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Pyrene lipids as markers of peroxidative processes in different regions of low and high density lipoproteins

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Abstract

Three different pyrene derivatives, pyrene decanoyl phosphatidylcholine ($P_{10}PC$), pyrene dodecanoyl sulfatide ($P_{12}CS$) and cholesteryl pyrenyl hexanoate (P_6Chol), were used to follow lipid peroxidation in low and high density lipoproteins. Probe-labelled lipoproteins were subjected to Cu^{2+} catalyzed peroxidation. In all cases the fluorescence of the probes progressively decreased due to the involvement of pyrene in the peroxidative reaction. Thus, we used the fluorescence decrease of P_6Chol to monitor the lipid peroxidation in the hydrophobic core of LDL and HDL, and that of the amphipatic probes, $P_{10}PC$ and $P_{12}CS$, to follow lipid peroxidation in the envelope of both lipoproteins. The possibility of following lipid peroxidation in individual lipoprotein regions could lead to more detailed information on the oxidative modifications that play an important role in the altered cholesterol homeostasis involved in the formation of atherosclerotic lesions. No differences were observed in the peroxidation kinetics of the hydrophobic core of HDL and LDL monitored with P_6Chol. On the contrary kinetics obtained with $P_{10}PC$ and $P_{12}CS$ demonstrated the HDL envelope to be more susceptible to Cu^{2+}-dependent lipid peroxidation than that of the LDL. This could be due to a greater radical generating capacity of the HDL envelope and can be explained on the basis of low vitamin E levels and large amounts of polyunsaturated fatty acids esterified on phospholipids determined in HDL, and on literature evidence that indicates HDL as the principal vehicle of circulating plasma lipid peroxides.

Keywords: Lipid peroxidation; Lipoprotein; Pyrene lipid; Atherosclerosis

1. Introduction

The peroxidation of low density lipoprotein (LDL) and the uptake of oxidized LDL by macrophages, via 'scavenger' receptors whose expression is not regulated by cellular cholesterol levels, have been implicated in the formation of lipid laden cells [1]. This is an early and important step in the development of atherosclerotic lesions [2,3]. Recent data indicate that not only does oxidized LDL actually exist in vivo [4–7] but it is also present in arterial atherosclerotic plaques [8].

In atherogenic processes the protective role of high density lipoproteins (HDL) is supported by the fact that plasma HDL levels have an inverse epidemiological correlation with the incidence of coronary artery disease [9]. The biochemical mechanisms of action of HDL in preventing the development of atherosclerotic lesions are not well established: nevertheless, experimental evidence supports that: (1) HDL serves as an acceptor of cholesterol efflux from foam cells (the cholesterol ester laden macrophages formed in atherosclerotic lesions) by a mechanism similar to that of 'reverse cholesterol transport' [10]; (2) oxidized phospholipids and lipid peroxidation products are rapidly exchanged between LDL and HDL [11,12]; (3) binding of transition metals by ApoAI inhibits the oxidation of LDL [13].

The presence of a specific binding protein for HDL, i.e., the HDL receptor, has been demonstrated in several cells such as parenchymal liver cells [14], sinusoidal liver cells [15], cells from steroidogenic tissues [16–18] and macrophages [19]. The mechanism hypothesized for HDL-dependent cholesterol efflux from macrophages is based on a receptor-mediated non-lysosomal endocytic–exocytic pathway for HDL [20–22]. Although there is still no evidence of the existence of oxidatively modified HDL in vivo, it was recently demonstrated that the in vitro oxidation of HDL significantly reduces the capacity of the

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lipoprotein to enhance cholesterol efflux from macrophage-derived foam cells [23]. This could depend on a reduced receptor affinity of oxidized HDL, as a consequence of ApoAI modification [24]. Endocytosis of modified HDL seems to be mediated by a 'scavenger' receptor: the internalized particle is thought to be degraded in the lysosomal district but not addressed to the retroendocytotic pathway [25].

Lipid peroxidation can occur in both the envelope and the core of plasma lipoproteins. Thus the possibility of following the lipid peroxidation of the individual regions of LDL and HDL (i.e., core and surface) could lead to more detailed information on the modifications brought about by oxidation, modifications that are the basis of the altered cholesterol homeostasis involved in the formation of atherosclerotic lesions.

We used three different pyrene derivatives, pyrene decanoyl phosphatidylcholine ($P_{10}PC$), pyrene dodecanoyl sulfatide ($P_{12}CS$) and cholesteryl pyrenyl hexanoate (P_6Chol), to selectively follow the kinetics of Cu^{2+} catalyzed peroxidation at the surface and core of human LDL and HDL. Pyrene was chosen as the 'peroxidation-sensitive fluorophore' on the basis of previous studies [26] that demonstrated that the decrease in pyrene fluorescence, due to lipid peroxidation, strongly depends on the membrane region in which the probe is located.

2. Materials and methods

Analytical grade chemicals, distilled solvents and doubly distilled water were used. Egg phosphatidylcholine, β-(pyren-1-yl) decanoyl γ-palmitoyl L-α phosphatidylcholine ($P_{10}PC$), cholesteryl (pyren-1-yl) hexanoate (P_6Chol) were purchased from Sigma Chemicals, 12-(1-pyrene) dodecanoic acid from Molecular Probes. This last was purified on a C_{18} reverse phase HPLC column [27]. N-(12-(-1-pyrene) dodecanoyl) galactosylsphingosine I^3-sulfate ($P_{12}CS$) was prepared from galactosylsphingosine I^3-sulfate following the procedure of Marchesini et al. [28].

2.1. Labelling of lipoproteins with fluorescent probes

Plasma was prepared from freshly obtained heparinized blood of overnight (o.n.) fasted, healthy and normolipidemic male and female subjects (30–40 years old). The fluorescent probes were incorporated into the lipoproteins by incubating the plasma for 60 min at 25°C in the presence of $P_{10}PC$, $P_{12}CS$ or P_6Chol. In the case of $P_{10}PC$ and $P_{12}CS$, each was dissolved in 154 mM NaCl and added to plasma to a final concentration of 10 nmol/ml; P_6Chol was mixed with egg phosphatidylcholine (1:10 mol: mol) and dissolved in 154 mM NaCl then added to plasma to a final fluorescent lipid concentration of 20 nmol/ml.

LDL and HDL were prepared from plasma, with or without fluorescent probes, by ultracentrifugation using a multistep discontinuous gradient in a Beckman TL-100 tabletop ultracentrifuge, adapting the conditions described by Havel et al. [29]. After separation LDL and HDL were dialyzed against 154 mM NaCl at 4°C for 12 h in order to eliminate KBr. Isolated and dialyzed lipoproteins were stored under argon gas in the dark at 4°C for a maximum of 2 weeks.

2.2. Lipoprotein characterization

The protein concentration of each lipoprotein fraction was determined with the Lowry method [30] using bovine serum albumin as standard.

Total lipids were extracted from each lipoprotein fraction following the Folch procedure [31]. The phospholipid content was determined according to Bartlett [32]; cholesterol was determined using a reagent kit from Boehringer Mannheim.

Pyrene lipids incorporated in LDL and HDL were analyzed by TLC using 20 × 20 silica gel 60 plates (Merck). TLC plates were developed using a monodimensional double solvent technique; solvents used were: (A) $CHCl_3$:CH_3OH:H_2O (65:25:4 by vol.) and (B) $CH_3(CH_2)_4CH_3$: $(C_2, H_5)_2O$: CH3COOH (70:30:1 by vol.). Plates developed with solvent A for 7 cm, were then dried and submitted to a second 15-cm long run in the same direction using solvent B as developing system. P_6Chol, $P_{10}PC$ and $P_{12}CS$ were used as standard lipids.

Lipoprotein neutral lipids were extracted with 50 volumes of hexane after addition of 10 volumes of methanol to the lipoprotein fraction; chloroform was then added to the water/methanol phase for the Folch extraction of phospholipids. Fatty acid composition of neutral lipids and phospholipids was determined after methylation of the lipid extracts; the methylation was carried out in 0.5 M HCl in degassed methanol at 80°C for 20 h. Fatty acid methyl esters were then extracted in hexane, the hexane phase was dried under nitrogen and fatty acid methyl esters resuspended in small volumes of hexane; a DANI HR PTV-2CH (flame ionization detector) connected with a 3390 Hewlett Packard integrator was used. The analysis was performed by injecting 2 μl of the sample into a 30 meter Supelcowax 10 polar capillary column. The temperature was maintained at 100°C for 3 min, then increased 30°C/min up to 180°C then 3°C/min up to 260°C, this was followed by an isotherm step of 10 min.

Vitamin E was extracted following the Vuilleumier method [33]. The amount of α-tocopherol of LDL and HDL was determined by HPLC using a Beckman system Gold 156 apparatus with a 4.6 mm ID × 100 mm column packed with 5 μm LiChrospher RP18 (Merck, Darmstadt, Germany) protected by a 4 mm ID × 4 mm (5 μm) LiChrospher RP18 Merck. The operating conditions were: methanol: acetonitrile (8:2 v:v), isocratic, 1.5 ml/min, spectrophotometric detection at 292 nm.

2.3. Lipoprotein peroxidation

Peroxidation of LDL and HDL was induced by incubating lipoproteins (0.1 mg protein/ml) suspended in 10 mM Tris-HCl, 154 mM NaCl (pH 7.4) at 37°C with different concentrations (0.5–20 μM) of copper sulfate.

The oxidation of LDL and HDL was monitored by measuring the thiobarbituric acid reactive substances (TBARS) following the procedure of Buege and Aust [34].

The oxidation of labelled LDL and HDL was monitored by the decrease in the pyrene's fluorescence emission intensity at 379 nm (excitation 343 nm) [26]. This decrease was expressed as $((F_0\text{-}F_t)/F_0) \times 100$, where F_0 represents the fluorescence intensity at zero time of peroxidation and F_t the residual fluorescence for a given peroxidation time.

The fluorimetrically determined peroxidation profile of each lipoprotein sample can be divided into latency, propagation and steady state phases and allows the evaluation of lag time, expressed in minutes, calculated as the intercept of the linear least square regression of the propagation phase with that of the latency phase.

2.4. Excimer to monomer ratio determinations

Unlabeled LDL and HDL, (0.1 mg of protein) resuspended in 10 mM Tris-HCl 154 mM NaCl (pH 7.4) were incubated separately for 60 min at 25°C with $P_{10}PC$, and $P_{12}CS$ (5% of endogenous phospholipids). The Excimer (475 nm) to Monomer (379 nm) fluorescence intensity ratio of fluorescent lipids was evaluated as a function of temperature.

The intensity of excimer fluorescence depends on the possibility of collisions between pyrene lipids. Thus, when the concentration of pyrene lipids in two different lipid structures is the same, the difference observed in the E/M vs temperature curves will indicate a different lateral mobility of the pyrene derivative in the lipid environment considered [35].

2.5. Fluorescence measurements

All fluorescence measurements were carried out in a Jasco spectrophotofluorimeter FP-777 equipped with a cuvette holder. The temperature maintained by a Haake GD3 thermostatic circulating bath, was monitored with a Subline PT 100 digital thermometer.

2.6. Statistics

The results were statistically evaluated by Student's *t*-test.

3. Results

The incubation of plasma with pyrene derivatives of cholesterol, phosphatidylcholine and sulfatide leads to their different distribution in the lipoprotein fractions (Fig. 1): the amount of P_6Chol found in LDL is 3-fold that in HDL; $P_{10}PC$ is almost equally distributed between LDL and HDL; $P_{12}CS$ is preferentially incorporated in HDL, the amount being 4-fold that in LDL. Thin-layer chromatography of the LDL and HDL lipid extracts excluded the presence of fluorescent compounds other than those used for the lipoprotein labelling (data not shown).

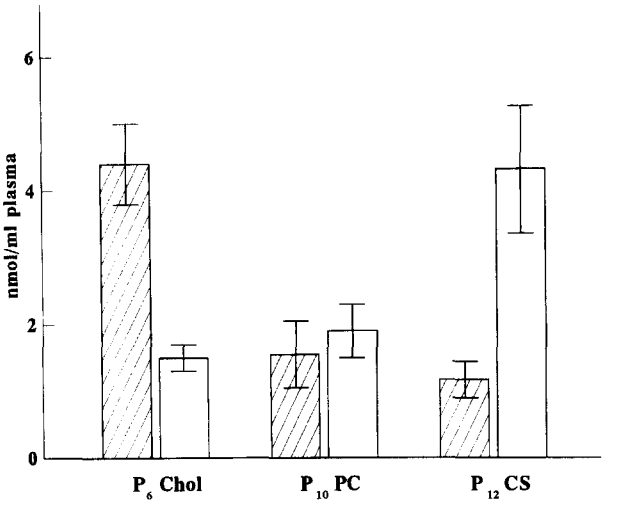

Fig. 1. Distribution of pyrene derivatives of cholesterol, phosphatidylcholine and sulfatide in LDL (striped bar) and HDL (open bar). Data are expressed as nmol/ml plasma. Measurements were done in triplicate on five donors. Data are means ± S.D. ($n = 5$).

The distribution of P_6Chol and $P_{10}PC$ mimicked that of corresponding natural compounds (Fig. 2). No comparison can be made of the preferential incorporation of $P_{12}CS$ in HDL since the plasma of healthy subjects shows only traces of the corresponding natural compound sulfatide. On the basis of their distribution in different lipoproteins and

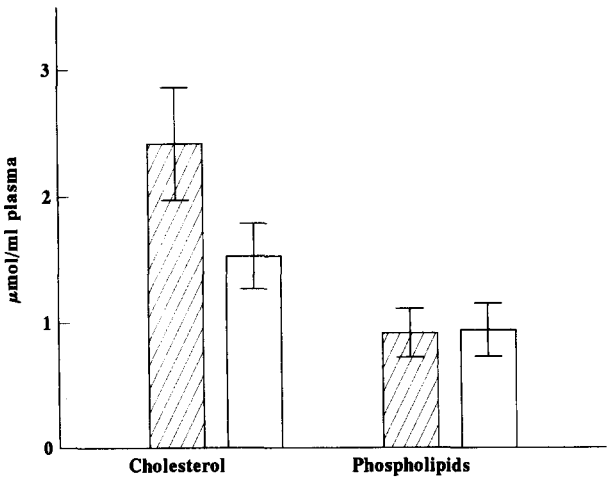

Fig. 2. Distribution of cholesterol and phospholipids in LDL (striped bar) and HDL (open bar). Data are expressed as μmol/ml plasma. Measurements were done in triplicate on five donors. Data are means ± S.D. ($n = 5$).

their chemical structure, it can be assumed that $P_{10}PC$ and $P_{12}CS$ are located in the stabilizing 'envelope' (a monolayer composed of specific apoproteins, phospholipids and unesterified cholesterol), and P_6Chol in the cholesteryl ester and triglyceride-rich 'core' of lipoproteins. Thus amphipatic probes $P_{10}PC$ and $P_{12}CS$ were used for monitoring the kinetics of Cu^{2+}-dependent lipid peroxidation in the envelope and P_6Chol for monitoring Cu^{2+}-dependent lipid peroxidation in the core of lipoprotein.

The sensitivity of the pyrene ring to Cu^{2+}-dependent lipid peroxidation was demonstrated by the absorption and emission spectra of pyrene recorded on the lipid extracts of HDL labelled with $P_{12}CS$. These spectra recorded before and after 40 min of lipid peroxidation clearly indicate (Fig. 3) a 30% reduction in fluorescence emission and a parallel strong modification of the absorption spectra of pyrene.

The time-dependent TBARS production in LDL and HDL is reported in Fig. 4A. After a latency phase of 40 min, the TBARS production in LDL linearly increases up to about 180 min, reaching a steady-state level with a maximal TBARS production of about 80 nmol/mg protein. In HDL after a latency phase of 13 min the TBARS production linearly increases up to about 60 min with a maximal TBARS production of about 50 nmol/mg protein.

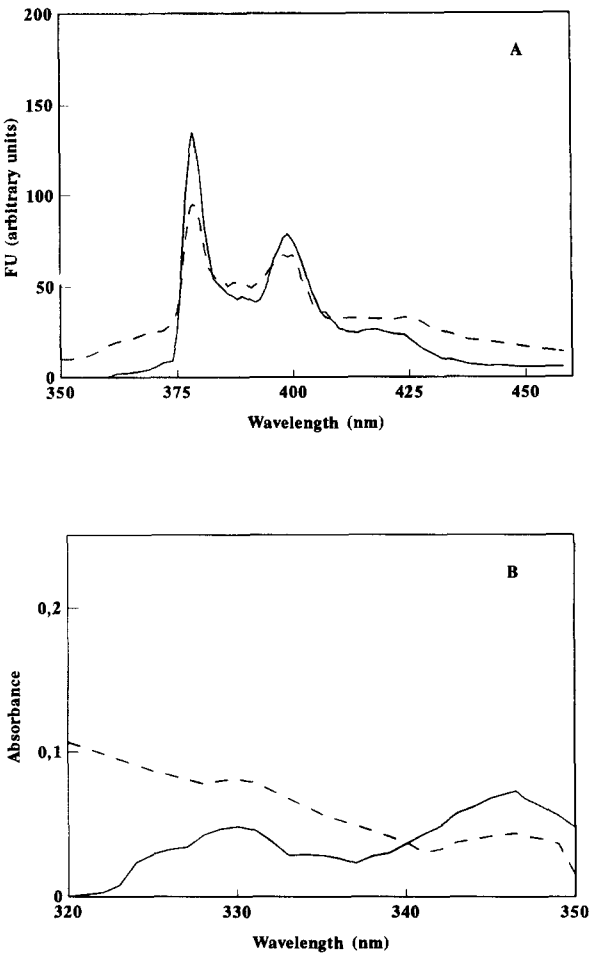

Fig. 3. Absorption (panel A) and emission (panel B) spectra of $P_{12}CS$ in HDL lipid extract before (unbroken line) and after (dotted line) 40 min Cu^{2+}-dependent lipid peroxidation.

Incubation of P_6Chol labelled LDL and HDL with 10 μM $CuSO_4$ determined, after a latency phase of 50 and 45 min (lag time) respectively, a progressive decrease in fluorescence emission intensity consequent to lipid peroxidation (Fig. 4B). The kinetic profiles obtained with labelled LDL and HDL were very similar: in fact the latency phase was followed in both cases by a rapid linear increase in lipid peroxidation up to 120 min, and a steady-state phase monitored up to 300 min. Peroxidation profiles obtained incubating $P_{10}PC$ labelled LDL and HDL with 10 μM $CuSO_4$ are reported in Fig. 4C. Kinetics of lipid peroxidation followed with $P_{10}PC$ in the envelope of HDL and LDL were very different. After a latency phase of 20 min, HDLs were rapidly peroxidized: a 70–80% decrease of pyrene fluorescence was reached after 60 min of incubation. Also in these cases the rapid linear increase of lipid peroxidation was followed by a steady-state phase monitored up to 300 min. On the contrary, when $P_{10}PC$ was used to follow the oxidation of the LDL envelope, a lag time of about 60 min was observed, the rate of lipid peroxidation after the latency phase being smaller than that observed in HDL labelled with the same probe; in fact a 60% reduction of fluorescence was reached only after 300 min of incubation. The kinetics obtained with $P_{10}PC$ in both LDL and HDL were confirmed by the data obtained with a different amphipatic probe, $P_{12}CS$ (Fig. 4D).

The lateral mobility of $P_{10}PC$ and $P_{12}CS$ in the envelope of LDL and HDL was evaluated by determining the excimer to monomer ratio (E/M) as a function of the temperature after incorporation of equal amounts of the two probes (5 mol% of total phospholipids) in unlabelled LDL and HDL (Fig. 5A,B). Slopes of E/M vs. temperature lines obtained with $P_{10}PC$ incorporated in LDL and HDL were 0.017 and 0.008 respectively, indicating a greater lateral mobility of the probe incorporated in the LDL envelope. Slopes of E/M vs. temperature lines obtained with $P_{12}CS$ were very similar, being 0.0009 for LDL and 0.0008 for HDL, thus indicating a similar lateral mobility of $P_{12}CS$ in the envelope of LDL and HDL.

Table 1 shows the LDL and HDL lipid composition and the vitamin E levels. The percentage of polyunsaturated fatty acids in the phospholipids was greater in HDL than in LDL: this is mainly due to arachidonic acid (Table 2). The association of vitamin E to LDL and HDL, expressed as mol vit. E/mol lipoprotein, was 15-fold greater to LDL than to HDL; as a function of lipoprotein phospholipids, the association to LDL is still significantly greater.

The effect of copper concentration on the kinetics of the oxidation of the core and surface of LDL and HDL was studied by measuring the P_6Chol and $P_{12}CS$ fluorescence decrease at copper concentrations ranging from 0.5 to 20 μM. In all cases increasing the copper concentration led to

a decrease in lag time and an increase in the velocity of propagation; see Fig. 6 for $P_{12}CS$-labelled HDL. Using the approach of Esterbauer and colleagues [36], we plotted the data as 1/lag time against copper concentration: the hyperbola function has the mathematical form of the Michaelis-Menten equation:

$$1/lag\,time = 1/lag\,time_{min} \times [Cu^{2+}]/K + [Cu^{2+}]$$

where lag time $_{min}$ is the theoretical value at infinite concentrations of copper.

This is demonstrated by the fact that data plotted in a double reciprocal manner (Figs. 7 and 8) give a strict linear equation which can be described:

$$lag\,time = K \times lag\,time_{min} \times 1/[Cu^{2+}] + lag\,time_{min}$$

Thus two additional parameters are available to describe the oxidation of the different domains of lipoproteins: lag time $_{min}$, the minimum latency time theoretically possible for oxidation at infinite copper concentration, and K, the copper concentration (in μM) that produces a lag time of twice the minimum value. Linear regression analysis showed a very high correlation between the experimental points. Values obtained from the fluorescence decrease of P_6Chol, that monitors the oxidation of the hydrophobic core of LDL and HDL, were respectively 4.83 and 6.7 μM; the corresponding lag time $_{min}$ were 27.3 and 27.4 min respectively. K-values obtained with $P_{12}CS$, that monitors the oxidation of the lipoprotein envelope, were 4.53 μM for LDL and 6.58 μM for HDL; the lag time $_{min}$ values were 51.6 and 13.66 min for LDL and HDL respectively.

4. Discussion

The importance of lipoprotein oxidation phenomena as cofactors of different cardiovascular pathologies has been stressed in many literature reports (for a review see Ref. [37]). The oxidation of LDL and, to a lesser extent, HDL has generally been studied considering the whole lipoprotein particle. In this study we evaluated the susceptibility of the different lipoprotein regions, i.e., the hydrophobic core and the stabilizing envelope, to oxidation by copper ions, using pyrene lipids selectively incorporated into the different domains of LDL and HDL. The use of pyrene derivatives to follow lipid peroxidation is based on our previous studies [26] that demonstrated that the fluorescence emission of pyrene incorporated in a lipid environment is strongly reduced by iron/ascorbate-induced per-

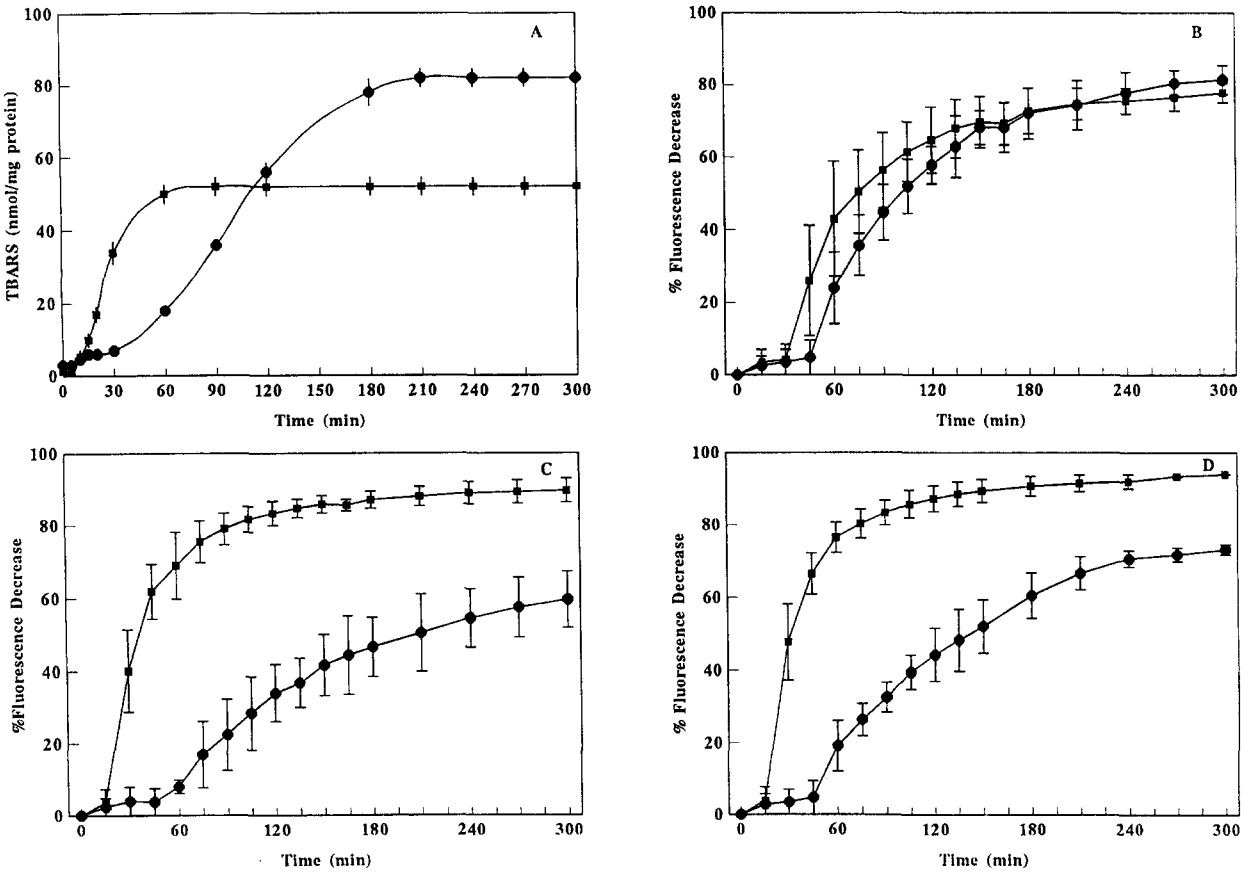

Fig. 4. Kinetics of lipid peroxidation of LDL (●) and HDL (■) followed as TBARS production (panel A) and decrease of fluorescence of P_6Chol (panel (B), $P_{10}PC$ (panel C) and $P_{12}CS$ (panel D). Peroxidation was induced by 10 μM $CuSO_4$ on 0.1 mg/ml lipoprotein aqueous suspension.

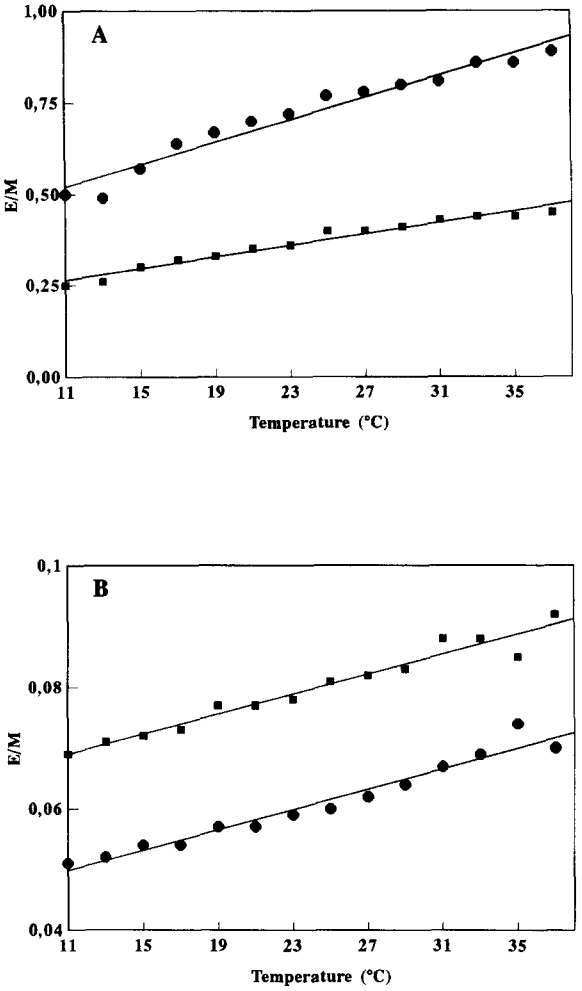

Fig. 5. Excimer (475 nm) to monomer (379 nm) ratio (excitation wavelength of 343 nm) vs temperature lines of $P_{10}PC$ (panel A) incorporated in LDL (●) and HDL (■) (r^2 values: 0.95 and 0.96 respectively) and $P_{12}CS$ (panel B) incorporated in LDL and HDL (r^2 values 0.95 in both cases).

oxidation. In those studies we demonstrated that the decrease in fluorescence is due to an involvement of pyrene in the radical reaction that propagates peroxidation. The fluorescence of a pyrene derivative incorporated in lipoproteins is also reduced in the case of cupric sulfate-dependent lipid peroxidation (Fig. 4). Also in these experimental conditions the pyrene ring seems to be involved in the peroxidative reaction, since the fluorescence recorded on the lipid extract is also decreased (Fig. 3A), excluding a reduction in fluorescence quantum yield due to end products of lipid peroxidation. Furthermore the absorption spectra of pyrene recorded on the lipid extracts after peroxidation are strongly modified, indicating that the pyrene ring is modified during peroxidation (Fig. 3B).

The incorporation of different pyrene derivatives in LDL and HDL was obtained by incubating whole plasma with fluorescent lipids; we used this procedure in order to minimize the possibility of oxidative processes during the incubation period. The different lipoprotein classes were then separated by ultracentrifugation and analyzed for protein, phospholipid and total cholesterol content (Table 1). Considering the chemical structure of fluorescent lipids and their distribution in the different lipoprotein fractions

Table 1
LDL and HDL composition

	LDL	HDL
Proteins (mg/ml plasma)	0.68 ± 0.03	1.1 ± 0.05
Phospholipids (μmol/ml plasma)	0.95 ± 0.04	1.01 ± 0.1
Cholesterol (μmol/ml plasma)	2.64 ± 0.1	1.59 ± 0.09
PUFA/TOT (%) Neutral lipids	7.60 ± 0.7	10.3 ± 2.2
Phospholipids	13.3 ± 3.9	18.7 ± 3.2
Vit E (mol/mol lipoprotein)	8.1 ± 1.60	0.53 ± 0.17
(nmol/ μmol PL)	8.1 ± 0.7 *	5.1 ± 1.2

* $P < 0.02$.
PUFA/TOT, polyunsaturated fatty acids/total fatty acids measurements were done in triplicate on five plasma samples; data are means ± S.D. ($n = 5$).

Table 2
Fatty acid profiles of phospho- and neutral lipids in LDL and HDL

	Phospholipids		Neutral lipids	
	LDL	HDL	LDL	HDL
16:0	29.1 ± 3.60	24.4 ± 1.60	15.2 ± 0.47	15.8 ± 1.40
16:1	1.9 ± 0.50	1.7 ± 0.40	3.4 ± 0.85	3.1 ± 0.81
18:0	14.2 ± 1.1	13.4 ± 0.91	2.9 ± 0.20	4.7 ± 0.26 **
18:1	12.0 ± 0.80	12.3 ± 0.97	28.4 ± 1.40	25.5 ± 0.53 *
18:2	19.7 ± 1.60	21.7 ± 0.65 *	40.3 ± 2.80	37.4 ± 3.10
18:4	1.1 ± 0.30	0.9 ± 0.15	0.4 ± 0.08	0.7 ± 0.14
20:0	1.2 ± 0.40	0.5 ± 0.11	1.0 ± 0.4	1.4 ± 0.4
20:4	8.4 ± 1.40	15.0 ± 1.58 **	6.3 ± 0.87	8.3 ± 2
20:5	3.3 ± 0.70	2.1 ± 0.63 *	0.5 ± 0.13	0.7 ± 0.15
24:0	8.1 ± 1.50	7.3 ± 1.10	1.2 ± 0.06	1.7 ± 0.26
22:6	0.7 ± 0.30	0.7 ± 0.40	0.6 ± 0.06	0.7 ± 0.10

* $P \leq 0.005$; ** $P \leq 0.0001$.
Measurements were done in triplicate on five plasma samples; Data are means ± S.D. ($n = 5$).

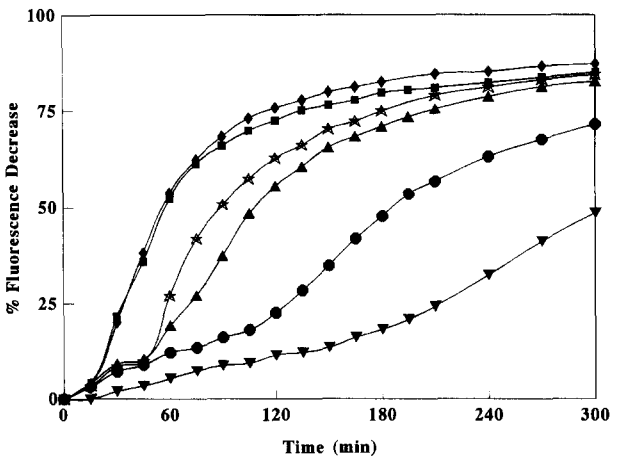

Fig. 6. Effect of copper concentration on the peroxidation kinetics of the HDL envelope measured as $P_{12}CS$ fluorescence decrease: 0.5 (▼), 1 (●), 2 (▲), 3 (★), 5 (■) and 10 (◆) μM $CuSO_4$.

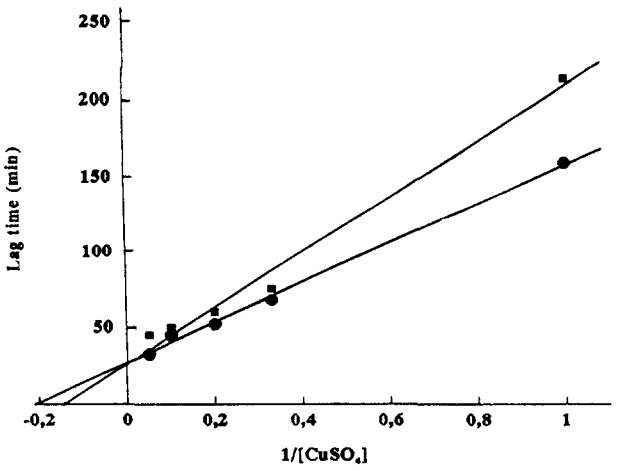

Fig. 7. Plots of lag time vs $1/[CuSO_4]$ determined in the hydrophobic core of LDL (●) and HDL (■) by measuring the kinetics of fluorescence decrease of P_6Chol at $CuSO_4$ concentration ranging from 1 μM to 20 μM; r^2 LDL = 0.99, HDL = 0.98.

(Fig. 1), it can be assumed that the fluorescent derivative of cholesteryl ester P_6Chol is incorporated in the hydrophobic core of LDL and HDL with a preferential location in LDL; the fluorescent derivative of phosphatidylcholine, P_{10}PC, is almost equally distributed between the phospholipid envelope of LDL and HDL; the fluorescent derivative of sulfatide P_{12}CS is incorporated in the envelope of lipoprotein with a preferential location in the HDL envelope. Thus P_{10}PC, P_{12}CS and P_6Chol were used to selectively monitor the kinetics of lipid peroxidation in the envelope and the core of LDL and HDL.

The response of hydrophobic core to an oxidative stress induced by 10 μM $CuSO_4$ is very similar in LDL and HDL, considering both the resistance to and the rate of peroxidation (Fig. 4B). On the contrary, the envelope of HDL is more rapidly peroxidized than that of LDL (Fig. 4C,D). In particular the latency time, which is an index of the antioxidant capacity of lipoprotein, is 3-fold shorter in HDL than in LDL; similarly, the rate of peroxidation in HDL is about 3-fold greater than in LDL. This evidence was confirmed by the use of two different amphipatic probes P_{10}PC and P_{12}CS.

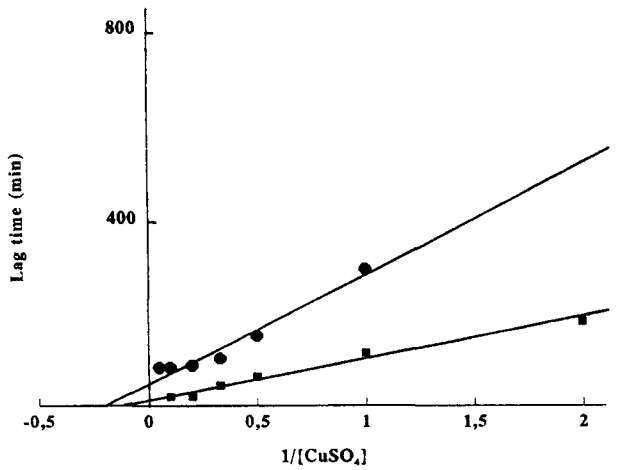

Fig. 8. Plots of Lag time vs $1/[CuSO_4]$ in the envelope of LDL (●) at $CuSO_4$ concentration ranging from 1 to 20 μM and in the envelope of HDL (■) at $CuSO_4$ concentrations ranging from 0.5 to 10 μM determined by measuring the kinetics of fluorescence decrease of P_{12}CS; r^2 LDL = 0.95, HDL = 0.98.

The higher oxidability of the HDL envelope could depend on several factors, including the frequency of radical collisions due to the fluidity of the lipid environment, the levels of peroxidable substrates, the concentration of antioxidants and a different catalyzing capacity of copper in the two lipoprotein classes.

In a recent study, Barenholz and colleagues [38] reported that the fluidity of the LDL envelope is greater than that of HDL, primarily as a consequence of a smaller protein to lipid ratio; in addition, the E/M vs. temperature lines obtained with P_{10}PC and P_{12}CS incorporated in HDL and LDL indicate, respectively, a greater and equal lateral mobility of pyrene lipids in the envelopes of LDL and HDL (Fig. 5). Thus the differences observed in the kinetics of lipid peroxidation cannot be ascribed to a greater frequency of collisions, neither between lipid radicals nor between lipids and pyrene derivatives.

The fatty acid profiles of phospholipids showed that the amount of polyunsaturated fatty acids, namely arachidonic acid, is higher in HDL than LDL (Table 2). Nevertheless, this difference is not sufficient to explain the different reactivity observed in the kinetics of lipid peroxidation. On the contrary, if we consider the levels of vitamin E, the quantitatively most important antioxidant present in lipoprotein, LDL is more protected than HDL; in fact moles of vitamin E associated to one mole of LDL are 15-fold that associated to one mole of HDL (Table 1). On the basis of its chemical structure, vitamin E is probably inserted in the phospholipid monolayer of lipoprotein [39], therefore it could be better to express the vitamin E concentrations as a function of lipoprotein phospholipid content. The vitamin E/phospholipid ratio is significantly lower in HDL than in LDL (Table 1); this could play an important role in the observed higher oxidability of the HDL envelope.

The existence of a finite number of copper binding sites, that can initiate lipid peroxidation in LDL, has recently been demonstrated by Esterbauer and colleagues [36]; we applied the approach described by these authors to evaluate the binding copper capacity of LDL and HDL. To calculate the copper concentration, in which half the LDL and HDL prooxidative binding sites are occupied, we used lag times determined at different copper concentrations in the envelope (Fig. 8) and core (Fig. 7) of both lipoproteins. As expected the calculated prooxidant copper binding sites within LDL and HDL did not depend on the probe used to follow lipid peroxidation, and LDL showed a greater affinity for oxidizing copper (K = 4.83 μM and K = 4.53 μM determined with P_6Chol and P_{12}CS respectively) than did

HDL (K = 6.7 μM and K = 6.58 μM determined with P_6Chol and P_{12} respectively). On the contrary, calculated lag time $_{min}$, which is inversely correlated to the radical generating capacity [36], is very similar when it is calculated for the hydrophobic core of LDL and HDL (23.7 and 27.4 min respectively), but dramatically different when calculated for the envelope of the two lipoproteins. In fact lag time $_{min}$ calculated for LDL (51.6 min) is about 4-fold that calculated for HDL (13.66 min). Thus the radical generating capacity of copper bound to HDL is about 4-fold that of copper bound to LDL. As mentioned above, HDL, if compared with LDL, is characterized by lower concentrations of vitamin E which is preferentially located in the lipoprotein envelope, and higher amounts of polyunsaturated fatty acids esterified to phospholipids; in addition, Bowry and colleagues [12] demonstrated that HDL is the principal vehicle for circulating plasma lipid hydroperoxides. Taken together, these observations could explain the higher radical generating capacity associated to the envelope of HDL.

In our experimental conditions LDL and HDL were 10- and 20-fold diluted with respect to their plasma concentrations; the physiological copper concentrations were about 10 μM, this being associated to ceruloplasmin, thus a 10- and 20-fold dilution of copper corresponds to 1 μM and 0.5 μM respectively. Comparing the kinetics of LDL and HDL peroxidation (Fig. 8) obtained with 1 and 0.5 μM $CuSO_4$ respectively, the resistance to oxidation of the HDL envelope (186 min) is 2-fold less than that of LDL (300 min). Ceruloplasmin at physiological concentrations can release, or express, enough copper ions to catalyze lipoprotein oxidation if exposed at moderately acid pH [40]. Although there is no experimental evidence for this, an acidic condition could theoretically occur within the atherosclerotic lesion, since the media of large arteries is paradoxically one of the most poorly perfused tissues in human body and hence experiences a low oxygen tension [41] which may result in a local acidosis. In addition, atherosclerosis is often described as a chronic inflammatory disease, and by analogy to other inflammatory sites, the pH could be expected to fall below pH 7.4. If low pH values were present within the atherosclerotic lesions, the HDL envelope could constitute the first target of the Cu-mediated peroxidative damage.

In conclusion, the modifications of the envelope of HDL consequent to oxidative stress could be important in the altered reverse cholesterol transport observed with oxidized HDL [23], and provide early markers for monitoring the initial stages of the oxidative imbalance that could be involved in the development of atherosclerotic lesions.

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