Low-density lipoprotein is the major carrier of lipid hydroperoxides in plasma

Relevance to determination of total plasma lipid hydroperoxide concentrations

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High-density lipoprotein (HDL) has been proposed as the principal carrier of hydroperoxides in plasma, based upon data gathered with an HPLC-chemiluminescence technique. To test this hypothesis we have measured total lipid hydroperoxides in native plasma using the ferrous oxidation in Xylenol Orange (FOX) assay and then fractionated plasma into very-low-density lipoprotein, low-density lipoprotein (LDL) and HDL fractions. Hydroperoxides were found to accumulate principally (more than 65%) in LDL, as judged by hydroperoxide content per amount of protein or cholesterol, or expressed as a proportion of total hydroperoxide in plasma. Plasma was also incubated at 37 °C in the presence and absence of 2,2′-azo-bis-(2-amidinopropane) hydrochloride (AAPH), an azo-initiator of lipid peroxidation. The majority of hydroperoxides generated in plasma were recovered in the LDL fraction. Furthermore, when isolated lipoproteins were subject to oxidation initiated by AAPH, very-low-density lipoprotein and LDL showed the greatest propensity for hydroperoxide accumulation, whereas HDL seemed relatively resistant. Estimates for plasma and LDL peroxidation based upon techniques which measure total lipid hydroperoxides suggest that levels of hydroperoxides in plasma and LDL are far higher than that those estimates generated by ostensibly more selective techniques. Higher levels of hydroperoxides in LDL than those reported by HPLC-chemiluminescence also seem in greater accordance with other available data concerning LDL oxidation.

INTRODUCTION

The peroxidation of lipoproteins is believed to play an important role in atherosclerosis [1,2]. First, aldehyde products of lipid peroxidation are believed to react with the amino groups of low-density lipoprotein (LDL), causing it to become 'modified' and prone to uptake by scavenger receptors [3,4]. Secondly, accumulation of oxidized phospholipids in the various fractions of lipoprotein may cause inappropriate, pathophysiological, responses within the cell types with which they come in contact. Precise measurement of lipid hydroperoxides would appear critical to the scrutiny of this oxidative stress hypothesis of atherosclerosis. There is, however, considerable disagreement concerning the levels of hydroperoxides in plasma, as well as with respect to their location.

Using comparable HPLC-chemiluminscence techniques, levels of plasma phospholipid hydroperoxide in healthy people have been reported to be undetectable or to lie in the range 10–500 nM [5–8]. Levels of cholesteryl ester (CE) hydroperoxides in normal plasma are reported to be as low as 3 nM [9] or as high as 920 nM [10]. HPLC-chemiluminescence estimates of the CE hydroperoxide content of purified LDL are variously reported to be 1 CE hydroperoxide molecule per 200 LDL particles [11], 1 per 2500 LDL particles [12] or 1 in every 8 LDL particles [13].

By contrast, techniques which measure total lipid hydroperoxides, rather than individual fractions using HPLC-chemiluminescence, suggest higher and consistent values for lipid hydroperoxides. Thus total lipid hydroperoxides in pooled plasma samples have been estimated to be 4.0 ± 1.7 μM [14] or in the range 2.1–4.6 μM ($n = 5$; mean = 3.1 μM) [15] by precise iodometric techniques. These values are in agreement with our own estimate for total plasma lipid hydroperoxides using the ferrous oxidation in Xylenol Orange (FOX) assay [16,17] with triphenylphosphine authentication of the signal or subsequent to total plasma lipid extraction using ethyl acetate/methanol [18]. We have reported values of 3.02 ± 1.85 μM ($n = 23$) and 3.76 ± 2.48 μM ($n = 23$) in two separate studies of apparently healthy individuals [18,19]. Marshall and colleagues, using a cyclo-oxygenase activation assay, have suggested that plasma hydroperoxide levels in healthy individuals are approx. 500 nM [20]. (Of course, it is not clear which classes of hydroperoxide activate cyclo-oxygenase, and the same authors record higher levels using their exact iodometric technique.) A recently developed sophisticated 'photon counting' chemiluminescence method has recently been applied to carefully isolated lipoproteins [21]. Using this technique Zamburlini and colleagues estimate, from the total hydroperoxide content of isolated individual lipoproteins, that native plasma contains 1.5–5.5 μM total lipid hydroperoxides [21]. Thus four distinct methods, of varying complexity, generate much the same answer for total lipid hydroperoxide content in plasma.

Further to the order-of-magnitude differences in levels of plasma lipid hydroperoxides determined by various techniques, there is conflict concerning the location of these oxidation intermediates. Since LDL is the fraction most closely associated with atherosclerosis risk, and becomes modified in a manner making it susceptible to uptake by scavenger receptors, it would seem natural to consider LDL as the principal carrier of lipid hydroperoxides. High-density lipoprotein (HDL), by contrast, is recognized to be a negative risk factor for atherosclerosis, so that

Abbreviations used: LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; PUFA, polyunsaturated fatty acids; FOX, ferrous oxidation in Xylenol Orange; TPP, triphenylphosphine; TBA, thiobarbituric acid; TBARM, TBA-reactive material; AAPH, 2,2′-azo-bis-(2-amidinopropane) hydrochloride; BHT, butylated hydroxytoluene; CE, cholesteryl ester.

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this fraction might be reasonably considered to contain only very low levels of hydroperoxides, if hydroperoxides are causative in atherosclerosis. By contrast, Bowry and colleagues [12], using data generated with the HPLC-chemiluminescence technique, suggested that HDL is the major carrier of plasma lipid hydroperoxides. The biochemical data concerning hydroperoxide location thus seem in conflict with the epidemiological data concerning risk associated with various cholesterol fractions.

In order to examine the location and level of hydroperoxides in plasma, we have fractionated native whole plasma into its constituent lipoprotein classes and determined the hydroperoxide content in these fractions. In addition, we have examined the *in vitro* susceptibility to oxidation of the various lipoprotein fractions and have also identified the lipoprotein location of hydroperoxides in whole plasma oxidized under basal or stimulated conditions *in vitro*.

MATERIALS AND METHODS

Reagents

Ammonium ferrous sulphate, H_2O_2, butylated hydroxytoluene (BHT), L-α-phosphatidylcholine (from egg yolk) and thiobarbituric acid (TBA) were obtained from Sigma Chemical Company (Poole, Dorset, U.K). Xylenol Orange [*o*-cresolsulphonphthalein-3,3-bis(methyliminodiacetic acid sodium salt)] and triphenylphosphine (TPP) were purchased from Aldrich (Gillingham, Dorset, U.K.). 2,2′-Azo-bis-(2-amidinopropane) hydrochloride (AAPH) was obtained from Polysciences (Warrington, PA, U.S.A.). All general chemicals and reagents were of the highest purity available.

Preparation of plasma

Blood was collected from fasted individuals by venipuncture into sampling vials containing heparin. Platelet-depleted plasma was prepared by centrifugation at 2000 ***g*** for 10 min at room temperature.

Preparation of FOX2-reagent

FOX2-reagent was prepared by dissolving Xylenol Orange and ammonium sulphate in 250 mM H_2SO_4 to yield final concentrations of 1 mM and 2.5 mM respectively. One volume of this concentrated reagent was added to 9 volumes of HPLC-grade methanol containing 4.4 mM BHT to make the working reagent, which comprised 250 μM ammonium sulphate, 100 μM Xylenol Orange; 25 mM H_2SO_4 and 4 mM BHT in 90% (v/v) methanol. The working reagent was routinely calibrated against solutions of H_2O_2 of known concentrations. Reagent was also obtained as the commercially available material from Pierce (Peroxoquant; methanol-compatible formulation).

Ultracentrifugal separation of plasma lipoproteins

The lipoprotein fractions [very-low-density lipoprotein (VLDL), LDL and HDL], with relative densities of < 1.006, 1.006–1.063 and 1.063–1.21 respectively, were isolated from plasma by sequential ultracentrifugation [22]. Weighed plasma samples (10 ml) containing 1 mg/ml EDTA were centrifuged at 100000 ***g*** for 18 h at 16 °C on an MSE Europa 55M preparative ultracentrifuge using a 6×14 ml swing-out titanium rotor (Kontron Instruments). Aliquots (2 ml) of the supernatants were made up to 5 ml with 150 mM NaCl containing EDTA (1 mg/ml) and stored at 4 °C prior to lipid analysis. The procedures for the subsequent ultracentrifugal separation of LDL and HDL were similar to that described for VLDL, but with the following modifications. The weight of the 10 ml volume of lipoprotein solution was used to calculate the amount of KBr required to raise its relative density to 1.073 or 1.21. The calculated amount of KBr was then dissolved in the lipoprotein solution. This adjusted lipoprotein solution was finally layered with the appropriate salt solution (density 1.063 or 1.21) prior to centrifugation. Lipoprotein fractions were dialysed against PBS (5 mM potassium phosphate, 150 mM NaCl, pH 7.4) for 12 h at 4 °C to remove EDTA that was added during preparation to diminish peroxidation. The protein content of the lipoprotein fractions was determined by the Lowry assay.

Plasma and lipoprotein peroxidation

Plasma samples (10 ml) were mixed with AAPH as oxidizing agent at a final concentration of 10 mM at 37 °C for 6 h. VLDL, LDL and HDL fractions were diluted to a final concentration of 200 μg/ml protein using PBS. Lipoprotein peroxidation was carried out by incubating the samples with copper sulphate or AAPH at final concentrations of 10 μM and 1 mM respectively at 37 °C for 24 h.

Measurement of lipid peroxidation products

Hydroperoxides

Aliquots (90 μl) of a plasma sample were transferred into 1.5 ml microcentrifuge vials together with 10 μl of methanol (in triplicate) or 10 μl of TPP (10 mM) in methanol (in triplicate). This generated triplicate blank and test samples respectively as described previously [18,19]. The samples were then vortexed and subsequently incubated for 30 min at room temperature. FOX2-reagent (900 μl) was then added and the samples were vortexed and incubated for a further 30 min. The samples were then centrifuged at 12000 ***g*** for 10 min prior to determination of the absorbance of the supernatants at 560 nm. The level of hydroperoxide in the plasma sample was then determined using the difference between the mean absorbances of samples with and without TPP pretreatment. The S.D. for the hydroperoxide concentration was determined from the larger of the S.D.s obtained for the sets with and without TPP pretreatment. In the case of lipoprotein suspensions or liposomes, samples (90 μl) were mixed with 10 μl of methanol or with 10 μl of TPP (10 mM) in methanol, incubated at room temperature for 30 min and then mixed with FOX2-reagent (900 μl) prior to incubation for a further 30 min at room temperature. After centrifugation at 12000 ***g*** for 10 min, the absorbance of the supernatants was monitored at 560 nm. Hydroperoxide content was determined using an molar absorption coefficient of $4.3 \times 10^4\ M^{-1} \cdot cm^{-1}$ or by reference to a H_2O_2 standard curve. Typical measured absorbance differences at 560 nm for plasma isolated from healthy volunteers are in the range 0.005–0.050 absorbance units. For example, plasma from subject 1 gave the following absorbance values when measured in triplicate: without TPP, 0.071, 0.070, 0.067 (mean ± S.D. 0.069 ± 0.002); + TPP, 0.047, 0.053, 0.050 (0.050 ± 0.002); difference between means 0.019 ± 0.002. Where required, confirmation of plasma signal authenticity as a ferric–Xylenol Orange complex can be established by scanning from 500 nm to 600 nm and identifying an absorbance peak at 560 nm.

TBA-reactive material (TBARM)

For the measurement of malondialdehyde and related aldehydes

in lipoprotein suspensions, samples (100 μl) were mixed with 0.67 % (v/v) TBA (1000 μl) and 20 % trichloroacetic acid (500 μl) and incubated at 100 °C for 20 min. After centrifugation at 12000 ***g*** for 10 min, the absorbance of the supernatants was measured at 532 nm. The total content of aldehydes capable of reacting with TBA to form chromophores absorbing at 532 nm was estimated using a molar absorption coefficient for the malondialdehyde–TBA complex of 1.56×10^5 $M^{-1} \cdot cm^{-1}$.

Vitamin E (α-tocopherol) analysis

Samples (200 μl) were mixed with 500 μl of ethanol (containing 50 ng/ml γ-tocopherol as internal standard), 1000 μl of hexane and 300 μl of water. The sample was centrifuged at 3000 ***g*** for 5 min and the upper (hexane) layer was transferred into glass vials. Hexane (1000 μl) was added to the residual aqueous layer and the extraction procedure was repeated. The hexane layers were pooled, the solvent was removed under nitrogen and the residue was redissolved in acetonitrile (100 μl). HPLC separation was carried out on a Hypersil-ODS column (20 cm × 5 mm; particle size 5 μm; Chrompack) using acetonitrile/tetrahydrofuran/water (80:14:6, by vol.). Tocopherols were monitored fluorimetrically (emission 295 nm; excitation 340 nm).

Fatty acid analysis

Samples (100 μl) were mixed with 250 μl of ethanol [containing 100 μg of heptadecanoic acid ($C_{17:0}$) as internal standard], ethyl acetate (500 μl) and water (250 μl). After centrifugation at 3000 ***g*** for 5 min, the upper (ethyl acetate) layers were tranferred into a glass vial and the solvents were removed under a stream of nitrogen. Boron trifluoride (14 %, w/v, in methanol) (500 μl) was then added and the samples were heated at 60 °C for 30 min. Fatty acid methyl esters were then extracted with 1000 μl of hexane and the solvent was removed under nitrogen. The residues were redissolved in 50 μl of hexane and 1 μl of each sample was injected on to a Stabilwax column (30 m × 0.53 mm; film thickness 1.0 μm). Gas chromatographic separation was carried out using a gradient of 140 to 210 °C at 2 °C/min. The signal was detected by flame ionization.

RESULTS

Distribution of plasma hydroperoxides in the major lipoprotein classes

We used the FOX assay to assess native plasma for total lipid hydroperoxide content prior to separation of the plasma into its constituent components and analysis of hydroperoxides in each fraction. As shown in Table 1 and Figure 1, LDL contained more than 65 % of the total hydroperoxide present in native plasma. VLDL contained 17 % of plasma hydroperoxide. By contrast, HDL contained only 11 % of total plasma lipid hydroperoxides, which was only slightly higher than the proportion (8 %) present in the fraction floating at a relative density greater than 1.21. This fraction contains albumin, other plasma proteins and 'very-high-density lipoproteins' consisting of lysophosphatidylcholine associated with albumin as well as lipid-poor apoprotein complexes [23]. When the hydroperoxide content in the major lipoprotein fractions was expressed on a per protein or per cholesterol basis, then LDL, HDL and VLDL were found to contain approx. 2.5, 0.75 and 0.75 nmol/mg of protein respectively and 2.0, 1.2 and 2.8 nmol/mg of cholesterol respectively.

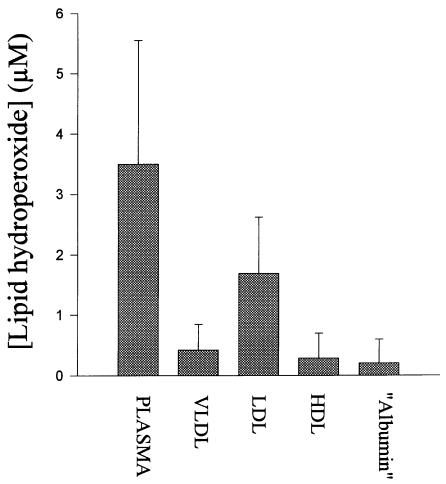

Figure 1 Contribution of plasma lipoproteins to total plasma hydroperoxide levels

Shown are the uncorrected summary data (means ± S.D.) from Table 1. Levels of hydroperoxide in LDL and HDL were significantly different (Mann-Whitney *U* test) at the 95% confidence level. 'Albumin' refers to that fraction with relative density > 1.21, consisting of protein and very-high-density lipoprotein.

Table 1 Location and sequestration of plasma hydroperoxides by lipoprotein class

The Table gives hydroperoxide concentrations (μM) for whole plasma and lipoprotein fractions. Percentage contributions of plasma lipoproteins to total plasma hydroperoxide levels were calculated from levels of hydroperoxides present in fractions generated by ultracentrifugation. It was assumed that loss of hydroperoxides during preparation was spread uniformly across the lipoprotein classes in calculating the location of hydroperoxides by lipoprotein class. Summary data are means ± S.D. Abbreviation: VHDL, very-high-density lipoprotein. The total plasma hydroperoxide levels reported are consistent with earlier studies. We have reported 3.02 ± 1.85 μM ($n = 23$) and 3.76 ± 2.48 μM ($n = 23$) in two separate studies of apparently healthy individuals [18,19].

	Hydroperoxide concn. (μM) in:					
Subject	Whole plasma	VLDL	LDL	HDL	Protein + VHDL	Recovery (%)
1	4.75	0.22	2.08	0.88	0.80	84
2	1.00	0.00	0.69	0.00	0.00	69
3	2.72	0.44	1.20	0.00	0.00	60
4	5.55	1.00	2.78	0.24	0.00	73
Summary data	3.50 ± 2.05	0.42 ± 0.43	1.69 ± 0.93	0.28 ± 0.42	0.20 ± 0.40	72 ± 10
Location (%)	100	17	67	11	8	

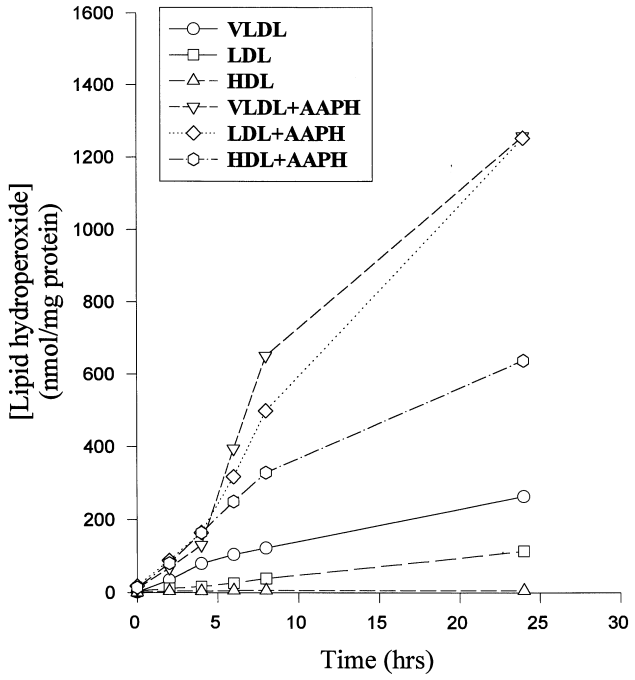

Figure 2 Susceptibility to peroxidation of isolated lipoproteins *in vitro*

Purified lipoproteins were exposed to AAPH (1 mM) or incubated in the absence of additions, with hydroperoxide content measured as a function of time.

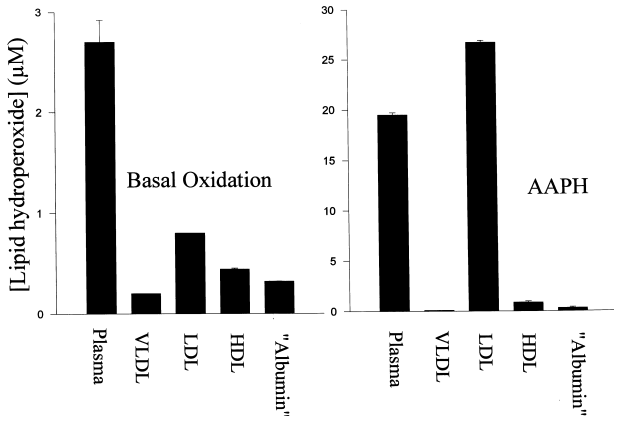

Figure 3 Susceptibility to peroxidation of lipoproteins in plasma

Whole pooled plasma from five individuals (distinct from those used in Table 1) was incubated at 37 °C in the presence or absence of AAPH (10 mM) for 6 h prior to measurement of hydroperoxide content. Lipoproteins were then isolated (as described in the Materials and methods section and in the legend to Table 1) and analysed for hydroperoxide content. 'Albumin' refers to that fraction with relative density > 1.21, consisting of protein and very-high-density lipoprotein. Results are means $\pm$ S.D. (Note that there is a small loss of hydroperoxide during lipoprotein isolation when summing basally oxidized lipoprotein, but a gain when isolating lipoproteins from highly oxidized plasma.)

Relative susceptibility to oxidation of lipids in the plasma major lipoprotein fractions

In order to probe the physicochemical mechanisms by which LDL and other lipoproteins accumulate hydroperoxides, we incubated isolated VLDL, LDL and HDL with the peroxidation initiator AAPH. We monitored hydroperoxide accumulation,

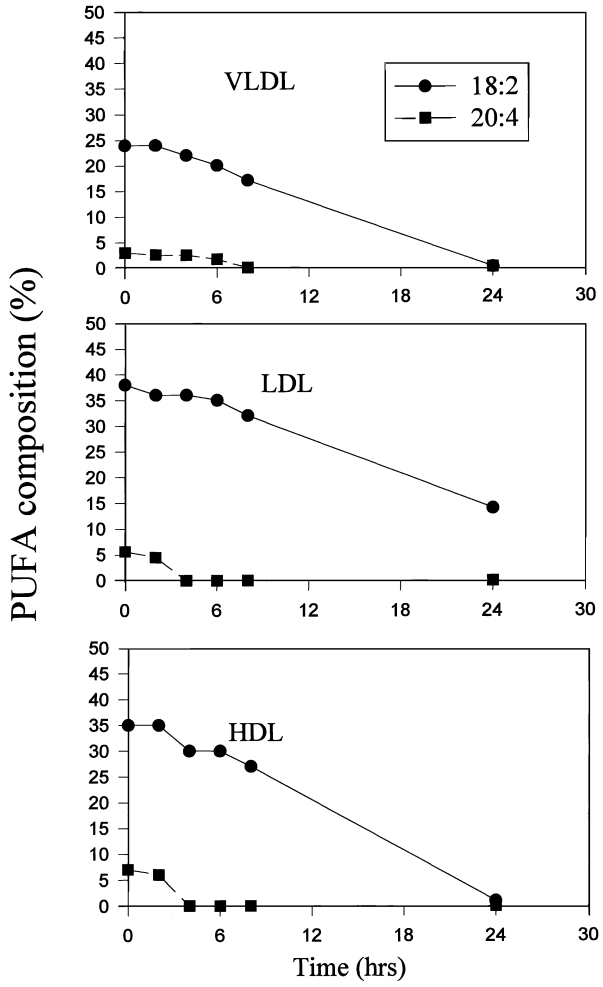

Figure 4 Consumption of PUFA during peroxidation by AAPH

Arachidonic (20:4) and linoleic (18:2) acids were measured as a percentage of the total fatty acid content of the lipoproteins during exposure to AAPH.

malondialdehyde accumulation (as measured with the TBA assay) and depletion of vitamin E and arachidonic and linoleic fatty acids. In the presence of AAPH (which undergoes thermolysis to yield an oxidizing radical capable of direct hydrogen abstraction), accumulation of hydroperoxides in all three fractions was approximately linear over time and lowest in HDL (Figure 2). In the absence of AAPH, but on incubation in buffer, LDL and VLDL showed smaller extents of hydroperoxide accumulation (in terms of hydroperoxide content per amount of protein) than in the presence of AAPH, as expected, whereas HDL appeared to be immune to oxidation (Figure 2). In a related experiment we also incubated whole pooled plasma at 37 °C for 6 h in the presence and absence of 10 mM AAPH (Figure 3) prior to separation of the lipoproteins and assignment of the hydroperoxide to individual lipoprotein fractions. In both cases (plasma oxidized under basal as well as stimulated conditions) the majority of hydroperoxide was found to be resident in the LDL fraction. Indeed, accumulation of hydroperoxide in AAPH-oxidized plasma was predominantly in the LDL fraction.

LDL and HDL contained approximately equal levels of arachidonic and linoleic acids prior to incubation with AAPH (approx. 40 % and 5 % respectively of total), and these levels

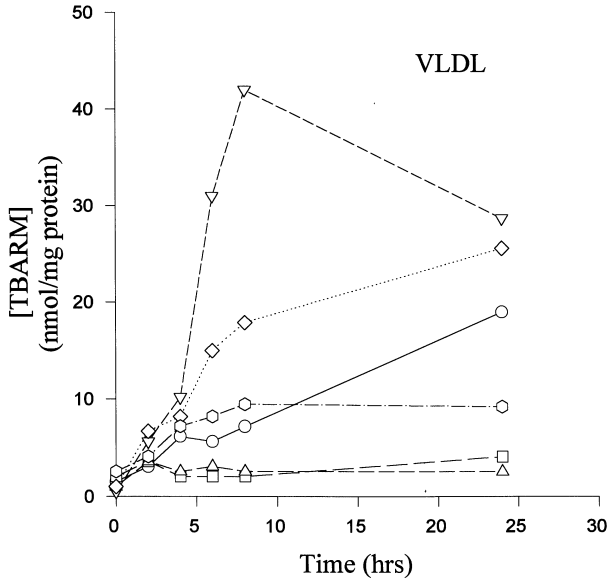

Figure 5 Appearance of TBARM in lipoproteins

Purified pooled lipoproteins were exposed to AAPH (1 mM) and TBARM content was measured as a function of time. ○, VLDL; □, LDL; △, HDL; ▽, VLDL + AAPH; ◇, LDL + AAPH; ⬡, HDL + AAPH.

were higher than in the case of VLDL (25 % and 3 % respectively). During oxidation the levels of $C_{18:2}$ and $C_{20:4}$ decreased rapidly in all lipoproteins (Figure 4). All lipoproteins rapidly lost arachidonic acid, whereas linoleic acid was still detectable in LDL at 24 h (Figure 4). Differences in the susceptibility of lipoproteins to peroxidation thus were not related to differences in composition of the lipoproteins with respect to arachidonic ($C_{20:4,n-6}$) and linoleic ($C_{18:2,n-6}$) acids (Figure 4). Nor was it possible to explain the differences in peroxidizability in terms of vitamin E content. Levels of vitamin E in LDL, VLDL and HDL were 1.1, 1.0 and 1.3 μg/mg of total lipid respectively. In all isolated lipoproteins exposed to AAPH, consumption of vitamin E was rapid and complete prior to total loss of arachidonic acid (results not shown).

The data on relative hydroperoxide accumulation in the isolated lipoproteins were consistent with trends in accumulation of aldehydes capable of reacting with TBA to form chromophores (Figure 5). As observed previously [16], accumulation of TBARM in the lipoproteins was 5–10-fold lower than accumulation of hydroperoxides, concordant with known low yields of malondialdehyde (and other aldehydes reactive to form chromophores with TBA) from lipid peroxidation. The overall trend was, however, similar to that observed for hydroperoxide accumulation in isolated lipoproteins oxidized *in vitro*. LDL and VLDL generated considerably more TBARM than HDL. In the absence of AAPH, LDL and VLDL showed smaller TBARM accumulation during the course of incubation, whereas HDL appeared to be immune to oxidation. Lipoprotein peroxidation patterns with copper ions as the pro-oxidant were comparable with those obtained with AAPH, despite differences in the mechanism of oxidation induced by this pro-oxidant (results not shown).

DISCUSSION

Native plasma from healthy individuals contains hydroperoxides which appear to be principally resident in LDL. Indeed, more than 65 % of hydroperoxides in native plasma are present in LDL. Our data also show that isolated LDL and VLDL are the lipoprotein groups that are most susceptible to hydroperoxide accumulation when oxidized *in vitro*. Further, when native plasma is oxidized *in vitro* the great majority of hydroperoxides which accumulate are found to be resident in the LDL fraction. HDL, by contrast, represents a small fraction of the total native plasma hydroperoxide pool, and also accumulates low levels of hydroperoxides during oxidation *in vitro*. The propensity of the lipoproteins to peroxidation appears to be unrelated to relative vitamin E levels and to the polyunsaturated fatty acid (PUFA) composition of the different lipoprotein fractions.

The reason for the greater propensity of LDL to peroxidation is not entirely clear. The triacylglycerol content of LDL [24], the presence of pre-existing lipid hydroperoxides [25,26], the ratio of $C_{18:1}$ to $C_{18:2}$ [27–29], the ubiquinol-10 content [30] and the ratio of vitamin E to cholesterol [31] have all been suggested to contribute to the peroxidizability of individual LDL samples. Similar factors may also provide an explanation for gross differences in behaviour of the major lipoprotein classes. Simple mass action effects may also play a role. For example, the higher concentration of LDL than HDL and VLDL in plasma could imply that initiators of lipid peroxidation are simply more likely to interact with LDL than with the other lipoproteins. However, it cannot be excluded that discrete physicochemical differences or differences in molecular architecture of the lipoproteins may also contribute to differences in susceptibility to peroxidation *in vivo* and *in vitro*.

The data presented here suggest that the total LDL hydroperoxide content in apparently healthy individuals is approx. 2.5 nmol of hydroperoxide/mg of LDL protein, or 2 nmol of hydroperoxide/mg of cholesterol. This is in good agreement with values obtained by Zamburlini and colleagues (2 ± 1 nmol/mg of cholesterol) using chemiluminescence modified by a 'photon counting' luminescence technique [21]. A concentration of approx. 3 μM total lipid hydroperoxide in plasma, mainly present in LDL, would imply that between one and two PUFA molecules out of 1200 per LDL particle are present as hydroperoxides; less than 0.2 % of the total. Furthermore, given that *in vitro* studies such as those reported here suggest that only one aldehyde molecule capable of forming a chromophore with TBA is generated for every 10–20 hydroperoxide molecules produced, it can be estimated that a level of 1.5 hydroperoxide molecules per LDL particle translates to a modification of only 1 in 15 LDL particles by peroxidation-derived aldehydes. This estimate obviously ignores the possibility that some aldehydes may be much more reactive with amino groups than others, but is in agreement with observations that 5 % of LDL from normocholesterolaemic monkeys is modified in a manner which affects its charge [32] and that 4 % is so affected in LDL isolated from normal human subjects [33].

Assuming that hydroperoxides are uniformly distributed throughout PUFA molecules present in the triacylglycerol, phospholipid and CE fractions of LDL, and taking an LDL hydroperoxide content of 3 nmol/mg of protein, then the triacylglycerol fraction in native LDL in apparently healthy individuals would contain 0.2 molecule of hydroperoxide per LDL particle (450 nmol/mg of LDL protein or 320 nmol/mg of total LDL cholesterol), the phospholipid fraction would contain 0.5 molecule of hydroperoxide (1 μmol/mg of protein; 750 nmol/mg of cholesterol) and the CE fraction would contain 0.75 molecule of hydroperoxide (1.5 μmol/mg of protein, 1.1 μmol/mg of cholesterol) per LDL particle. However, *in vitro* peroxidation of LDL is associated with a 5-fold higher accumulation of hydroperoxide in CE than in phospholipid, suggesting that the CE fraction may

contain in excess of 2 molecules of hydroperoxide per LDL particle [34]. It is thus surprising that Bowry and colleagues [12] found such low levels of CE hydroperoxides (0.0004 molecules per LDL particle) and undetectable levels of phospholipid hydroperoxides in their preparations of LDL.

Such multiple-order-of-magnitude differences in levels of plasma lipid hydroperoxides as determined by various techniques have not been adequately explained. Zhang and colleagues [35], studying *in vitro* lipid peroxidation, have pointed out that some of the disagreement may arise from inadequate preparation of standards, differential reactivity of various classes of hydroperoxide in the chemiluminescence cocktails, or gross artefact. Miyazawa [6] also suggests that variable recoveries of lipid hydroperoxides using the many lipid extraction techniques available prior to HPLC-chemiluminescence analysis may be a large source of error. Zamburlini and colleagues [21] suggest that oxidative side-reactions and the presence of unrecognized quenchers of chemiluminescence may also contribute to the underestimation of hydroperoxides by some research groups using HPLC-chemiluminescence.

The focus on CE and phospholipid classes may also lead to underestimation, since with this approach the plasma triacylglycerol fraction, which contains a large proportion of peroxidizable fatty acid, is not separately scrutinized for hydroperoxide content but may possibly contain hydroperoxides which are poorly reactive in the chemiluminescence assays. An alternative explanation is that there are components present in LDL (such as free cholesterol) which are neglected by the HPLC-chemiluminescence assays but are extremely rich in hydroperoxides, so that the low estimates for CE and phospholipid hydroperoxides obtained by some authors are realistic in the context of a high background level of cholesterol hydroperoxides. However, cholesterol hydroperoxides are extremely unstable and rapidly degrade to oxysterols (J. Nourooz-Zadeh, unpublished work).

All current available methods for the direct determination of hydroperoxides are dependent upon the ability of hydroperoxides to oxidize other molecules. The FOX assay is dependent upon the ability of hydroperoxides to oxidize ferrous to ferric ions under acidic conditions where ferrous ions auto-oxidize slowly. The chemiluminescence techniques rely upon chemistry which is less well defined, but are based on the ability of some higher oxidation state of cytochrome *c* or 'microperoxidase' (proteolytically cleaved cytochrome *c*), generated by hydroperoxides, to further oxidize isoluminol with the emission of light. The iodometric techniques depend upon the ability of hydroperoxides to oxidize iodine ion to iodine atom. Critical characteristics of all these assays are: (a) the efficiency of the oxidation process by different hydroperoxides, (b) the influence of interfering factors and (c) the sensitivity of the detection method.

The data we have gathered here, using the FOX assay as a measure of total lipid hydroperoxide formation, suggest that LDL is the major carrier of plasma lipid hydroperoxides. This conclusion, which contrasts with conclusions drawn using the HPLC-chemiluminescence technique [12], is supported by physicochemical considerations of the peroxidizability of various lipoprotein classes, by other considerations of the mechanism of lipoprotein peroxidation, and by various estimates of plasma and lipoprotein peroxidation. We conclude that apparently sensitive HPLC-chemiluminescence techniques may grossly underestimate the extent of peroxidation in biological samples. This may lead to the development of erroneous conclusions about mechanisms of lipid peroxidation as they relate to atherosclerosis.

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