel film electrophoresis using the two following procedures depending on the LDL constituents: (1) with a lipoprotein electrophoresis kit, P/N 655910, using the Paragon electrophoresis system (Beckman Instruments, Fullerton, CA) and stained for lipid with Sudan black B; (2) with a Hydragel Protein kit, using an automatized electrophoresis system Hydrasys (Sebia, Issy-les-Moulineaux, France) and stained for protein with amidoschwarz. Aliquots (50 μg of protein/well) of LDL were also submitted to a polyacrylamide gradient (2–13%) gel electrophoresis with a set of standard proteins and stained with Coomassie blue or a silver staining kit protein (Pharmacia Biotech, Orsay, France). Albumin and

PAF-AH were identified by immunoblotting and immunodetection by using standard protocols and specific antibodies: a mouse monoclonal antibody against human albumin (clone HSA-11, Sigma, Saint Quentin Fallavier, France), and a rabbit polyclonal antibody against human PAF-AH (SPI Bio, Massy, France), revealed with polyclonal peroxidase-labeled anti-mouse and anti-rabbit antibodies from goat (Life Technology, Gibco BRL, Cergy-Pontoise, France), respectively.

Assessment of Associated Albumin

The total protein content (TProt) and the apoB content of LDL were significantly different depending on the UC procedures. The molar ratio of human serum albumin to apoB (HSA/apoB) was assessed as follows: [(TProt (g/l) − apoB (g/l))/0.069]/[apoB (μmol/l)].

LDL Resistance to Oxidation

We measured the formation of conjugated dienes (CD), due to the 5 μmol/l copper chloride-mediated peroxidation of a LDL solution (0.1 μmol/l apoB). CD formation is indicative of the early step of the polyunsaturated fatty acid peroxidation. The LDL solution was prepared by dilution in air-saturated PBS without DTPA at 37°C. The DTPA concentration after dilution was <0.50 μmol/l, which has previously been verified to be without influence on the Cu^{2+}-CD production.[12] Continuous monitoring was carried out at 234 nm using a Kontron (Uvikon 930) spectrophotometer equipped with a 12 position automated sample changer (Kontron Instruments SA, Montigny Le Bretonneux, France). We assessed the lag time, the maximal oxidation rate and the maximal level of the CD production[40] with the Cu^{2+}/apoB molar ratio of 50/1.[27,33] Oxidation rate was expressed as mol of CD formed per mol apoB per min (mol/mol/min) and maximal level of the CD production was expressed as mol of CD formed per mol apoB (mol/mol).

The protein effects on the *in vitro* LDL oxidation were tested after incubation in the presence of human serum albumin (HSA: essentially fatty acid- and globin-free) or nonproteolytically degraded caeruloplasmin (both from Sigma, Saint Quentin Fallavier, France) for 1 h at 37°C. LDL was then submitted to Cu^{2+}-oxidation immediately or after an UC in the HS-4 h condition and subsequent dialysis.

LDL-PLA_2 (PAF-AH) Activity and LysoPCho Production

The PAF-AH activity was assessed using the fluorescent probe 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4- yl) amino) hexanoyl-1-hexa-decanoyl-*sn*-glycero-3-phosphocholine (NBD-C6-HPC; Interchim, Montluçon, France) as a substrate.[41] The fluorescence production was compared with that obtained with NBD-hexanoic acid (NBD-X; Interchim, Montluçon, France).[42] The PAF-AH inhibition was estimated by incubating LDL (0.1 μmol/l) with diethyl *p*-nitrophenyl phosphate (1 mmol/l) (DENP; Sigma, Saint Quentin Fallavier, France) prior to Cu^{2+}-oxidation. The lysophosphatidyl choline (lysoPCho) production was determined by HPLC using a light diffusion detector (DDL 21, Eurosep Instrument, Cergy-Pontoise, France) equipped with a DOS Chemstation, after LDL lipid extraction as previously described.[42]

Statistics

All results are presented as means ± SEM. The statistical significance of results was determined by using ANOVA analysis with the Statview 4-5 Software (Alsyd, Meylan, France). The correlation coefficients in univariate and multiple regression analyses were compared with a test of slope (*F*-test). $P < 0.05$ was considered statistically significant.

RESULTS

Lipoprotein Composition

The weight composition of the following lipoproteins: VLDL·HS-4 h, IDL.HS-4 h, LDL·HS-4 h and LDL·LS-24 h, were characterized by a TG/TC ratio of 1.9 ± 0.3, 0.8 ± 0.3, 0.33 ± 0.06 and 0.22 ± 0.02, respectively. In LDL, apoB, TC and TG+PL were unaffected when the two procedures were compared (i.e. LDL·HS-4 h to LDL·LS-24 h), whereas TG was higher and PL was lower in LDL·HS-4 h than in LDL·LS-24 h, as accounted for by a TG/PL mass ratio of 0.63 ± 0.13 and 0.34 ± 0.01, respectively.

Table I goes into details as regards the LDL composition in relation with the five UC procedures used. Significant differences were observed between short centrifugation time (t_{cent})—i.e. HS-4 h—and long t_{cent}—i.e. HS-6 h, HS-8 h or HS-16 h—. There was a decrease in TG, no modification in TC and a decrease in TG/TC molar ratio (from 0.3 to 0.14). On the other hand, TProt/apoB weight ratio progressively decreased from 3.96 to 1.01 when LDL·HS-4 h, LDL·HS-6 h, LDL·HS-8 h, and LDL·HS-16 h were successively examined. The value of 0.89 in LDL·LS-24 h was surprising and will be discussed. There was no significant variation in the molar ratios TC/apoB, PL/apoB and Vitamin E/apoB. No significant difference was observed in FA composition when expressed as the FA to apoB molar ratio (data not shown). In conclusion, only TG and TProt

TABLE I Characteristics of LDL depending on the ultracentrifugation procedure. (Results are given as the mean ± SEM; *statistical significance vs HS-4 h: $P < 0.05$; TProt: total proteins; HSA: human serum albumin; vit E: Vitamin E; TC: total cholesterol; TG: triglycerides; PL: phospholipids)

Centrifugation procedure	TProt/apoB (g/g)	HSA/apoB (mol/mol)	vit E/apoB (mmol/mol)	TC/apoB (mol/mol)	TG/apoB (mol/mol)	PL/apoB (mol/mol)
HS-4 h	3.96 ± 0.91	23.5 ± 6.5	7.75 ± 1.21	1873.0 ± 196.4	560.4 ± 197.1	854.2 ± 117.6
HS-6 h	1.56 ± 0.35*	5.57 ± 2.50*	7.10 ± 1.00	2049.7 ± 160.8	360.4 ± 80.4*	742.9 ± 77.6
HS-8 h	0.97 ± 0.02*	0.03 ± 0.03*	7.32 ± 1.40	2389.9 ± 250.3	359.7 ± 85.0*	844.3 ± 75.9
HS-16 h	1.01 ± 0.07*	0.43 ± 0.76*	7.40 ± 1.18	2166.4 ± 223.5	360.7 ± 63.1*	809.4 ± 56.1
LS-24 h	0.89 ± 0.02*	0*	9.76 ± 3.61	2109.1 ± 168.8	299.1 ± 8.2*	975.7 ± 9.1

were found to be affected by UC procedure, and they were affected in the same way.

Agarose gel electrophoresis with lipid staining (Fig. 1A) illustrates that LDL, whatever the isolation procedure used, migrated as a single band with β-mobility and clearly shows that there was no band with preβ- and α-mobilities, providing evidence for the absence of contamination by other lipoproteins. Protein staining (Fig. 1B), however, demonstrates that an albumin fraction was present in LDL·HS-4 h amounting to 42.3 ± 11.6% of proteins contrasting with the absence of this fraction in all other LDL preparations. The protein composition of LDL·HS-4 h was characterized by a HSA/apoB molar ratio of 23.5 ± 6.5. Figure 1B also shows trace amounts of proteins in this type of LDL, with α1- and α2-mobilities.

Eluting LDL·HS-4 h onto Sephacryl-400 column did not succeed in dissociating albumin from LDL (not shown). By contrast, dissociation was successful when blue Sepharose 6FF was used (Fig. 2A) as highlighted by nondenaturing gradient polyacrylamide gel electrophoresis (see peaks of albumin numbers 7 and 8, Fig. 2B). Albumin identification was confirmed by immunodetection (not shown). However, no separation of PAF-AH was carried out as supported by immunodetection procedure. In order to confirm the presence of PAF-AH in LDL, we

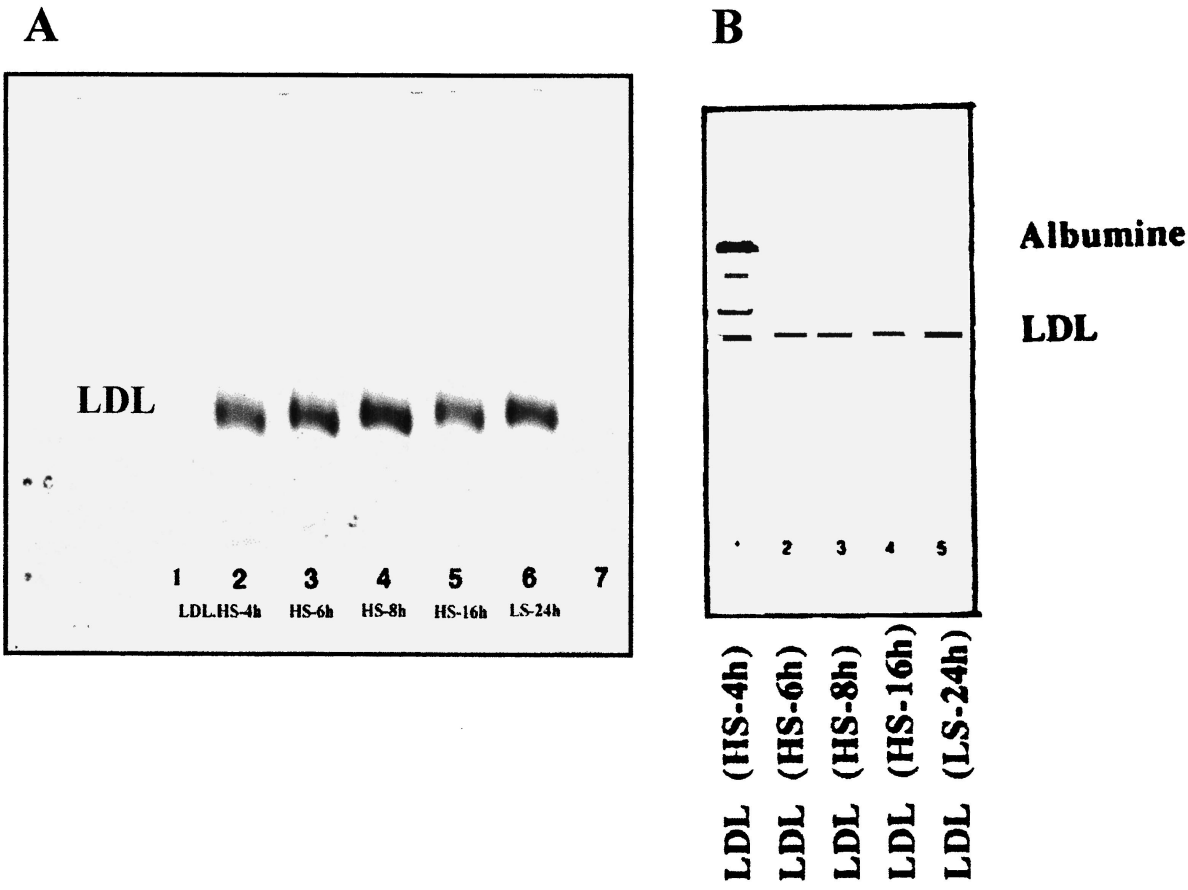

FIGURE 1 LDL electrophoretic analyses on agarose gel films depending on the five ultracentrifugation procedures used. Electrophoresis was performed in 1% agarose gel with lipoprotein electrophoresis systems and stained for lipids with Sudan black B (A) and proteins with amidoschwarz (B). Five bands were present in LDL·HS-4 h: albumin, α1-, α2-, β- and γ-mobility (42.3 ± 11.6, 2.5 ± 1.0, 9.0 ± 1.4, 42.5 ± 10.0, 3.7 ± 2.2%, respectively, calculated as the relative area of each band as compared to the total band area).

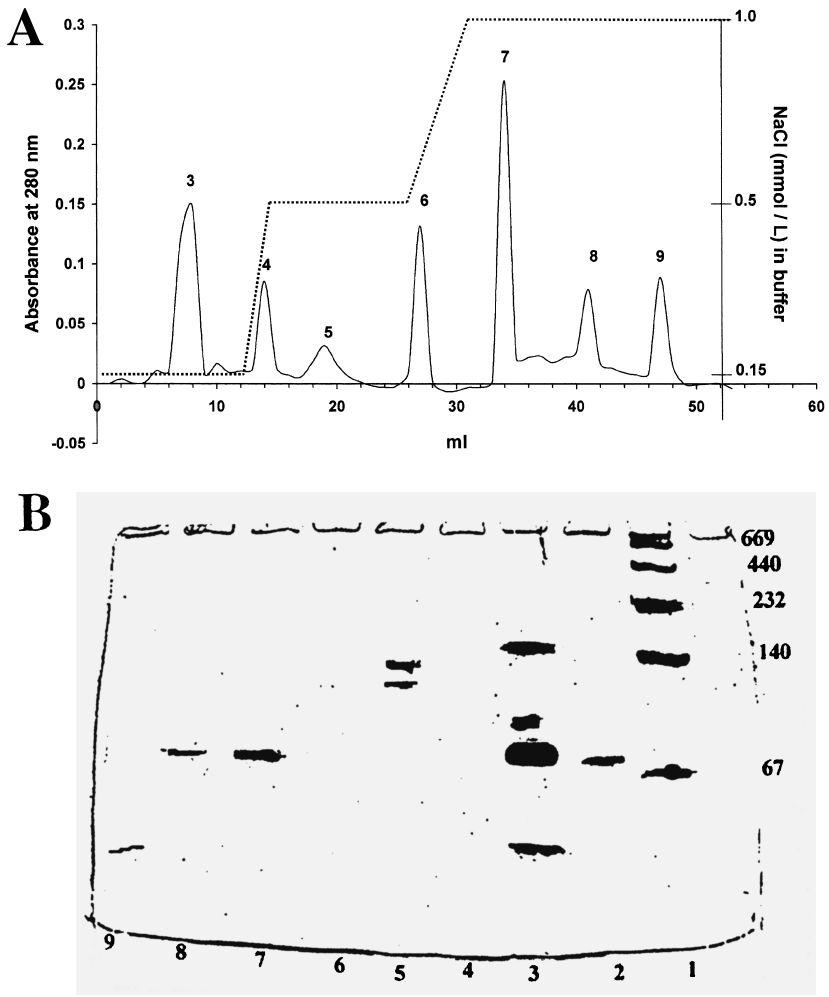

FIGURE 2 Dissociation of albumin from LDL·HS-4 h on a blue Sepharose 6FF column. Elution profile was obtained by applying a 50 mmol/l Tris buffer pH 8.0 containing a discontinuous gradient from 0.15 to 1 mol/l NaCl (A). The different protein fractions collected were then examined by a nondenaturing polyacrylamide 2–13% gradient gel electrophoresis (B). A set of marker proteins (from the top to the bottom: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; and albumin, 67 kDa) was applied to well 1. The parent LDL·HS-4 h preparation from which the subfractions were derived was applied to well 2. The seven subfractions collected in (A) were then successively applied to wells 3–9. Gel was stained with a silver staining kit protein.

carried out a denaturing gradient polyacrylamide gel electrophoresis (Fig. 3). It clearly appears that there were two bands with anti-PAF-AH activity at 60 kDa (the less intensive peak) and at 56 kDa (the most intensive one).

Albumin–LDL Association

In order to confirm that the albumin–LDL association we presently found was dependent on the intrinsic characteristics of LDL in plasma (and did not result from relative concentrations in LDL and albumin at any time all along the isolation procedure), the two following incubations were carried out for 1 h at 37°C: aliquots of LDL·HS-4 h at the same apoB concentration in the presence of increasing amounts of HSA; and aliquots of HSA at the same concentration in the presence of increasing amounts of LDL·HS-4 h. LDL·HS-4 h was then re-isolated by using the same UC procedure. The TProt/apoB weight ratio obtained after re-isolation was measured and plotted against the TProt/apoB ratio present in the incubation medium (Fig. 4). It is clearly found that in both experiments weight ratio remained constant and similar to the values in LDL·HS-4 h before incubation (3.44 ± 0.15 and 1.12 ± 0.07, respectively, corresponding to an HSA/apoB molar ratio of 19.0 ± 1.2 and 1.2 ± 0.4), showing that the albumin–LDL association in LDL·HS-4 h was not affected significantly by the relative concentration in LDL and free albumin.

Consequence of the UC Procedure on the PAF-AH Activity

The specific PAF-AH activity was expressed either per gram of apoB or per gram of TProt (Fig. 5). Whereas the activity per apoB appeared to be constant regardless of the TProt/apoB weight ratio, the activity per TProt

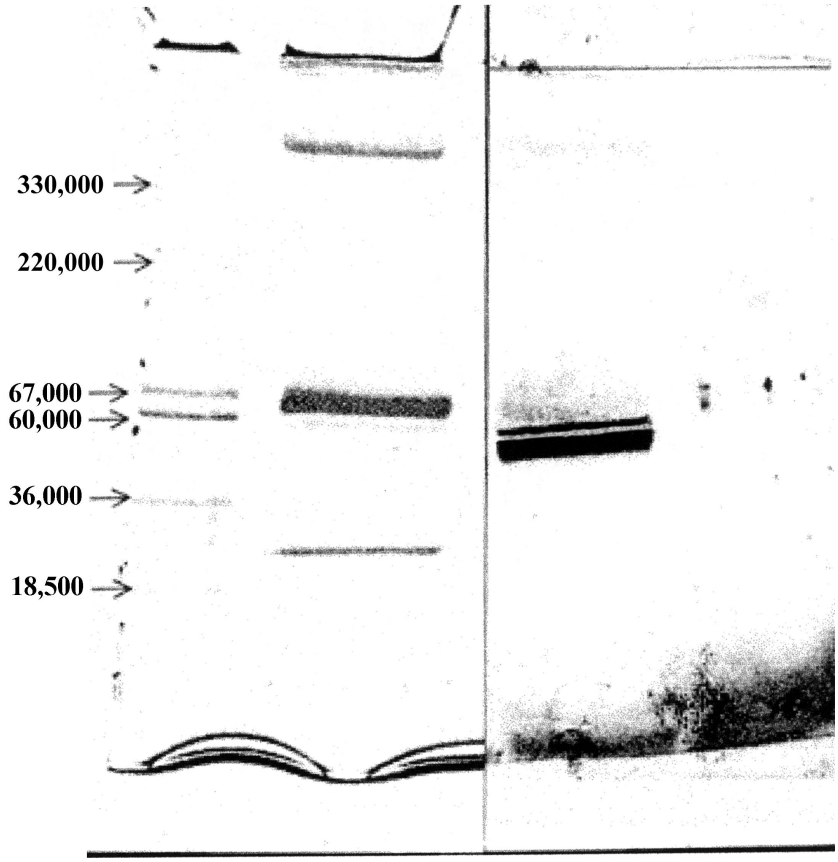

FIGURE 3 SDS–polyacrylamide 2–13% gradient gel electrophoresis of the LDL·HS-4 h proteins. A set of marker proteins (from the top to the bottom: thyroglobulin, 330 kDa; albumin, 67 kDa; catalase, 60 kDa; lactate dehydrogenase, 36 kDa; and ferritin, 18.5 kDa) was applied to wells 1 and 4. 50 μg of LDL protein was applied to wells 2 and 3. Lanes 1 and 2 were stained with Coomassie blue. Lanes 3 and 4 were identified by immunoblotting with a rabbit polyclonal antibody against human PAF-AH and immunodetection with an anti-rabbit polyclonal peroxidase-labeled antibody from goat.

increased with decreasing values of TProt/apoB. The relationship was of the form $Y = k/X$ with k equal to the specific activity related to apoB and $X =$ (non apoB + apoB)/apoB, leading to the experimental equation: $Y = 458[1 -$ non apoB/(non apoB + apoB)], which shows that the only variable was apoB. It can be drawn that activity was practically dependent on the particle concentration and independent of the non apoB proteins. These results also clearly show that PAF-AH activity per particle was unaffected with regard to the UC procedure. The specific PAF-AH activity expressed as mole of product per mole of apoB per min is 104.2 ± 0.9 mol/mol/min ($n = 8$).

Cu^{2+}-mediated Oxidation of LDL

We firstly verified that, in the presence or absence of HSA, the difference between oxidized and non-oxidized LDL spectra showed a peak at 234 nm as expected from the presence of CD in ox-LDL, showing that HSA did not interfere with the CD determination (data not shown).

Lag Time and Characteristics of CD Formations

As shown in Table II, a higher lag time and a lower maximal rate of CD production were found with albumin-associated LDL (LDL·HS-4 h) submitted to Cu^{2+}-oxidation. In contrast, the maximal CD formation was unaffected. DENP inhibition of PAF-AH (Fig. 6A) slightly reduced the lag time and the maximal rate of CD formation, did not modified significantly the Vitamin E consumption, and resulted expectedly in the complete loss of lysoPCho production. When the effect of DENP was examined using albumin-free LDL instead of albumin-associated LDL we found an increase in lag time ($P < 0.05$; $n = 4$) whereas the diminishing effect on the maximal rate of CD production which was found with albumin remained without albumin. (Fig. 6B).

Instead of comparing the lag time of albumin-associated LDL to albumin-free LDL, we decided to study the effect of adding albumin to albumin-free LDL. Figure 7 shows that albumin did increase the lag time of CD production which became similar to that of LDL·HS-4 h. Lag time was found to be highly correlated with albumin ($r = 0.88$; $n = 19$;

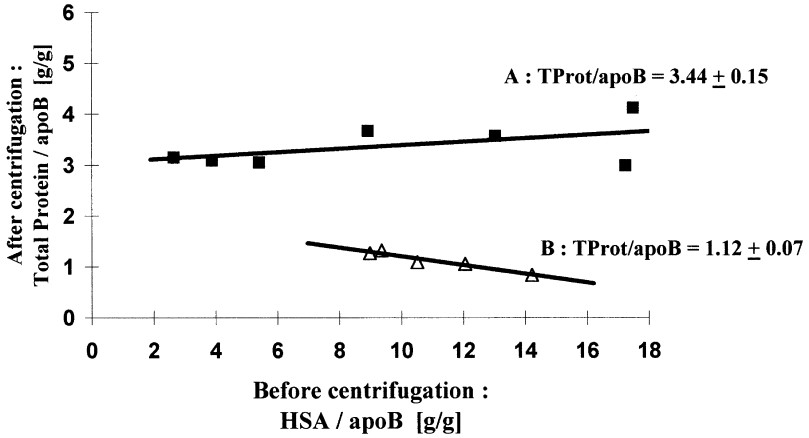

FIGURE 4 Effect of high speed/short time (4 h) ultracentrifugation procedure on albumin–LDL association. Aliquots of LDL·HS-4 h (apoB=2.11 ± 0.4 μmol/l; TProt/apoB=2.64 g/g) were incubated in the presence of increasing amounts of HSA (0–13 g/l) for 1 h at 37°C (■); aliquots of HSA (13 g/l) were incubated in the presence of increasing amounts of LDL·HS-4 h (apoB=1.56–2.62 μmol/l; TProt/apoB=1.29 g/g) in the same conditions of temperature and duration (△). LDL was then re-isolated by using the HS-4 h ultracentrifugation procedure. The final TProt/apoB ratios are reported for each correlation straight line.

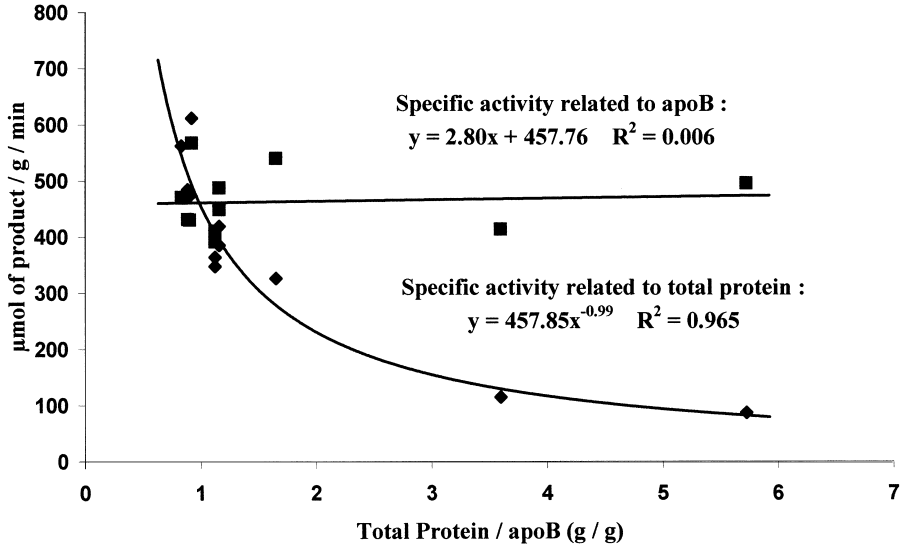

FIGURE 5 Effect of ultracentrifugation procedure on the specific activity of PAF-AH. Specific activity of LDL-associated phospholipase A_2 (PAF-AH) expressed per gram apoB (■) or TProt (◆) was measured using the fluorescent probe NBD-C6-HPC (0.58 μmol/l, ε_{466}=21,100 l/mol/cm) as a substrate. Assays were performed at room temperature in Tris buffered saline (TBS: 10 mmol/l Tris–HCl, pH 7.4, 0.1 mol/l KCl). NBD-C6-HPC and LDL (0.04 nmol) were added to TBS (3 ml) and the fluorescence production (excitation at 466 nm; emission at 534 nm) was measured at room temperature with NBD-X used as a standard.

TABLE II Oxidizability parameters of LDL depending on the ultracentrifugation procedure. (Results are given as the mean ± SEM; *statistical significance vs HS-4 h: $P < 0.05$; CD: conjugated dienes)

Centrifugation procedure	Lag time (%)	CD/apoB (mol/mol)	Maximal rate (mol/mol/min)
HS-4 h	100[†]	359.0 ± 13.6	5.1 ± 1.5
HS-6 h	64.5 ± 0.5*	349.5 ± 7.0	8.9 ± 0.9*
HS-8 h	62.0 ± 2.9*	345.7 ± 4.3	8.9 ± 0.9*
HS-16 h	64.9 ± 4.7*	343.8 ± 6.0	9.1 ± 0.9*
LS-24 h	55.2 ± 3.4*	357.8 ± 8.2	8.8 ± 0.7*

[†] Lag time of LDL·HS-4 h=134.1 ± 22.6 min.

$P < 0.01$) (data not shown). Lag time was approximately doubled for 20 molecules of albumin per particle of LDL. Adding caeruloplasmin (0–0.25 μmol/l) decreased the lag time in a dose dependent manner and without significant differences between albumin-free and albumin-associated LDL, showing that albumin was without effect on the caeruloplasmin pro-oxidant activity (data not shown).

The lag time of the Cu^{2+}-oxidation was also assessed in LDL·HS-4 h (see composition and

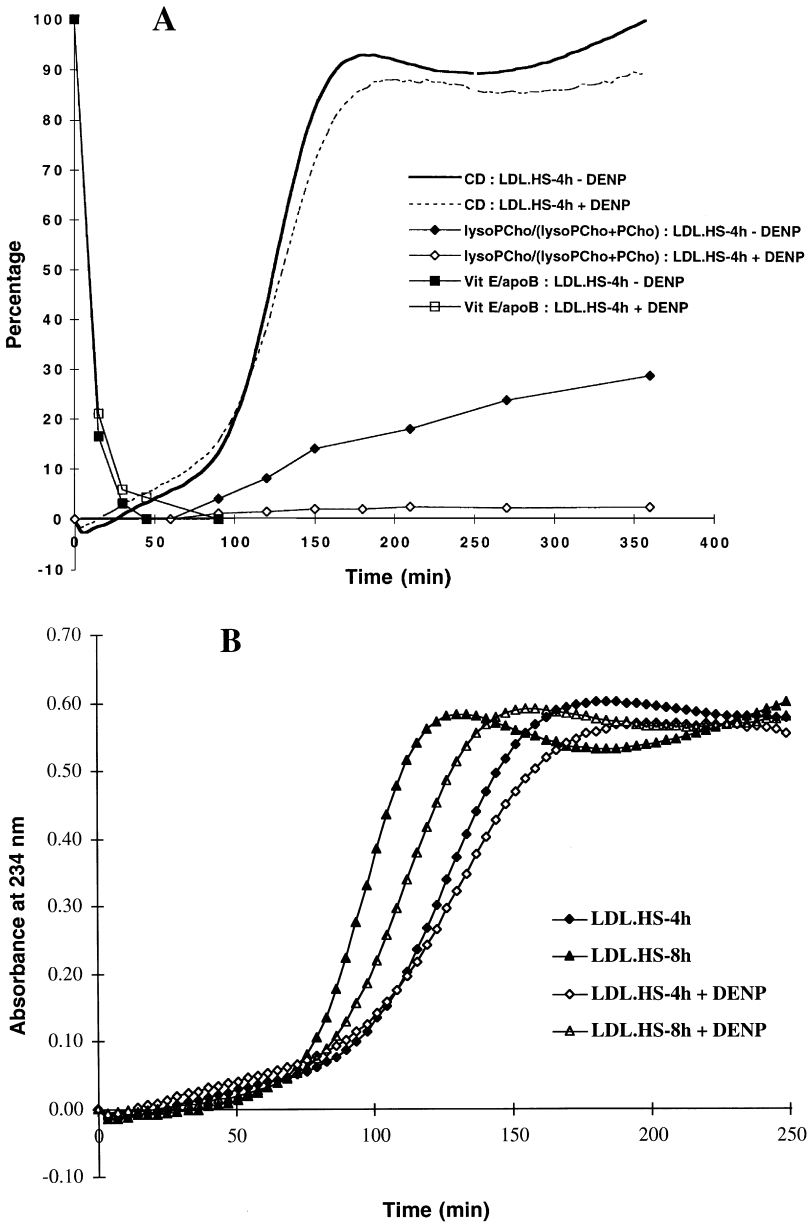

FIGURE 6 Effect of DENP on LDL Cu^{2+}-oxidation. Time course of CD production, Vitamin E consumption and lysoPCho production during Cu^{2+}-oxidation of LDL·HS-4 h (A). LDL was preincubated without or with DENP (10 mmol/l) for 1 h at 37°C under N_2 and then diluted at 0.1 μmol/l in PBS buffer for Cu^{2+} ($CuCl_2$ = 5 μmol/l)-oxidation. Kinetics of CD production were recorded at 37°C by continuous monitoring of the 234 nm-absorbance against the reference cuvette containing LDL supplemented with 10 μmol/l-EDTA and 0.2 μmol/l-butylated hydroxytoluene to prevent oxidation. Results are presented as percentages of the initial value for Vitamin E/apoB molar ratio, the maximal value for CD production and the lysoPCho/(lysoPCho + PCho) molar ratios. Comparison of the effect of DENP on LDL·HS-4 h and LDL·HS-8 h oxidation (conditions of preincubation and oxidation are as above) (B). LDL·HS-4 h: TProt/apoB = 1.95 g/g, lag time = 88.5 min (without DENP) and 86.4 min (with DENP) and LDL·HS-8 h: albumin-free LDL, lag time = 74.1 min (without DENP) and 84.3 min (with DENP).

oxidation characteristics in Table III) from healthy volunteers. Table IV shows the correlations between the protein content or lag time, on the one hand, and data regarding lipid constituents, on the other. Albumin content was the most highly positively correlated with lag time and was also positively correlated with TG content and the TG/TC ratio. We found, therefore, the albumin protection against oxidation was previously observed using different centrifugation procedures or different conditions for albumin incubation. It is worth mentioning that there was no correlation between the oxidation lag time and either the Vitamin E content or the ubiquinol content of LDL. However, Vitamin E normalization by TC or, to a lesser extent (TC + TG), allowed us to positively correlate Vitamin E with lag time (which was not true for ubiquinol). In order to gain further insight into the characteristics of LDL resistance to Cu^{2+}-oxidation, multiple regression analyses were

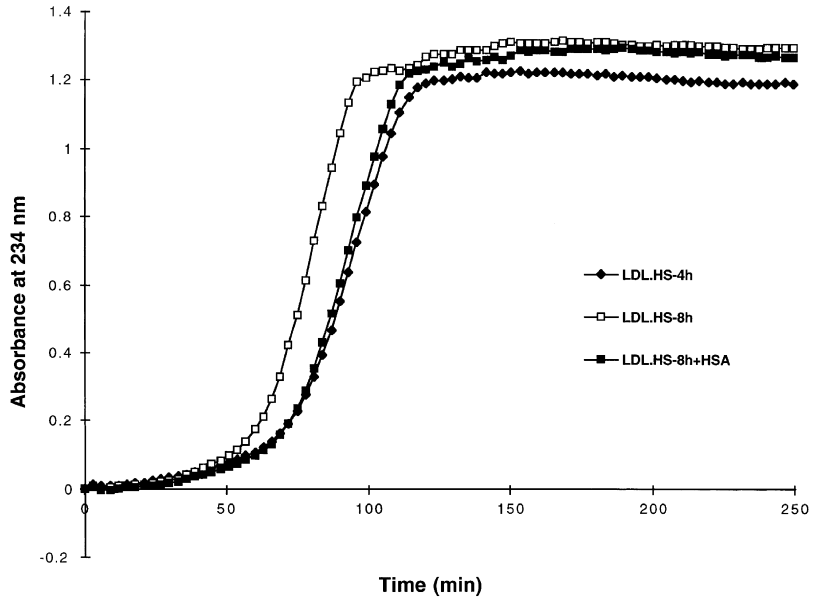

FIGURE 7 Effect of albumin on LDL Cu^{2+}-oxidation. LDL·HS-4 h (apoB=1 μmol/l) was preincubated without albumin and LDL·HS-8 h (apoB=1 μmol/l) was preincubated without or with albumin prior to Cu^{2+}-oxidation (conditions of preincubation and oxidation are as in Fig. 6A). (LDL·HS-4 h: TProt/apoB=2.04 g/g, lag time=68.7 min; LDL·HS-8 h: albumin-free LDL, lag time=60 min and LDL·HS-8 h supplemented with HSA: HSA/apoB=1.5 g/g, lag time=69.8 min).

TABLE III LDL·HS-4 h characteristics (composition and oxidation) originating from healthy subjects. (Tprot: total proteins; HSA: human serum albumin; TC: total cholesterol; CE: cholesterol esters; C: free cholesterol; TG: triglycerides; PL: phospholipids; bisal C: number of bis allylic carbons in polyunsaturated fatty acids)

	Means ± SEM (min − max)		Number
Lag time (min)	47.8 ± 1.3 (24.0 − 100)		122
Oxidation rate*	9.68 ± 0.61		122
	Weight percentage	Mol/mol	
Tprot	29.0 ± 0.9 (20.0 − 78.1)		125
HSA	15.8 ± 1.1 (0 − 68.5)		125
HSA/apoB		9.45 ± 0.88 (0 − 58.0)	125
α-Tocopherol/apoB		8.51 ± 0.24 (3.18 − 14.01)	125
α-Tocopherol/TC		$[3.37 \pm 0.12\ (1.25 - 8.85)] \times 10^{-3}$	125
α-Tocopherol/(TC+TG)		$[3.03 \pm 0.10\ (1.21 - 7.80)] \times 10^{-3}$	125
α-Tocopherol/bisal C		$[3.09 \pm 0.11\ (0.96 - 6.72)] \times 10^{-3}$	125
Ubiquinol/apoB		0.4 ± 0.03 (0.03 − 2.04)	96
Ubiquinol/TC		$[0.15 \pm 0.11\ (0.01 - 0.71)] \times 10^{-3}$	96
Ubiquinol/(TC+TG)		$[0.14 \pm 0.10\ (0.01 - 0.67)] \times 10^{-3}$	96
TC	43.3 ± 0.7 (13.0 − 56.5)	2638.5 ± 46.8 (1306.6 − 4041.9)	125
CE/TC		0.71 ± 0.02 (0.50 − 0.97)	32
TG	6.7 ± 0.2 (2.8 − 15.4)	272.3 ± 10.2 (130.5 − 661.1)	125
TG/TC	0.16 ± 0.01 (0.08 − 0.58)	0.106 ± 0.005 (0.053 − 0.376)	125
PL	18.0 ± 0.3 (1.9 − 33.3)	808.2 ± 14.2 (67.1 − 1380.3)	125
PL/C†		1.07 ± 0.02 (0.14 − 2.14)	125

*Mol of DC per mol of apoB per min.
†Estimated value with C/TC ≠ 0.29 mol/mol.

performed. Only HSA, TG and TC were significant predictors of the LDL resistance to oxidation, accounting for 29% of the total variance. The equation was:

$$\text{Lag time (min)} = 62.1 + 0.67(\text{HSA/apoB}) + 0.02(\text{TG/apoB}) - 0.01(\text{TC/apoB})$$

(with a multiple regression coefficient $r = 0.54$, $P < 0.0001$, $n = 121$).

Of interest is the fact that the most saturated lipid classes were TG, then PL, whereas CE was highly unsaturated, as accounted for by the (SFA+MUFA)/PUFA ratio of 12.6 ± 3.4, 3.2 ± 0.7, 0.6 ± 0.05, respectively.

Propagation Rate

Using LDL·HS-4 h, we first confirmed that the maximal rate of CD formation (which can be

assimilated to the propagation rate of chain lipid peroxidation) was maximal for Cu^{2+} concentrations superior to 5 μmol/l (for apoB=0.1 μmol/l).[27,33] Assuming that Cu^{2+} dependence was of the form of the Michaelis–Menten kinetics[27]

$$V = V_{max}[Cu^{2+}]/(K_M + [Cu^{2+}])$$

we then assessed K_M and V_{max} for albumin-free LDL in the presence of HSA or in the absence of HSA (Fig. 8A). The K_M was 3.1 and 1.2 μmol/l and the V_{max} was 9.7 and 14.6 mol/mol/min, respectively.

K_M and V_{max} were also calculated for different LDL preparations. Figure 8B shows that there was a positive correlation ($r = 0.94$; $P < 0.0001$; $n = 11$) between K_M and the TProt content of LDL, with an extrapolated value of K_M equal to 2.14 μmol/l for albumin-free LDL. By contrast, no significant variation of V_{max} was found with regard to TProt content. The mean V_{max} value was 13.2 ± 0.8 mol/mol/min. These results show that albumin has a diminishing effect on the Cu^{2+} affinity for LDL, as it was the case in Fig. 8A, and no effect on the maximal rate of CD production.

DISCUSSION

In vitro Cu^{2+}-oxidation of LDL has been frequently used to assess the effects of antioxidants on oxidized-LDL formation. Even though Vitamin E does contribute to the LDL resistance to oxidation, numerous studies have shown that LDL Vitamin E is not correlated with the LDL resistance to Cu^{2+}-oxidation in subjects consuming Vitamin E from food origin (i.e. in conditions of no Vitamin E supplementation).[12,13,17,43] By contrast, other observations have underscored that LDL-surrounding substances present in the plasma or the intercellular fluids are of great importance in protecting LDL against oxidation.[20,21,28]

Since LDL oxidation occurs in the subendothelial fluids of the vessel wall in the presence of albumin[28,44,45] all along the atherosclerotic process, the eventual association of albumin to LDL and the albumin effect on LDL oxidation are of pathophysiological interest. Some reports have already been concerned with the role of albumin added to LDL *in vitro*.[19,27] This issue has been approached here using another way. We decided to systematically show that, starting from the same plasma, LDL can be prepared with different lipid and protein compositions resulting from UC conditions (time and *g* number), with consequences as regards the LDL susceptibility to oxidation.

We presently established that the LDL resistance to Cu^{2+}-oxidation depended on the UC procedure used for LDL isolation. Short t_{cent} (4 h), even at high speed (300,000 *g*), led to LDL (LDL·HS-4 h) with enhanced amounts of TG and proteins and high oxidation resistance without any change in Vitamin E as compared to LDL prepared at higher t_{cent} (6, 8 and16 h). Among proteins, LDL-associated albumin was found to be profoundly affected by the UC procedure, since the highest molecule number of albumin per LDL particle was greater than 20 in LDL·HS-4 h whereas no albumin was found at higher t_{cent}. By contrast, PAF-AH, another protein linked to LDL, was found not to be affected. As regards the LDL-associated albumin found in LDL·HS-4 h, it is of interest to stress that the amount was only dependent on the inherent, plasma-occurring characteristics of LDL (see Fig. 4), strengthening the use of this type of LDL as representative of the lipoparticles which really occur in the plasma. In this respect, our results support that the LDL-associated albumin in

TABLE IV Correlation coefficients (*r*) between LDL·HS-4 h protein content (HSA/apoB molar ratio) or oxidation parameter (lag time) and LDL·HS-4 h lipid composition in human healthy subjects. (HSA: human serum albumin; TC: total cholesterol; C: free cholesterol; TG: triglycerides; PL: phospholipids; NS: not significant; statistical significance: *$P < 0.05$, **$P < 0.01$

Molar ratio	HSA/apoB		Lag time	
	r	*P*	*r*	*P*
HSA/apoB	–		+ 0.393	<0.0001**
α-Tocopherol/apoB	NS		+ 0.063	0.490
α-Tocopherol/TC	NS		+ 0.241	0.0078**
α-Tocopherol/(TC+TG)	NS		+ 0.204	0.025*
α-Tocopherol/C bisal	NS		− 0.102	0.270
Ubiquinol/apoB	NS		− 0.185	0.071
Ubiquinol/TC	NS		− 0.123	0.232
Ubiquinol/(TC+TG)	NS		− 0.128	0.214
TC/apoB	− 0.006	0.942	− 0.282	0.0017**
TG/apoB	+ 0.286	0.0013**	+ 0.239	0.0084**
TG/TC	+ 0.274	0.0021**	+ 0.330	0.0002**
PL/apoB	+ 0.147	0.103	+ 0.102	0.267

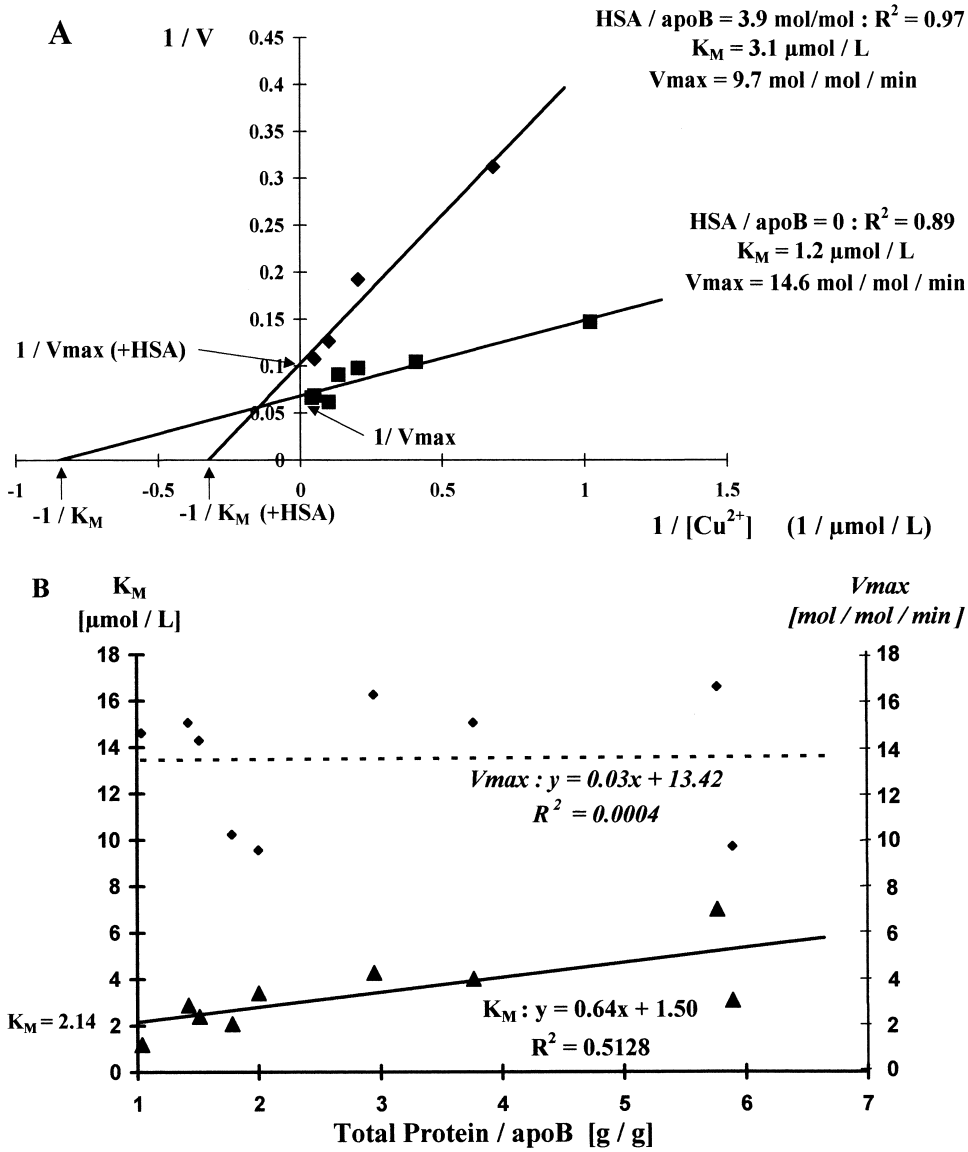

FIGURE 8 Effect of Cu^{2+} concentration on the oxidation propagation rate. Linear relationships were obtained by plotting $1/V$ versus $1/[Cu^{2+}]$ for LDL·HS-4 h (◆) and LDL·HS-8 h (■). The respective values for V_{max} and K_M were determined by using the linear regression (A). Linear relationship was also obtained by plotting the K_M values for different LDL·HS-4 h preparations versus protein composition expressed as the TProt/apoB weight ratio (B).

LDL·HS-4 h would be highly variable, depending on the donor. The determinants of this variability remain to be explored. In addition, it is likely from the present results that the albumin effect is explained (see Fig. 8A, B) by a decreased apparent affinity (higher K_M) of Cu^{2+} for its site of fixation in apoB.[46] This is in accordance with the fact that Cu^{2+} binding components are well known for having a pronounced effect on LDL susceptibility to Cu^{2+}-oxidation.[47–49] An interesting point is that the K_M and V_{max} values extrapolated to zero albumin were in complete agreement with those obtained by others with albumin-free LDL.[33,46]

The role of PAF-AH in general, and in LDL oxidation in particular appears to be not clear.[50–53] This is probably due to its dual action, since the enzyme decreases the pro-inflammatory substances such as PAF and oxidized phospholipids and increases the release of oxidized products from the LDL particle, on the one hand, and the formation of pro-atherogenic lysoPCho,[54] on the other. Interestingly, the inhibition experiments we presently carried out led to conclude that the pro-oxidant role of PAF-AH appeared only in the absence of albumin. In other words, albumin could play a role as a scavenger of oxidized products released by the enzyme.[25] This hypothesis is supported by the impairment of the maximal rate of CD formation by DENP, confirming the capacity of PAF-AH at releasing oxidized products such as

alkylperoxides known to participate in the propagation of lipid peroxidation. This also underlines the need for albumin in order to protect LDL against the oxidation due to PAF-AH activity. Contrasting with the complementary actions of albumin and PAF-AH, the pro-oxidant role of caeruloplasmin we presently showed appeared to take place regardless of the albumin amounts remaining associated to LDL. This suggests that albumin did not interfere with the pro-oxidant effect of caeruloplasmin and the histidine site involved in this effect.[55]

PAF-AH was presently found in the form of two bands with an apparent molecular mass of 60 and 56 kDa. This appears to be different from the molecular weight of 43 kDa previously found.[56,57] More recently, Tew *et al*.[26] found a molecular weight within the range of 43–67 kDa, and a deglycosylated enzyme of 47 kDa. However, a PAF-AH with a molecular weight of 60 kDa has also been described in human kidney.[58] Given that the present plasma was obtained from patients submitted to plasmapheresis sessions with a nephrotic dysfunction, it can be thought that the present molecular mass was due either to two glycosylated forms of the hepatocyte- and monocyte/macrophage-secreted enzyme,[59,60] or to peculiar forms possibly originating in other tissues (kidney in particular). This remains to be elucidated.

To conclude on the quality of the LDL prepared, increasing the t_{cent} results in a decrease in TG and albumin whereas the dissociation of PAF-AH from LDL is not possible in conditions for which albumin was dissociated, showing that this enzyme is more strongly associated to the lipoparticle. This confirms that a nondissociable form of the enzyme is present in LDL.[61,62]

Regarding the paradoxical value found inferior to one for the ratio TProt/apoB in Table II, it is noteworthy that this could be due to the overestimated apoB in conditions using the very long (24 h) t_{cent}, i.e. that producing the most delipidated LDL. This indeed could lead to apoB with unmasked epitope(s) as suggested by different approaches[63–65] then becoming able to respond to the polyclonal antibody used in the nephelometry assessment. It is worth mentioning that, by contrast, the ratio was close to one for t_{cent} values of 8–16 h.

LDL·HS-4 h from 125 healthy subjects was also treated to verify the influence of lipid, protein and antioxidant characteristics of LDL. Strikingly, we found that the most significant correlations took place between albumin and TG (see Table IV). This result has to be compared to that shown in Table I concerning the simultaneous decrease in albumin and TG due to the prolonged t_{cent}. The mean albumin content of LDL was about 10 mol/mol and the data confirm that this characteristic was highly variable. According to the equation accounting for the parameters playing a role in the LDL resistance to oxidation, albumin and TG (the highly saturated lipid class) were found to be the major determinants for oxidation resistance and TC (probably because of the highly unsaturated CE) was a determinant for oxidation sensitivity. By contrast, LDL oxidation resistance was confirmed not to be correlated with its intrinsic antioxidants (see the α-tocopherol/apoB and the ubiquinol/apoB molar ratios),[66,67] but to be positively correlated, after lipid normalization, with α-tocopherol/TC and to a lesser extent α-tocopherol/(TC+TG) molar ratios, contrasting with other results.[68,69] This may be explained partly by the negative correlation of TC with oxidation resistance we have just mentioned above. That the increased LDL resistance to oxidation in normolipidemic subjects took place with increased LDL-albumin and increased LDL-TG could cause some confusions between the respective role of TG and albumin in the oxidation resistance and suggests that the link between LDL size and oxidizability deserves to be more thoroughly explored.[70] In particular, it remains to elucidate as to whether the higher susceptibility to oxidation of the most dense LDL (i.e. the smallest particle) mentioned by others[43,71] can be explained either by reduced TG or by the loss of associated albumin. Let us simply suggest that LDL protein composition may account for some discrepancies between the oxidation resistance and the biochemical characteristics of LDL.[72,73]

In conclusion, both protein and lipid moieties are of prime importance in determining the LDL resistance to Cu^{2+}-oxidation, both clearly depending on the centrifugation procedure used for LDL isolation. We also specified the procedure leading to dissociate apoB from other proteins, which protein can be dissociated and the respective role of albumin and PAF-AH. This considerations are of great interest for assessing LDL resistance to oxidation in a physiological context, and have to be taken into account in evaluating the antioxidant capacity of substances through LDL oxidation tests.

Acknowledgements

We wish to thank Professor J.P. Cristol (Department of Biochemistry, Hospital Lapeyronie, Montpellier, France) for his valuable contribution, Dr C. Bouloux (Department of Hematology, Hospital Lapeyronie, Montpellier, France) for providing plasmapheresis bags used in this study and C. Lauret for her excellent technical assistance.

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