Generation in Human Plasma of Misfolded, Aggregation-Prone Electronegative Low Density Lipoprotein

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ABSTRACT Human plasma contains small amounts of a low density lipoprotein in which apoprotein is misfolded. Originally identified and isolated by means of anion-exchange chromatography, this component was subsequently described as electronegative low density lipoprotein (LDL)(−), with increased concentrations associated with elevated cardiovascular disease risk. It has been recognized recently as the trigger of LDL amyloidogenesis, which produces aggregates similar to subendothelial droplets observed in vivo in early atherogenesis. Although LDL(−) has been produced in vitro through various manipulations, the mechanisms involved in its generation in vivo remain obscure. By using a more physiological model, we demonstrate spontaneous, sustained and noticeable production of LDL(−) during incubation of unprocessed human plasma at 37°C. In addition to a higher fraction of amyloidogenic LDL(−), LDL purified from incubated plasma contains an increased level of lysophospholipids and free fatty acids; analysis of LDL lipids packing shows their loosening. As a result, during plasma incubation, lipid destabilization and protein misfolding take place, and aggregation-prone particles are generated. All these phenomena can be prevented by inhibiting calcium-dependent secretory phospholipases A2. Our plasma incubation model, without removal of reaction products, effectively shows a lipid-protein interplay in LDL, where lipid destabilization after lipolysis threatens the apoprotein's structure, which misfolds and becomes aggregation-prone.

INTRODUCTION

Subendothelial retention of low density lipoproteins (LDL), mainly in the form of lipid droplets, activates a series of biological events culminating in an inflammatory response, which is considered the first hallmark of atherogenesis (1). A high level of LDL in plasma is widely regarded as a major risk factor for developing vascular disease, and can reasonably be assumed to be responsible for the accumulation of lipids in the subendothelial space. However, a mechanistic link between LDL levels and atherogenesis remains lacking, and this gap in our knowledge has inspired the search for a modification that transforms an otherwise innocuous cholesterol carrier—the native LDL—into a new species able to trigger an inflammatory response. To produce an LDL toxic to arterial walls and able to reproduce early biological disease events, several modifications have been attempted in vitro, the most popular of which was massive lipid peroxidation (2,3). Such drastic modifications in LDL elicit inflammatory and unspecific toxic responses in cells in culture, and result frequently in the production of aggregates that are taken up by macrophages, thereby mimicking the early formation of foam cells. A major limitation of the oxidative theory of atherogenesis is the difficulty encountered in unequivocally detecting oxidatively modified LDL in the bloodstream (4,5); this drawback prompted the search for alternative, nonoxidative modifications.

Modified LDL that is toxic to cultured endothelial cells, and that induces events compatible with a cell response-to-injury has, in fact, been identified in human plasma (6). This form of LDL was isolated by means of anion-exchange chromatography, and subsequently named electronegative LDL (LDL(−)). Many reports have described the relationship between an increase in LDL(−) and increased cardiovascular disease risk. Indeed, several risk markers correlate with LDL(−) levels, namely hypercholesterolemia, diabetes mellitus types 1 and 2, kidney failure, exhausting physical exercise, and postprandial lipemia (7–12). Finally, it has been found that LDL(−) lipids are oxidized more readily in vitro (13) and, significantly, that its apoprotein is misfolded, displaying structural and conformational modifications (14).

In the course of our effort to determine further characteristics of this intriguing LDL(−), we recently obtained clear evidence that it is capable of promoting LDL amyloidogenesis (15). The misfolded character of apoB-100 in LDL(−), where the content of the β-sheet structure increases, represents the driving force behind LDL aggregation, whereas the protein misfolding in native LDL is propagated through a paradigmatic domino process. Consistent with protein amyloidogenesis in general, a chaperon protein actually

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inhibits misfolding and aggregation that, conversely, are boosted in the presence of the β-sheet-rich amyloid β peptide. Results obtained from a computational analysis of the apoB structure confirms the ease with which these changes take place by identifying a few peculiar loops that are extremely prone to an α-to-β shift. On the other hand, the relevance of the role played by oxidation in promoting LDL aggregation was ruled out by the analysis of initial clusters (16). Indeed, in LDL aggregates the individuality of initial LDL particles is preserved, whereas oxidatively-modified LDL fuses. Finally, in LDL fibrillogenesis, intermediate aggregates are impressively similar to subendothelial droplets observed in early atherogenesis (15).

Modified LDL with increased affinity for proteoglycans was produced in vitro by secretory phospholipase A2 (17). LDL(−) can also be produced, in vitro, from native LDL (nLDL) by incubation in the presence of vascular cells (18,19), or cobra venom phospholipase A2 (20). However, the mechanism/s responsible for in vivo generation of LDL(−) is/are still unknown.

We have already reported that LDL isolated from plasma that was previously incubated overnight at 37°C is prone to amyloidogenic aggregation (16). In this study, we set out to clarify some of the processes able to generate this aggregation-prone LDL in plasma.

MATERIALS AND METHODS

LDL isolation

LDL was isolated from venous blood following routine procedures (16), either from freshly collected plasma (LDL-F), or from plasma that had been incubated previously at 37°C for different durations (LDL-I). In some of the experiments, LDL was isolated from serum. When specified, 0.1 mM of the inhibitor of the family secretory-PLA2s, 4-bromophenacyl bromide (21,22) (Sigma-Aldrich, Milan, Italy), or alternatively that of the calcium-dependent lipoprotein-associated PLA2 (Lp-PLA2), Pefabloc (23,24) (Sigma-Aldrich), was added to plasma or serum at the beginning of incubation. The use of serum instead of plasma was a test for the chelating efficiency of the small amount of EDTA present in the Vacutainer tubes used to collect blood, particularly when activity of the calcium-dependent or independent PLA2 had to be tested. From our results (not shown), this EDTA amount did not exert any role on PLA2s activities. Blood donors were healthy, normolipemic, and normal fasting volunteers in the age range from 29 to 55 years. LDL concentration was determined as the apoprotein concentration by using the Bradford reagent. In total LDL, subclasses were analyzed by using anion-exchange chromatography in an ÄKTA-FPLC system (GE Healthcare, Amersham Biosciences, Piscataway, NJ) using a MonoQ 5/50 GL column and a multistep gradient from 0 to 0.3 M NaCl. Percent amount of subclasses was calculated from the elution profiles by integrating the absorbance of each eluted peak using the Origin software (Microcal Software, Northampton, MA).

Lipid analysis

Lipids were extracted from LDL by adding 3 mL of $CHCl_3$/MeOH = 1:2 to 0.8 mL of LDL samples. After vortexing, 0.8 mL of a 0.88% KCl solution, plus 3 mL of $CHCl_3$ were added, the samples were vortexed again, centrifuged, and the upper aqueous phase removed and discarded. The organic phase was dried under a gentle stream of nitrogen and redissolved in 0.5 ml of $CHCl_3$/MeOH = 1:1; samples were stored at −20°C until undergoing analysis. All solvents contained 10 mg/L of butylated hydroxytoluene. TLC analysis was carried out on silica gel TLC plates (Merck Kieselgel 60, Merck, Darmstadt, Germany). Lipid classes were separated in chloroform/methanol/30% ammonia (65:25:5 v/v). After elution, the lipids were examined and identified using authentic standards. The spots were scraped and methylated in 2 mL of 5% acetyl chloride (in MeOH) at 80°C for 2 h in the presence of 15:0 FA internal standard. The resulting fatty acid methyl esters were injected into a GC-MS system (GC-2010, Shimadzu Deutschland GmbH, Duisburg, Germany) fitted with a BPX70 capillary column (MS Wil GmbH, Wil, Switzerland).

Fluorescence measurements

LDL samples (50 μg of protein/ml) were equilibrated at 37°C in the fluorimeter cell holder and blank spectra were acquired for subtraction later on. The LDL was then labeled with 2-dimethylamino-6-lauroylnaphthalene (Laurdan, Molecular Probes, Eugene, OR) by adding 0.3 μL of a 1.3 mM solution of the probe in dimethylsulfoxide, incubated for 15 min. After this, emission spectra were acquired from 430 nm to 500 nm, using 360 nm excitation and 8 nm bandwidths. A GREG 200 fluorometer (ISS, Champaign, IL) fitted with a xenon arc lamp and photon counting electronics (PX01; ISS) was used. The cell holder was thermostatically maintained at 37.0 ± 0.1°C using a circulating water bath. After subtraction of the blank, the Laurdan generalized polarization (GP) value was calculated from the emission spectra according to

$$\mathrm{GP} = (I_{440} - I_{490})/(I_{440} + I_{490}), \quad (1)$$

where I_{440} and I_{490} are the emission intensities at 440 nm and 490 nm, respectively (25).

Circular dichroism

Spectra from the different LDL samples (with apoprotein concentrations of 0.1 μM) were recorded on a JASCO spectropolarimeter (Tokyo, Japan) using a 0.1 cm quartz cuvette. The cell holder compartment was maintained at 37 ± 0.1°C. Six spectra were averaged for each measurement.

Light scattering

The light scattered from 1 mg/mL of LDL protein was monitored using a commercial light scattering ALV spectrometer set-up (ALV, Langen, Germany), consisting of a CGS-5000 rotating arm goniometer, a photomultiplier tube (PMT) (EMI, Ruislip, UK), an ALV 5000 multi-tau digital correlator operating at a sampling time of 200 ns, and an Innova 70 argon ion laser (Coherent, Santa Clara, CA) operating at 488 nm and 100 mW. The scattering cell was immersed in a refractive index matching fluid (toluene) kept at 37 ± 0.1°C. Light scattering data were collected simultaneously from a scattering volume of ~100 μm^3, and analyzed using specially developed software. Angular runs were carried out with angles logarithmically scaled in $\sin(\theta)$, where θ is the scattering angle, ranging from 30° to 150°.

RESULTS

LDL(−) is produced in plasma

LDL isolated from LDL-F is composed of a main fraction, called nLDL, and of a minor, more electronegative fraction, known as LDL(−), which accounts for <10% of total LDL (Fig. 1 *A*, *solid curve*) (6). When averaged over the 30 healthy individuals used in this study, LDL(−) levels measured 8.2 ± 0.5%. This fraction dramatically increased when LDL was isolated from plasma – or serum – that had been previously incubated at 37°C (LDL-I) for ~20 h, in

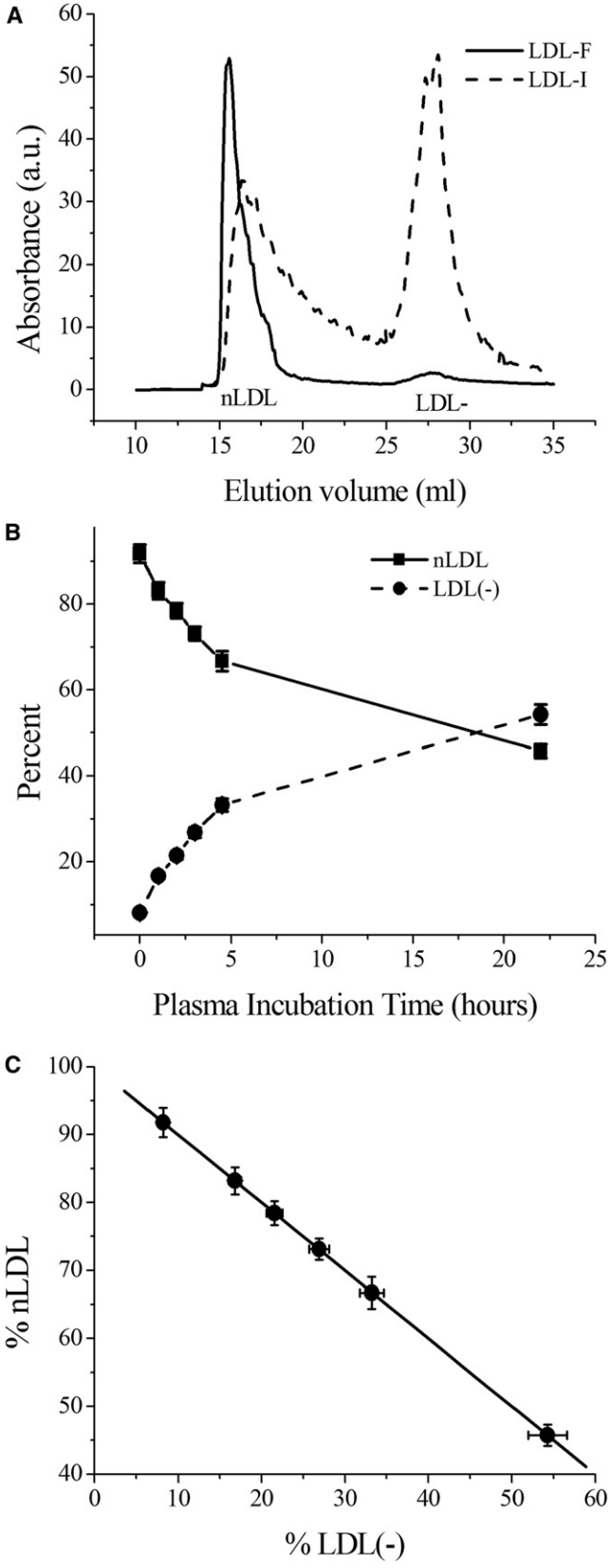

FIGURE 1 Conversion of nLDL in LDL(−) during plasma incubation. (*A*) Representative FPLC chromatograms of LDL-F (*solid curve*) and LDL-I after an incubation of 20 h (*dashed curve*) is reported. (*B*) Percent amount of nLDL (■, *solid curve*) and of LDL(−) (●, *dashed curve*) versus plasma incubation time. Average values of three independent readings, with SE. (*C*) Percent amount of nLDL versus LDL(−). The solid line represents a linear fit. Slope = 1.

parallel with a decrease in nLDL (Fig. 1 *B*, *dotted curve*). Averaged over all subjects, the amount of nLDL decreased to 42.1 ± 2.5%, with a corresponding increase in LDL(−) to 57.9 ± 1.6%. This increase in LDL(−) was not affected by the addition of antioxidants to plasma, such as 0.1 mM Trolox C or 0.1–0.3 mM vitamin C (not shown), thus ruling out any involvement of oxidative mechanisms. No LDL(−) formation was observed when plasma was incubated at 4°C or, as reported (14), when LDL purified from fresh plasma was subsequently incubated at 37°C.

The time-course of the process (Fig. 1 *B*) suggests a direct and quantitative conversion of nLDL into LDL(−), confirmed by the linear plot of nLDL versus LDL(−) percentages (Fig. 1 *C*).

Identification of the enzymatic activity that primes LDL(−) formation

Based on previous in vitro evidence (20), the most likely candidate enzyme responsible for the formation of LDL(−) was a phospholipase A2 present in plasma. In fact, present evidences show that with respect to LDL-F, both lysophosphatidylcholine (LPC) and free fatty acid (FFA) content increased in LDL-I (Fig. 2 *A*), consistent with the LPC/PC ratio (Fig. 2 *B*) calculated from GC-MS analysis. No relevant changes were observed in sphingomyelin, cholesterol esters or triglycerides (not shown). Various PLA2s are present in plasma: the family of the 10 secretory, calcium-dependent PLA2s (sPLA2s), and the calcium-independent PLA2, also known as Lp-PLA2, or platelet-activating factor acetylhydrolase. The effect that these enzymes exert in the formation of LDL(−) in plasma—or in serum, as detailed in Materials and Methods—was tested by using specific inhibitors. The results, reported in Fig. 2 *C*, unequivocally show that only the inhibitor of sPLA2s hinders LDL(−) formation, and prevents the production of LPC and FFA (Fig. 2, *A* and *B*). On the other hand, the involvement of Lp-PLA2 in the conversion process can be ruled out on account of the absence of any effect exerted by its specific inhibitor.

Effect of phospholipid hydrolysis on LDL lipid structure

The observed increase in LPC—and in FFA—is expected to modify the packing of surface lipids in LDL. To verify and characterize this anticipated modification we used the spectral sensitivity of the lipophilic fluorescent probe Laurdan, widely used as a sensitive detector of changes in lipid dynamics (25,26). We acquired Laurdan spectra and calculated the GP value (Eq. 1) in LDL purified after different plasma incubation times. A progressive spectral shift toward

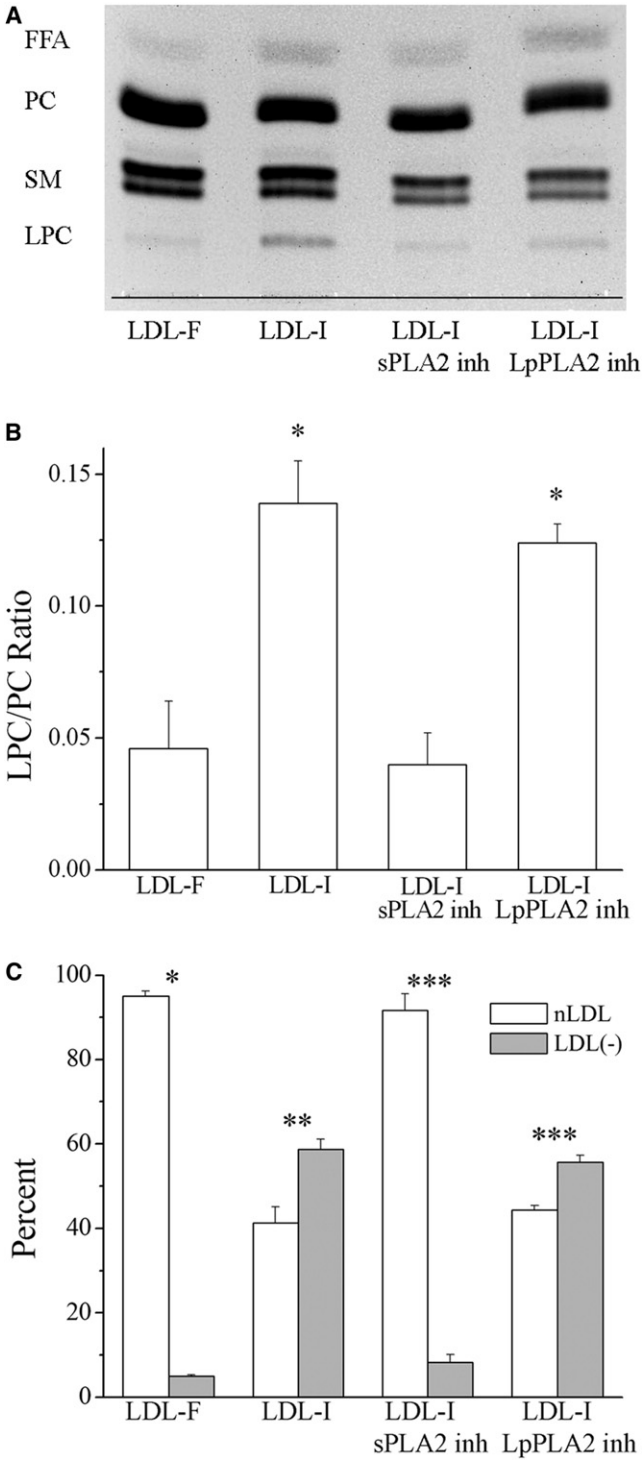

FIGURE 2 Lysophospholipids in LDL(−) and the role of phospholipases. (*A*) Representative chromatogram of lipid extracted from LDL-F and from LDL-I purified after 20 h of plasma incubation, also in the presence of the sPLA2 or LpPLA2 inhibitor. LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; FFA, free fatty acids. (*B*) LPC/PC ratio calculated in the same samples as in *A*, determined by GC-MS. Average with SE calculated from three independent experiments; *significance $p \leq 0.05$. (*C*) Percentage of nLDL (*white*) and LDL(−) (*gray*) subfractions in total LDL determined in the above-mentioned samples. Averages of: *, 23; **, 31; and ***, three independent experiments, with SE.

longer wavelengths was observed with increased plasma incubation times (Fig. 3 *A*), resulting in progressively lower GP values (Fig. 3 *B*). As has been reported previously (25), this is related to an increased water penetration into lipids, caused by a loosening of their packing. The kinetics of GP decrease was consistent with the increase in LDL(−) content (Fig. 3 *B*). In agreement with the variation in the production of LPC (Fig. 2), in the presence of the sPLA2s inhibitor no significant GP modification was observed, whereas a GP decrease still occurred when using the LpPLA2 inhibitor (Fig. 3 *C*).

Effect of plasma incubation on LDL apoprotein misfolding and on aggregation

In agreement with its higher content in LDL(−), a decreased ellipticity was observed in LDL-I, isolated after a plasma incubation time of 20 h, as compared to that seen in LDL-F (Fig. 4 *A*) (14). Due to the presence of a consistent fraction of nLDL in LDL-I (Fig. 1 *B*), its CD absorbance was still higher than that of isolated LDL(−). The consequences of the presence of PLA2 inhibitors during plasma incubation could also be detected through the CD absorbance spectra. Inhibition of the sPLA2s prevented the ellipticity loss, resulting in a CD spectrum that overlapped the LDL-F's corresponding spectrum. Inhibition of Lp-PLA2, on the other hand, had no effect (Fig. 4 *A*).

Light scattering intensity results showed that isolated LDL-I further incubated at 37°C forms aggregates, whereas LDL-F does not (Fig. 4 *B*) (14). In a previous study, we showed that the trigger for LDL aggregation resides in the misfolded apoB-100 present in LDL(−) (15). Hence, the final problem we sought to resolve regarded the effects of phospholipase inhibition on LDL aggregation. Inhibitors were added to plasma during incubation; we then monitored the light scattering intensity versus time on isolated LDL samples. In agreement with the lack of an increase in LDL(−) and of a structural modification in its apoprotein, LDL-I isolated from plasma in which the sPLA2s inhibitor was present did not show any aggregation. Conversely, inhibition of Lp-PLA2 in plasma had no effect, and LDL-I aggregated in a way similar to that seen in the sample without an added inhibitor (Fig. 4 *B*).

These findings also confirm that LDL(−) produced by sPLA2s activity in plasma is in all respects identical to the LDL(−) isolated in small amounts from fresh plasma.

DISCUSSION

Almost 30 years after the groundbreaking identification of the electronegative LDL subfraction (6), LDL(−), which lately has been associated with several markers of cardiovascular disease risk (7–12), in our quest for the mechanism responsible for LDL(−) generation in vivo we have succeeded in obtaining evidence for a spontaneous lipolytic

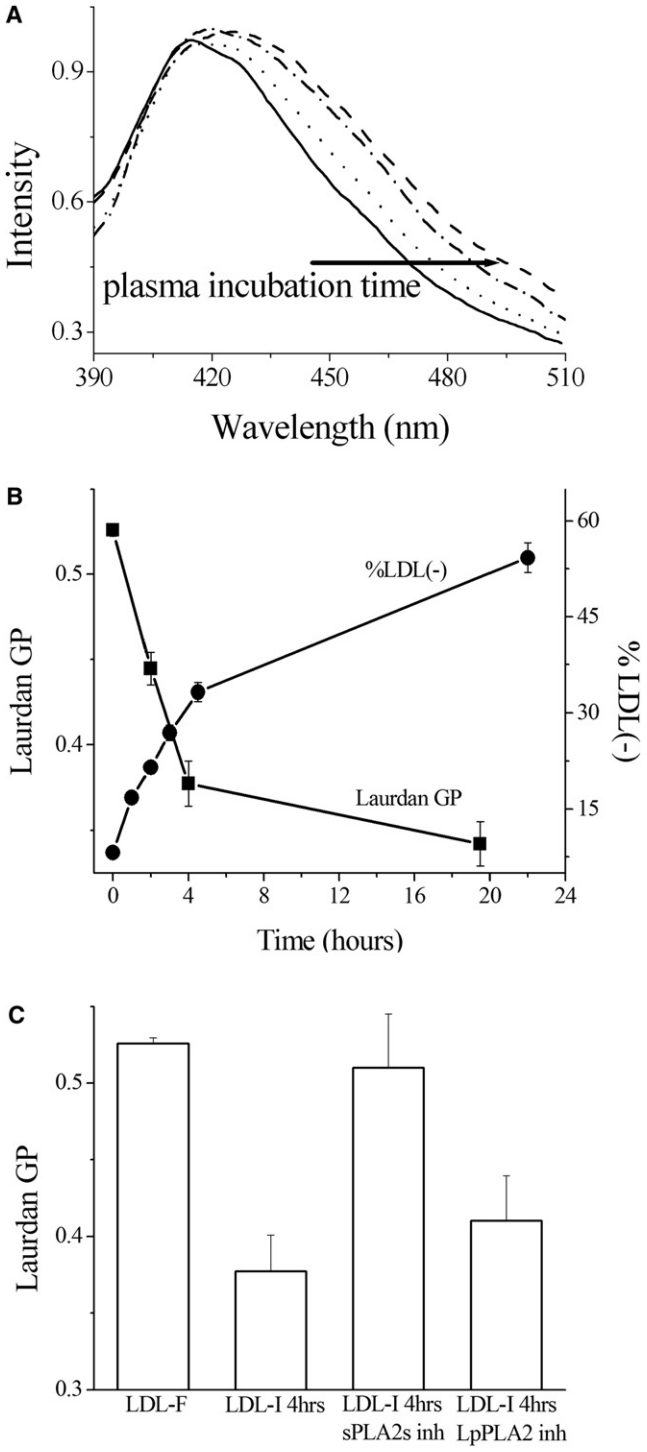

FIGURE 3 Loosening of LDL lipid structure after phospholipid hydrolysis. (*A*) Representative normalized Laurdan fluorescence emission spectra measured in LDL purified from plasma after various incubation times: 0, 2, 4, and 20 h. (*B*) Average GP values of similar samples, as a function of plasma incubation time, from three independent experiments with SE (■). For comparison, the FPLC data on the percentage amount of LDL(−) are reproduced (●). (*C*) Laurdan GP values measured in LDL-F, in LDL-I isolated after 4 h of plasma incubation, and after the same length of time with incubation carried out in the presence of the sPLA2s, or of the LpPLA2 inhibitor. Average of three independent experiments with SE.

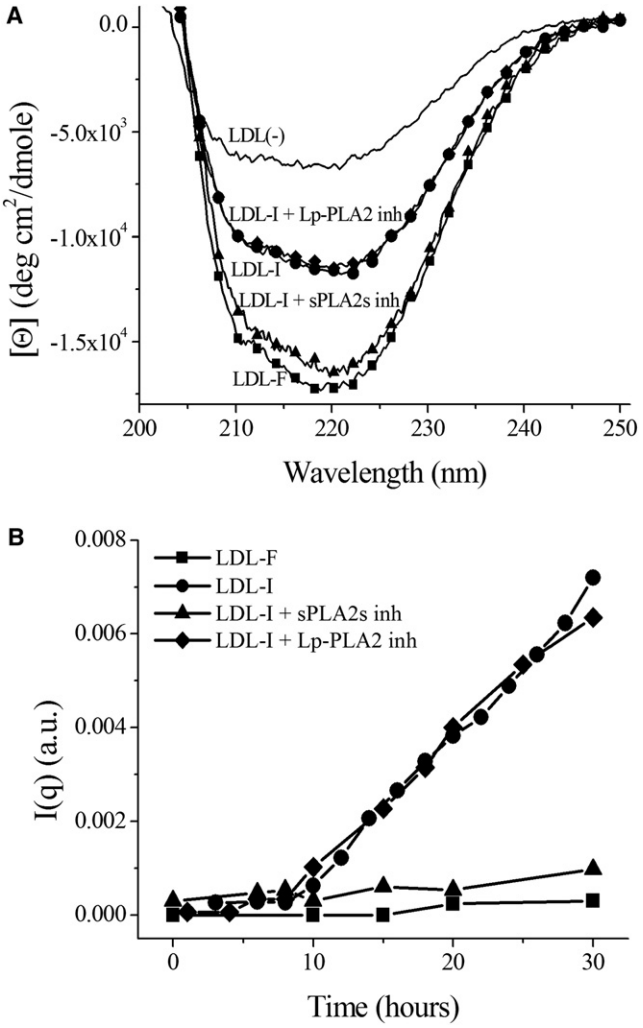

FIGURE 4 Ellipticity loss of LDL apoprotein and LDL aggregation after phospholipid hydrolysis. (*A*) CD spectra, and (*B*) light scattering intensity curves obtained from: LDL-F (■), LDL-I isolated after 20 h of plasma incubation (●), also in the presence of sPLA2s (▲) or of Lp-PLA2 inhibitor (◆). In *A*, the CD spectrum of LDL(−) (*solid curve*, *no symbol*), isolated and collected by means of anion exchange chromatography, is also reported.

conversion of nLDL into LDL(−) in human plasma. We have demonstrated this process by means of the simple incubation of plasma at 37°C, resulting in the generation of LDL(−) that has all the features of a misfolded protein prone to amyloid aggregation, commensurate with those described previously for LDL(−) isolated from fresh plasma (14–16). In our ex vivo conditions, this prompt and massive production of LDL(−) is a nonequilibrium process, in the absence of the organism's removal mechanisms. Our procedure also excludes the possibility that LDL(−) undergoes a lipolytic modification in the artery wall—either intracellular or extracellular—and then returns to the plasma compartment; an unsolved problem concerning instead the small quantity of circulating oxidized LDL (4).

The report that, in vitro, cobra venom PLA2 efficiently promotes the formation of LDL(−) from nLDL (20),

inspired our search for a similar mechanism functioning in vivo. Our model of unprocessed plasma incubation shows unequivocally that hydrolysis of PC in LDL can occur in plasma and is accompanied by LDL(−) formation. The temperature dependence of this process is a major argument in favor of an enzymatic reaction: no increase in LDL(−) could be detected when plasma was incubated at 4°C.

Among lipolytic enzymes involved in intravascular LDL remodeling, particular attention has been paid recently to some of the 10 components of the family of secretory, calcium-dependent PLA2s (sPLA2s), and to the lipoprotein associated, calcium-independent PLA2 (Lp-PLA2 or platelet-activating factor acetylhydrolase) (17,27–29). LDL treated in vitro with these enzymes has not been structurally characterized in the past, but has been shown to elicit cellular responses that mimic the early phases of atherogenesis, and to enhance the binding of lipoproteins to proteoglycans.

Type II, V, and X sPLA2 have been suggested to play a role in cardiovascular diseases. Data from a study concerning the Type II suggest the presence of this phospholipase both in control and in stable/unstable angina patients (30). Data on the Type X indicate that expression of this sPLA2 is constitutive rather that inducible (27) and that it has a potent lipolytic effect on LDL, with consequences on an increased negative charge (31). Although several studies exist on the quantitative evaluation of other sPLA2 classes in the vascular tissue or in the atherosclerotic lesions, little is reported on specific activity measurement in plasma. Significantly, the products of their activity are proinflammatory lipids such as lysophospholipids and free unsaturated fatty acids. Clinical observations also strongly support an association between high levels of circulating sPLA2s and increased risk of cardiovascular disease (17,32,33), thus highlighting two possible sites where these enzymes act: in the subendothelium, or alternatively on circulating lipoproteins.

Without excluding other possible mechanisms for LDL modification that take place in the subendothelium, our data in this study shows that, in plasma, phospholipase activity on circulating LDL results in an increased concentration of lysophospholipids. The straightforward effect of a sPLA2s inhibitor strongly supports the involvement of one or more members of this family of enzymes. Instead, Lp-PLA2 is apparently ruled out by the absence of any effect of its specific inhibitor. This lipoprotein-associated phospholipase would also be expected to generate LDL(−) during the incubation of isolated LDL, an effect that, consistent with our previous data (15,16), was not in fact observed. Indeed, no LDL(−) generation was observed incubating at 37°C LDL after its isolation.

Laurdan GP served well for the detection of changes induced on LDL lipids by an increased LPC content. LPCs possess a structure similar to that of most detergents and therefore exert a loosening effect on the packing of LDL surface lipids. Laurdan GP value showed quite a significant diminution, from 0.53 to 0.34. Although this occurrence cannot be translated into a quantitative evaluation of lipid compositional change, it serves to provide an idea of the structural effect when compared with what observed in membranes containing cholesterol. In phospholipid vesicles containing 30% cholesterol—a concentration close to that present in cell plasma membranes—such a GP decrease occurs with a temperature increase of >15°C (25). LDL(−) signature chiefly consists in a misfolding of apoB-100, with an α-to-β structural shift. Given that apoB-100 is a multipotential protein that interacts closely with lipids, and possesses several functional regions that can all respond to key modifications in selected domains (34–36), it is not surprising that significant lipid loosening is able to affect apoB-100 conformation.

An interesting question concerns the relationship between phospholipid hydrolysis and protein misfolding. Following a consensus model for LDL structure (37), the apoB-100 is located in the outer surface together with phospholipid polar heads, but at the same time seeps through the interfacial layer and down to the core. In this situation, conformational modifications in the protein have already been reported as a consequence of lipid modification (for a comprehensive review see Hevonoja et al. (36)). Worthy of note, a treatment that uses bee venom PLA2 has been reported to alter the immunoreactivity of a protein site (38). A further example of this interplay between protein and lipid structure is provided by our previous demonstration that the binding to the protein of a single estradiol molecule per LDL particle is able to affect the structure of both the protein and the lipid's interfacial and core regions (39). We can therefore conclude that phospholipid hydrolysis/structural loosening, on one hand, and the apoprotein misfolding, on the other hand, represent an additional clear example of the tight lipid-protein structural interaction in this complex and relevant particle. In addition, our results propose a mechanism for the α-to-β switch in the apoB-100 few loops, identified previously through computational analysis as extremely aggregation-prone (15).

The LDL(−) produced on a massive scale in incubated plasma is in all respects identical to LDL(−) present in small amounts in fresh plasma, the amyloidogenic character of its protein included. The possibility that aggregation might be primed directly by LPC rather than by misfolded apoB-100 is extremely unlikely. Although LPCs possess structural characteristics similar to those of detergents and can therefore promote fusion, LDL aggregates do not contain fused LDL. We have shown previously the presence, in LDL clusters, of readily identifiable particles of the initial LDL size. Fractal analysis and the determination of a reaction-limited interaction mechanism both suggested a few protein sites as the drivers that produce aggregation (16).

Subsequent to our previous report on the aggregation propensity of LDL isolated from plasma that was previously incubated overnight at 37°C (16), we now identified in LDL(−) the modified particle that is produced in plasma,

able to trigger the amyloidogenic process, and a mechanism for its generation.

Although the possibility that other mechanisms capable of affecting the lipids or protein structure in LDL exists, the picture emerging from this evidence involves an active lipolytic process occurring in plasma that is able to destabilize the structure of LDL lipids. Concurrently, a misfolding in apoB-100 takes place, which renders the LDL particle aggregation-prone by means of a process that has already been described as typically amyloidogenic. Intriguing questions that remain open concern the regulation of sPLA2s concentration and/or activity in vivo, together with the metabolic processes able to remove the apparently continuously formed LDL(−). Answer to these questions will add to the role of LDL(−) in vascular disease and possibly to pharmaceutical interventions for regulating its concentration.

Lipid analysis was done in Laszlo Vigh's laboratory, Biological Research Centre, Institute of Biochemistry, Szeged, Hungary. The authors declare that no conflict of interest exists.

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