Novel Phospholipolytic Activities Associated with Electronegative Low-Density Lipoprotein Are Involved in Increased Self-Aggregation†

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ABSTRACT: Electronegative low-density lipoprotein (LDL(−)) is a minor LDL subfraction present in plasma with increased platelet-activating factor acetylhydrolase (PAF-AH) activity. This activity could be involved in the proinflammatory effects of LDL(−). Our aim was to study the presence of additional phospholipolytic activities in LDL(−). Total LDL was fractionated into electropositive (LDL(+)) and LDL(−) by anion-exchange chromatography, and phospholipolytic activities were measured by fluorometric methods. Phospholipolytic activity was absent in LDL(+) whereas LDL(−) presented activity against lysophosphatidylcholine (LPC, 82.4 ± 34.9 milliunits/mg of apoB), sphingomyelin (SM, 53.3 ± 22.5 milliunits/mg of apoB), and phosphatidylcholine (PC, 25.7 ± 4.3 milliunits/mg of apoB). LDL(−), but not LDL(+), presented spontaneous self-aggregation at 37 °C in parallel to phospholipid degradation. This was observed in the absence of lipid peroxidation and suggests the involvement of phospholipolytic activity in self-aggregation of LDL(−). Phospholipolytic activity was not due to PAF-AH, apoE, or apoC-III and was not increased in LDL(+) modified by Cu^{2+} oxidation, acetylation, or secretory phospholipase A_2 (PLA_2). However, LDL(−) efficiently degraded phospholipids of lipoproteins enriched in LPC, such as oxidized LDL or PLA_2-LDL, but not native or acetylated LDL. This finding supports that LPC is the best substrate for LDL(−)-associated phospholipolytic activity. These results reveal novel properties of LDL(−) that could play a significant role in its atherogenic properties.

Growing evidence indicates that qualitative modification of lipoproteins is a key step in atherogenesis. Oxidation, glycosylation, desialylation, or enzymatic modification of LDL[1] renders modified lipoparticles with atherogenic, inflammatory, and immunogenic properties (*1*). Most of these studies with modified lipoproteins were conducted using "in vitro" modification procedures. However, several groups have studied the properties of modified lipoproteins isolated from plasma. One of these lipoproteins is electronegative LDL (LDL(−)), a minor subfraction of LDL that presents inflammatory, apoptotic, and cytotoxic properties "in vitro" (*2–4*) and whose proportion is increased in subjects with high cardiovascular risk (*5, 6*). The inflammatory action of LDL(−) includes the release by endothelial cells of interleukins (IL-6), chemokines (IL-8, MCP-1,GROs, ENA78, GPC2), growth factors (GM-CSF, PDGF), and vascular adhesion molecules (VCAM) (*7–10*). A specific difference between LDL(−) and native LDL is the increased content in the former of proteins other than apolipoprotein (apo) B-100, such as apoC-III, apoE, and platelet-activating factor acetylhydrolase (PAF-AH) (*11, 12*). The role of these proteins is unclear; however, it has been suggested that PAF-AH could play a role in the inflammatory properties of LDL(−). LDL(−) has increased content of the degradation products of PAF-AH activity, lysophosphatidylcholine (LPC), and nonesterified fatty acids (NEFA), with both molecules being known to promote the expression of inflammatory molecules in cells from the artery wall (*13*). On the other hand, LDL(−) presents a high susceptibility to aggregation (*7*), a property that could favor its subendothelial retention. On the basis of these findings, our group studied the presence in LDL(−) of additional enzymatic activities related to lipid metabolism, inflammation, and lipoprotein aggregation, specifically focused on sphingomyelinase (SMase) and other phospholipolytic activities. SMases are a group of phospholipases that catalyze the hydrolysis of sphingomyelin into

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[1] Abbreviations: apo, apoprotein; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; ENA, epithelial neutrophil activator; GRO, growth-related oncogene; GGE, nondenaturing gradient gel electrophoresis; GM-CSF, granulocyte/monocyte-colony stimulating factor; GPC, granulocyte chemotactic protein; HPODE, hydroperoxide; IL, interleukin; LDL(+), electropositive low-density lipoprotein; LDL(−), electronegative low-density lipoprotein; LPC, lysophosphatidylcholine; lysoPLC, lysophospholipase C; MCP, monocyte chemotactic protein; NEFA, nonesterified fatty acid; PAF-AH, platelet-activating factor-acetylhydrolase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; $sPLA_2$, secretory phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SM, sphingomyelin; SMase, sphingomyelinase; VCAM, vascular cell adhesion molecule.

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ceramide and phosphorylcholine. The role of ceramide as a lipid second messenger in apoptosis, cell differentiation, and cell proliferation emphasizes the role of SMases in atherogenesis (*14*). Holopainen et al. previously reported that LDL present SMase activity and suggested that this activity could be a link between atherosclerosis and apoptosis (*15*). On the other hand, a key role in lipoprotein retention in the artery wall has been suggested for secretory SMase through aggregation and fusion of LDL (*16, 17*). SMase activity could be involved in several distinctive properties of LDL(−), including apoptosis and susceptibility to aggregation. Our results suggest that LDL(−) presents one or several enzymatic activities generating phosphorylcholine (phospholipase C activity) that degrade with high-affinity LPC and sphingomyelin (SM) and only poorly phosphatidylcholine (PC). The involvement of these findings in the atherogenic characteristics of LDL(−), such as increased susceptibility to aggregation and inflammatory properties, is discussed.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma (Madrid, Spain) unless otherwise stated.

LDL Subfraction Isolation. Plasma samples were obtained from healthy normolipemic subjects from the hospital staff, as described (*7*). The Ethics Committee of the hospital approved the study, and volunteers gave their informed consent. Total LDL ($1.019 < d < 1.050$ g/mL) was isolated by flotation sequential ultracentrifugation at 4 °C and in the presence of 1 mM EDTA. LDL was then subfractionated into nonmodified LDL (LDL(+)) and LDL(−) by anion-exchange chromatography, as described (*7*). Composition of LDL subfractions was assessed as previously described (*7, 11, 13*). Lipoperoxide content in LDL subfractions was determined by the leucomethylene blue method (*18*), and 8-isoprostane was quantified by commercial enzyme immunoassay (Cayman Chemicals).

LDL Modification. LDL was oxidized by incubation of PBS-dialyzed LDL(+) (at 0.5 g/L of apoB) with $CuSO_4$ (2.5 μmol/L) at 37 °C for increasing time periods. On the other hand, LDL(+) was lipolyzed with secretory phospholipase A_2 ($sPLA_2$) (20 ng/mL), as described (*13*). Acetylated LDL was obtained by modifying Lys residues of apoB by sequential addition of acetic anhydride (*9*). LDL(+) was enriched with apoE or apoC-III by incubating LDL(+) (at 0.5 g/L) with apoE (15 and 60 mg/L) or apoC-III (5 and 20 mg/L) for 2 h at 37 °C, and LDL(+) was then reisolated by ultracentrifugation at 40000 rpm for 6 h at 4 °C. Finally, LDL(+) (0.5 g/L) was aged by incubation at 4 °C with PBS containing 2 μM BHT up to 40 days.

Characterization of Modified LDLs. Major lipids (free and esterified cholesterol, triglyceride, total phospholipids, and NEFA) and apoproteins (apoB, apoE, apoC-III) were measured by commercial methods in a Hitachi 911 autoanalyzer (*7*). Phospholipid subclasses, including PC, SM, and LPC, were measured by normal-phase HPLC (Gold System, Pump module 126, detector 168, Beckman Coulter, Fullerton, CA) using a Luna 5 μm silica 250 × 4.6 mm column (Phenomenex, Torrance, CA) with photodiode array detection, as described (*13, 20*). Main peaks of phospholipids were detected at 205 nm, and conjugated dienes in the peak corresponding to PC (indicating oxidation of PC) were detected at 234 nm. The aggregation level of LDL was determined by measuring absorbance at 450 nm and by quantifying the proportion of precipitable apoB at low-speed centrifugation (15000*g*, 10 min) (*16, 21*). The content of apoE or apoC-III in apo-enriched LDL(+) was measured by commercial immunoturbidimetric methods (Kamiya, Thousand Oaks, CA). Electrophoretic characterization included agarose electrophoresis (Midigel, Biomidi, Toulouse, France), denaturing acrylamide gradient gel electrophoresis in the presence of SDS (SDS−PAGE) (4−20% gradient gels; Bio-Rad, Madrid, Spain) (*22*), and nondenaturing acrylamide gradient gel electrophoresis (GEE) (*23*).

Phospholipolytic Activity Assays. Phospholipolytic activities in LDLs were measured by a commercial fluorometric method (Amplex Red; Molecular Probes, Leiden, The Netherlands). The method is an enzyme-coupled assay based on the following sequence: (1) phospholipid (substrate) + PLC or SMase (assay sample) → diacylglycerol or ceramide + phosphorylcholine; (2) phosphorylcholine + alkaline phosphatase → P_i + choline; (3) choline + choline oxidase → betaine + H_2O_2; (4) H_2O_2 + peroxidase + Amplex Red → resorufin (highly fluorescent). This method detects phospholipase C (PLC) or sphingomyelinase (SMase) activities and, when alkaline phosphatase is not included, also detects phospholipase D (PLD) activity. The substrate was alternatively PC, LPC, or SM. LDLs (100 μL at 0.3 mg/mL), apoE (100 μL at 15 and 60 mg/L), or apoC-III (100 μL at 5 and 20 mg/L) were mixed with 10 μL of substrate (final concentration 0.25 mmol/L) and with the appropriate amount of Amplex Red and enzymes (final volume 200 μL), according to the manufacturer. *Staphylococcus* sp. SMase was used as a standard curve. Fluorescence production (λ excitation 530 nm, λ emission 590 nm) was monitored for 3 h, and results (milliunits of SMase equivalents/mg of apoB) were calculated from the maximum curve slope.

Several phospholipase inhibitors were tested to investigate whether phospholipolytic activity was due to the presence of a previously characterized enzyme attached to LDL(−); specifically, manumycin A (neutral SMase inhibitor), chlorpromazine (acidic SMase inhibitor, Calbiochem/Merck, Darmstadt, Germany), methyl-γ-linoleyl fluorophosphonate and tricyclodecanoyl dithiocarbonate (D-609) (PC-dependent PLC inhibitors), and Pefabloc (inhibitor of $sPLA_2$ and PAF-AH; Roche, Basel, Switzerland) were incubated with LDL subfractions or *Staphylococcus* sp. SMase (50 milliunits/mL) for 30 min at 37 °C in the presence of 2 μmol/L butylated hydroxytoluene (BHT) before SMase quantification.

The Amplex Red method is not suitable for evaluating the effect of pH and Mg^{2+}, since the mixture of enzymes requires neutral pH and Mg^{2+}. Hence, the effect of pH and Mg^{2+} was determined using a fluorescently labeled substrate, according to Holopainen et al. (*15*). SMase activity in LDL subfractions (0.3 mg/mL) was evaluated by incubation with Bodipy-FL-C12-SM at 0.025 mmol/L, (Molecular Probes, Leiden, The Netherlands) as substrate for 3 h at 37 °C in acid (50 mmol/L sodium acetate, pH 5.0) and neutral (10 mmol/L Tris, pH 7.4) buffer and in the presence or absence of 10 mM $MgCl_2$ and separation by thin-layer chromatography, as described (*15*).

$sPLA_2$ activity was measured by a commercial colorimetric method using 1,2-dithiodiheptanoylphosphatidylcholine as substrate (Cayman Europe, Tallinn, Estonia), according to

Table 1: Composition of LDL Subfractions ($n = 10$) and Oxidized LDL ($n = 4$)

	LDL(+)	LDL(−)	oxidized LDL
total cholesterol (%)	37.8 ± 0.5	36.5 ± 1.9	ND[a]
free cholesterol (%)	10.1 ± 0.2	10.7 ± 0.3	ND
triglyceride (%)	5.7 ± 0.1	9.5 ± 2.4[b]	ND
phospholipid (%)	32.8 ± 0.3	32.2 ± 0.6	ND
NEFA (mol/mol of apoB)	16.3 ± 3.2	35.3 ± 2.5[b]	ND
apoB (%)	23.7 ± 0.3	21.8 ± 1.8	ND
lipoperoxides (nmol/mg of apoB)	5.10 ± 3.86	6.10 ± 4.47	318 ± 283[b]
8-isoprostane (pg/mg of apoB)	289 ± 264	388 ± 314	9185 ± 5476[b]

[a] ND, not determined. [b] $p < 0.05$ vs LDL(+).

the manufacturer's instructions. PAF-AH activity was measured by a commercial colorimetric method using 2-thio-PAF as substrate (Cayman Europe, Tallinn, Estonia), as described (*12*).

Platelet-Activating Factor Acetylhydrolase (PAF-AH) Isolation. PAF-AH was purified from total LDL according to a shortened protocol based on that of Tew et al. (*24*), using sequentially Blue Sepharose and MonoQ HR5/5 columns (AmershamPharmacia, Uppsala, Sweden). PAF-AH active fractions were pooled, concentrated, and stored at 4 °C. SDS−PAGE with silver staining (Silver Stain Plus; Bio-Rad, Spain) and Western blot (*12*) confirmed the purity of isolated PAF-AH.

ApoB Secondary Structure Determination. Far-UV circular dichroism spectra were performed with LDL samples in PBS at 0.05 g/L in a JASCO J-715 spectropolarimeter, as previously described (*25*). Twenty spectra were averaged for each measurement, and the buffer blank was subtracted. Spectra were deconvolved by the analysis program K2D from the DICHROWEB server (*26, 27*).

Statistical Analysis. Results are expressed as mean ± SD. The SPSS 11.5.2 statistical package was used. Intergroup differences were tested with Wilcoxon's *t* test. A *p* value <0.05 was considered significant.

RESULTS

Phospholipolytic Activities in LDL(−). Composition of LDL(+) and LDL(−) is shown in Table 1. Results are similar to those previously reported (*7, 11*), with increased triglyceride and NEFA content in LDL(−). No evidence of increased oxidative modification was observed in LDL(−) compared to LDL(+). A high phospholipolytic activity on choline-containing phospholipids was shown by LDL(−). The best substrate was LPC (lysoPLC activity) followed by SM (SMase activity) and PC (PLC activity) (Table 2, Figure 1A). LDL(+) lacked the ability to degrade LPC, SM, or PC. SMase activity in LDL(−) was confirmed by an alternative method using Bodipy-SM as substrate and separation by thin-layer chromatography (Figure 1B). These differences between LDL(+) and LDL(−) were observed both in acid and in neutral conditions (Figure 1B). SMase activity was Mg^{2+}-independent (Figure 1B). LDL(−)-associated lipolytic activities were of phospholipase C and/or SMase type since phosphorylcholine, but not choline, was generated by LDL(−). This was demonstrated because when alkaline phosphatase (which hydrolyzes phosphorylcholine) was not included in the reaction mixture, no fluorescence was detected (Table 2). This observation indicated that phospholipase D activity was not present in LDL(−). $sPLA_2$ activity was also absent in LDL(−) in contrast to high PAF-AH activity (Table 2), in agreement with previous observations (*12*).

Phospholipolytic Activities in Several Modified LDLs. LDL(+) modified by oxidation, acetylation, or $sPLA_2$-induced lipolysis showed no increased phospholipolytic activity (Table 2). The chemical characteristics of these modified LDLs are shown in Table 3. Each modification induced a typical increase in electrophoretic mobility LDL and different changes in composition. This included increased LPC and decreased PC in PLA_2-modified LDL, decreased SM and PC, and increased dienes and LPC in oxidized LDL and no changes in acetylated LDL.

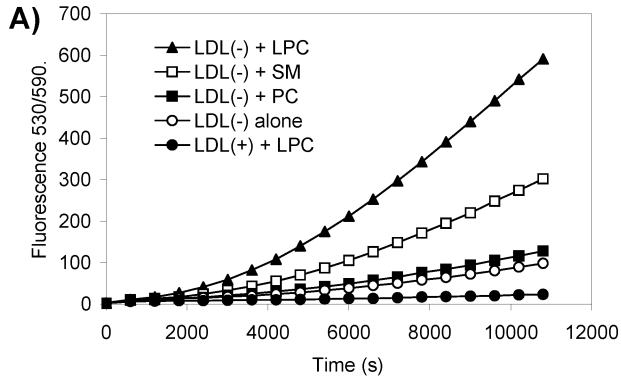
Effect of Phopholipase Inhibitors. The effect of different phospholipase inhibitors on LDL(−)-associated phospholipolytic activities, using SM or LPC as substrates, is shown in Table 4. None of the inhibitors were able to completely inhibit phospholipolytic activities at the concentrations used, although chlorpromazine and methyl-γ-linoleyl fluorophosphonate had mild effects. Chlorpromazine was the most effective inhibitor of phospholipolytic activities of LDL(−) (up to 39% at 400 μM) whereas methyl-γ-linoleyl fluorophosphonate showed a lower inhibitory capacity (16% at 400 μM). In contrast, manumycin A, D-609, and Pefabloc had no significant effect on LDL(−)-associated phospholipolytic activity. The behavior of all inhibitors was similar when LPC or SM was used as substrate, which suggests that lysoPLC and SMase activities of LDL(−) are caused by the same enzymatic activity. *Staphylococcus* sp. SMase was partially inhibited at the concentrations used by chlorpromazine, manumycin, and Pefabloc. PAF-AH activity was efficiently blocked (almost 100%) by Pefabloc and methyl-γ-linoleyl fluorophosphonate (Table 4).

Self-Degradation of LDL(−) Phospholipids and Self-Aggregation. Interestingly, LDL(−) not only degraded exogenous phospholipids added to the reaction mixture but also degraded its own phospholipids. When phospholipolytic activity of LDL subfractions was measured without exogenous substrate, a higher increment in fluorescence was observed in LDL(−) compared to LDL(+) (19.0 ± 3.6 versus 0.7 ± 0.3 milliunits of SMase equivalents/mg of apoB, respectively, $n = 4$). This value was only slightly lower than that observed when PC was used as substrate (22.8 ± 5.6 milliunits of SMase equivalents/mg of apoB; see Table 2), suggesting that PC was a poor substrate for phospholipolytic activity of LDL(−). To confirm the effect of LDL(−) on its own phospholipids, LDL(+) and LDL(−) were incubated for 24 h at 37 °C in the presence of 1 mmol/L EDTA and 2 μmol/L BHT to avoid oxidation. In contrast to LDL(+), whose phospholipid content was unchanged, LDL(−) decreased its PC and SM content to approximately 85% of the original content (Figure 2A). This decrease resulted in increased aggregation of LDL(−) after 24 h of incubation as indicated by the increase in absorbance at 450 nm, decrease in apoB content after high-speed centrifugation, and presence of higher sized bands in GGE (Figure 2B,C). In the same experiment, PBS-dialyzed LDL(+) was oxidized with 5 μM $CuSO_4$. The degradation of phospholipids in LDL(−) was not due to oxidation since absorbance at 234

Table 2: Phospholipolytic Activities of LDL Subfractions and Modified LDLs[a]

	lysoPLC ($n = 6$)	SMase ($n = 6$)	PLC ($n = 6$)	PLD ($n = 2$)	$sPLA_2$ ($n = 2$)	PAF-AH ($n = 6$)
LDL(+)	1.2 ± 0.5	0.9 ± 0.7	1.4 ± 0.6	0.1 ± 0.1	0.3 ± 0.1	1.5 ± 0.4
LDL(−)	85.4 ± 33.7^b	50.3 ± 19.1^b	22.8 ± 5.6^b	0.1 ± 0.2	0.2 ± 0.3	11.9 ± 4.9^b
oxidized LDL ($n = 4$)	2.2 ± 0.8	2.1 ± 0.7	2.2 ± 0.9	ND[c]	ND	ND
acetylated LDL ($n = 2$)	1.4 ± 0.4	1.3 ± 0.3	1.4 ± 0.1	ND	ND	ND
$sPLA_2$-LDL ($n = 4$)	1.2 ± 1.0	1.0 ± 1.1	1.1 ± 0.5	ND	ND	ND

[a] Results (mean ± SD) are expressed as milliunits of SMase equivalents/mg of apoB for lysoPLC, PLC, SMase, and PLD. $sPLA_2$ and PAF-AH are expressed as μmol min^{-1} (mg of apoB)$^{-1}$. [b] $p < 0.05$ vs LDL(+). [c] ND, not determined.



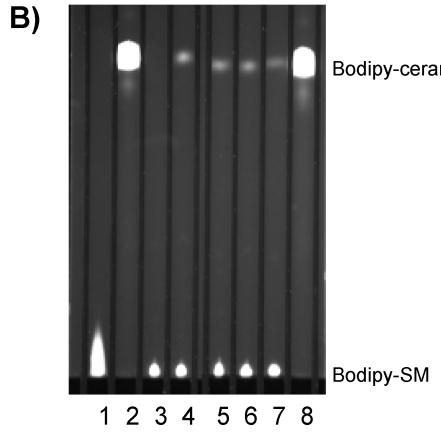


FIGURE 1: (A) Phospholipolytic activity of LDL subfractions alone or in the presence of different substrates. LDL(+) or LDL(−) (100 μL at 0.3 g/L) were incubated alone or with PC, SM, or LPC (0.25 mmol/L, final concentration), and Amplex Red assay was performed as described in Materials and Methods. Increment in fluorescence was monitored for 3 h at 37 °C. The figure shows a representative experiment. (B) Effect of pH and Mg^{2+} on phospholipolytic activity of LDL(−) using Bodipy-labeled SM as substrate. LDLs (0.3 g/L) were incubated with Bodipy-SM (0.025 mmol/L, final concentration) for 3 h at 37 °C. After lipid extraction, Bodipy-SM was separated from its degradation product (Bodipy-ceramide) by thin-layer chromatography and visualized in a Chemi-Doc densitometer (Bio-Rad), as described in Materials and Methods. Lane 1: Bodipy-FL-C12-SM alone. Lane 2: Bodipy-FL-C5-ceramide alone. Lane 3: LDL(+) with Bodipy-SM at pH 7.4 and $MgCl_2$. Lane 4: LDL(−) with Bodipy-SM at pH 7.4 and $MgCl_2$. Lane 5: LDL(−) with Bodipy-SM at pH 7.4 without $MgCl_2$. Lane 6: LDL(−) with Bodipy-SM at pH 5.0 and $MgCl_2$. Lane 7: LDL(−) with Bodipy-SM at pH 5.0 without $MgCl_2$. Lane 8: Bodipy-FL-C5-ceramide alone. The figure shows a representative from five independent experiments.

nm of the peak corresponding to PC did not increase after 24 h of incubation (increment 3.5 ± 2.4% of the PC peak area at 234 nm), in contrast to oxidized LDL(+) (increment 685 ± 52% of the PC peak area at 234 nm). Moreover, phosphorylcholine, the molecule measured by the Amplex Red method, is not a product that should be yielded by lipid peroxidation. A further difference between LDL(−) self-aggregation and $CuSO_4$-induced LDL oxidation was that the latter resulted in preferential degradation of PC (51% of PC molecules were degraded) versus SM (only 18% of SM molecules were degraded) after 24 h (Figure 2A). This observation concurs with the preferential degradation of SM versus PC after LDL(−) self-aggregation.

Effect of Phospholipolytic Activity of LDL(−) on Other Modified LDLs. Extensive modification of LDL(+) by oxidation, acetylation, or $sPLA_2$-mediated lipolysis induced no increase in phospholipolytic activities (Table 2). However, when oxLDL or $sPLA_2$-modified LDL was added to LDL(−) in the absence of other substrates, phospholipolytic activity increased dramatically (Figure 3). In contrast, the increment in lipolytic activity was much lower when LDL(+) or acetylated LDL were added to LDL(−), which suggests that it depended on LPC content in LDLs. This finding supports the notion that the main substrate for LDL(−) is LPC, with the amount of LPC molecules being the major determinant of the phospholipolytic activity of LDL(−). Other characteristics of LDLs, such as oxidation level or electric charge, appeared to be irrelevant (Table 3).

Role of ApoE, ApoC-III, and PAF-AH on Phospholipolytic Activity of LDL(−). The possibility that lysoPLC, PLC, and/or SMase activities displayed by LDL(−) could be due to proteins in which this subfraction is enriched, including apoE, apoC-III, and PAF-AH, was studied. LDL(+) incubated with apoE or apoC-III was enriched in these apolipoproteins. At the low concentration of apolipoprotein (5 and 15 mg/L for apoC-III and apoE, respectively), the content was similar to that observed in LDL(−) (0.23 ± 0.05 mol of apoE/mol of apoB and 0.15 ± 0.03 mol of apoC-III/mol of apoB, respectively, $n = 2$), and at the high concentration of apolipoprotein (20 and 60 mg/L for apoC-III and apoE, respectively), the content in LDL(+) was approximately 4-fold higher (0.95 ± 0.14 mol of apoE/mol of apoB and 0.65 ± 0.16 mol of apoC-III/mol of apoB at $n = 2$) than described in LDL(−) (*7, 11*). However, even at the highest content of apolipoproteins, no increase in phospholipolytic activity was observed in LDL(+) (Figure 4). Neither apoE nor apoC-III alone presented increased phospholipolytic activity. On the other hand, PAF-AH, highly purified by chromatography (tested by SDS−PAGE/silver staining and Western blot), lacked phospholipolytic activity (Figure 4). This observation concurs with results of inhibitors and confirms that PAF-AH was not involved in the phospholipolytic activities displayed by LDL(−).

Effect of LDL(+) Aging on Phospholipolytic Activity. LDL(+) incubated up to 40 days at 4 °C progressively increased its phospholipolytic activity. The progressive increase in activity measured by the Amplex Red method is

Table 3: Chemical Characteristics of LDL(+), Oxidized LDL, Acetylated LDL, and $sPLA_2$-LDL[a]

	LDL(+)	oxidized LDL	acetylated LDL	$sPLA_2$-LDL
relative electrophoretic mobility	1	1.7 ± 0.3^b	2.1 ± 0.3^b	1.5 ± 0.2^b
PC	100	40.1 ± 17.9^b	98.7 ± 2.3	29.6 ± 15.4^b
conjugated dienes in PC peak	100	825 ± 142^b	101 ± 1	103 ± 5
SM	100	85.2 ± 3.4^b	101.2 ± 0.8	103.7 ± 2.9
LPC	100	384.5 ± 40.0^b	99.7 ± 2.1	281.1 ± 35.9^b

[a] Results are the mean ± SD of four independent experiments. PC, SM, and LPC are expressed as the relative content (%) versus LDL(+) after normalization using dipalmitoyldimethylethanolamine as internal standard. Peaks were integrated at 205 nm. Conjugated dienes in the PC peak were measured at 234 nm and expressed as the relative content (%) versus LDL(+). [b] $p < 0.05$ vs LDL(+).

Table 4: Inhibition of LDL(−)-Associated SMase, LysoLPC, and PAF-AH Activities and SMase from *Staphylococcus* sp.[a]

	inhibition LDL(−)-SMase activity	inhibition LDL(−)-lysoPLC activity	inhibition LDL(−)-PAF-AH activity	inhibition SMase from *Staph.*⊄ sp.
CPM, 50 μM	24.8 ± 12.9	14.5 ± 2.1	4.0 ± 3.9	17.5 ± 24.7
CPM, 400 μM	38.6 ± 15.9	35.0 ± 4.2	11.3 ± 12.1	31.0 ± 43.8
MMA, 50 μM	4.0 ± 3.1	6.5 ± 2.1	4.5 ± 3.5	5.0 ± 7.1
MMA, 400 μM	7.2 ± 12.3	7.5 ± 2.1	4.5 ± 6.4	24.0 ± 33.9
Pefabloc, 50 μM	3.5 ± 3.3	3.0 ± 0.1	96.5 ± 4.9	31.3 ± 32.0
Pefabloc, 400 μM	4.3 ± 5.1	9.5 ± 3.5	95.0 ± 7.1	44.3 ± 38.9
D-609, 50 μM	1.8 ± 2.2	1.1 ± 1.1	4.0 ± 3.7	ND[b]
D-609, 400 μM	1.7 ± 2.9	2.3 ± 4.0	3.5 ± 3.9	ND
MLFP, 50 μM	4.2 ± 2.7	4.9 ± 3.3	96.5 ± 5.1	ND
MLFP, 400 μM	16.3 ± 11.2	15.3 ± 12.0	97.5 ± 5.7	ND

[a] Results are expressed as % of inhibition versus LDL(−) or SMase from *B. cereus* in the absence of inhibitor. Abbreviations: CPM, chlorpromazine; MMA, manumycin A; D-609, tricyclodecanoyl dithiocarbonate; MLFP, methyl-γ-linoleyl fluorophosphonate. [b] ND, not determined.

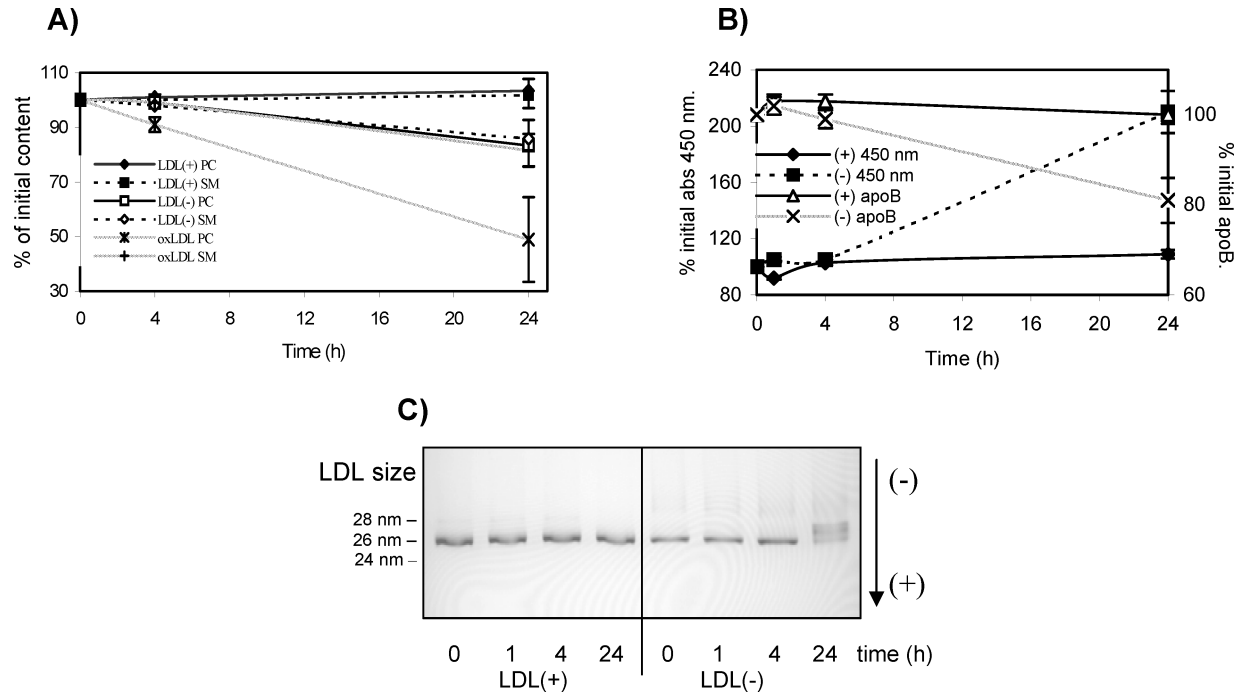


FIGURE 2: Self-degradation of PC and SM and self-aggregation of LDL subfractions. LDLs (0.3 g/L apoB) were incubated for 24 h at 37 °C in the presence of 1 mmol/L EDTA and 2 μmol/L BHT. (A) Phospholipid self-degradation. PC and SM content in LDLs was measured by normal-phase HPLC. Results are expressed as the proportion of PC or SM remaining after 24 h of incubation. (B) Self-aggregation of LDL subfractions. Self-aggregation was determined by measuring absorbance at 450 nm (left axis) or by quantifying the proportion of soluble apoB (nonprecipitable) after high-speed centrifugation (15000*g*, 15 min) (right axis). (C) Nondenaturing gradient gel electrophoresis of LDL subfractions after 24 h of incubation at 37 °C. Results are the mean of six independent experiments, except (C) which shows a representative gel.

shown in Figure 5A. This was confirmed using Bodipy-SM as substrate (Figure 5B). The increase in phospholipolytic activity was accompanied by changes in circular dichroism spectra (Figure 5C), showing a progressive loss of ellipticity signal. This loss was concomitant with an increased aggregation, as measured by absorbance at 450 nm (Figure 5D). LDL(−) presented a spectrum similar to that by LDL(+), with a mild decrease of around 10% in the ellipticity signal at the 215−225 nm region (Figure 5C), which would indicate a partial loss of regular secondary structure. Finally, the proportion of LDL(−) measured by analytical anion-exchange chromatography increased very slowly in aged LDL(+) (Figure 5D), indicating that electric charge is not a major cause of phospholipolytic activity.

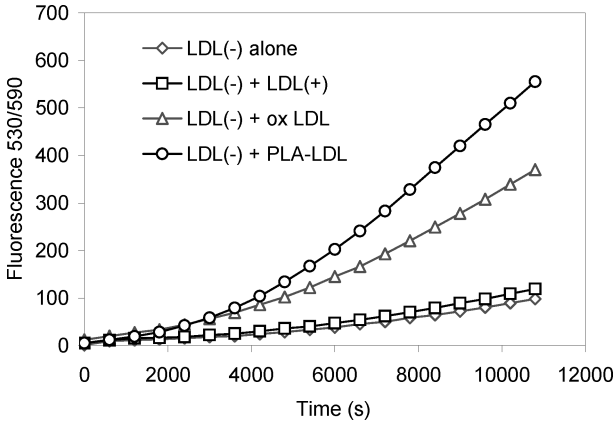


FIGURE 3: Phospholipolytic activity of LDL(−) using native or modified LDLs as substrate. LDL(−) (100 μL at 0.3 g/L) was incubated alone or with LDL(−), oxidized LDL of $sPLA_2$-LDL (100 μL at 0.3 g/L), and Amplex Red assay was performed as described in Materials and Methods. Increment in fluorescence was monitored for 3 h. The figure shows a representative experiment of three independent experiments.

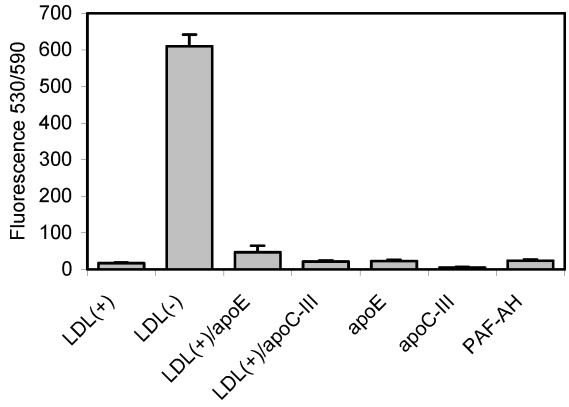


FIGURE 4: Effect of apoE, apoC-III, and PAF-AH on phospholipolytic activity in LDL subfractions. LDL(+) was enriched in apoE or apoC-III as described in Materials and Methods. Phospholipolytic activity was measured in LDLs or in purified apoE, apoC-III, and PAF-AH by Amplex Red assay, using SM as substrate. Results are the mean of three independent experiments.

DISCUSSION

Current results demonstrate that LDL(−) presents novel phospholipolytic activities that could play a relevant role in the atherogenic characteristics of this subfraction of plasma LDL. Holopainen and colleagues previously described the presence of SMase activity in human plasma LDL (*15*). Herein, we show that SMase activity is almost completely associated with LDL(−), but not with LDL(+). Moreover, this is the first description of phospholipase C activity associated with lipoproteins that degrades more efficiently LPC than PC. The implications of these LDL(−)-associated phospholipolytic activities could involve several atherogenic properties observed in LDL(−). SMase activity appears to be directly involved in the increased susceptibility to aggregation of LDL(−) (*7*) but could also be related to LDL(−)-mediated apoptotic processes (*4, 28*). Holopainen et al. suggested that SMase activity could be a link between atherosclerosis and apoptosis (*29*) since ceramide is a major mediator of apoptosis (*14*). This concurs with reports describing apoptosis induction mediated by LDL(−) through caspase activation (*4, 28, 30*), which supports a link with the ceramide-dependent apoptosis pathway.

LDL(−) presents high susceptibility to aggregation (*7*), a property that is induced in total LDL by SMase treatment (*16*). Current results suggest that self-aggregation of LDL(−) could be mediated by its intrinsic phospholipolytic activity, since the formation of LDL aggregates was accompanied by SM degradation. Thus, trapping of LDL(−) in the subendothelial space would be more atherogenic than that of LDL(+) since LDL(−)-associated SMase activity could promote aggregation and fusion, thereby increasing lipoprotein retention. Moreover, increased phospholipolytic activity exerted by LDL(−) on lipoproteins enriched in LPC, such as oxidized LDL or $sPLA_2$-modified LDL, could also favor their aggregation and retention in the artery wall.

On the other hand, lysoPLC activity could modulate the inflammatory capacity of LDL(−) that is related to the presence of PAF-AH. Our group previously reported that PAF-AH could be responsible for the inflammatory activity of LDL(−) (*12, 31*). The mechanism involved in such an inflammatory role of LDL-bound PAF-AH should be the formation of products such as LPC and oxidized fatty acids known to promote inflammation (*32*). Findings reported in the current work suggest a picture in which lysoPLC activity present in LDL(−) could counteract the effect of LPC generated by PAF-AH, thereby promoting its degradation. This could be protective action since the resulting products, phosphorylcholine and monoacylglycerol, are not known to be involved in inflammatory processes. Thus, a complementary action of PAF-AH and lysoPLC activities, with both being present in LDL(−), could attenuate the possibility of high inflammatory activity of mildly oxidized LDL. The question arising from these assumptions is whether LDL(−) could be generated as a mechanism to limit the deleterious effects exerted by minimal LDL oxidation on vascular cells; hence, LDL(−) could be a consequence rather than a cause of inflammation. On that score, the effect of LDL(−) on oxidized LDL or on PLA_2-modified LDL degrading its LPC content supports a protective role for LDL(−). Further studies are required to define the role of the putative coordination between PAF-AH and lysoPLC activities present in LDL(−).

Our results did not reveal the origin of phospholipolytic activity of LDL(−). Inhibitors of well-characterized SMase and PLC activities present in plasma had a poor inhibitory effect, suggesting that these enzymes are not responsible for the phospholipolytic activity. On the other hand, neither PAF-AH nor apoC-III or apoE is involved, since purified PAF-AH, apoE-enriched, or apoC-III-enriched LDL(+) presented no increase in choline generation. Lipid extracts from LDL(+) and LDL(−) were also tested for SMase activity, but no lipolytic activity was observed (data not shown). With apoE, apoC-III, PAF-AH, and lipid moiety ruled out, two explanations are possible. One is that a currently unknown protein could be responsible for phospholipolytic activity. The other possibility is that apoB could be the protein responsible for this enzymatic activity. Regarding the first possibility, Karlsson et al. reported the presence of 10 minor proteins in total LDL: apoE, apoC-III, apoC-II, apoA-I, apoA-IV, apoJ, apoM, serum amyloid A-IV, calgranulin A, and lysozyme C (*33*). To our knowledge, none of these proteins have been reported to present phospholipase activity. Moreover, the content of apoE, apoC-III, apoC-II in VLDL and of apoA-I, apoA-IV, apoJ, apoM, and serum amyloid A-4 in HDL is much higher than that detected in LDL(−).

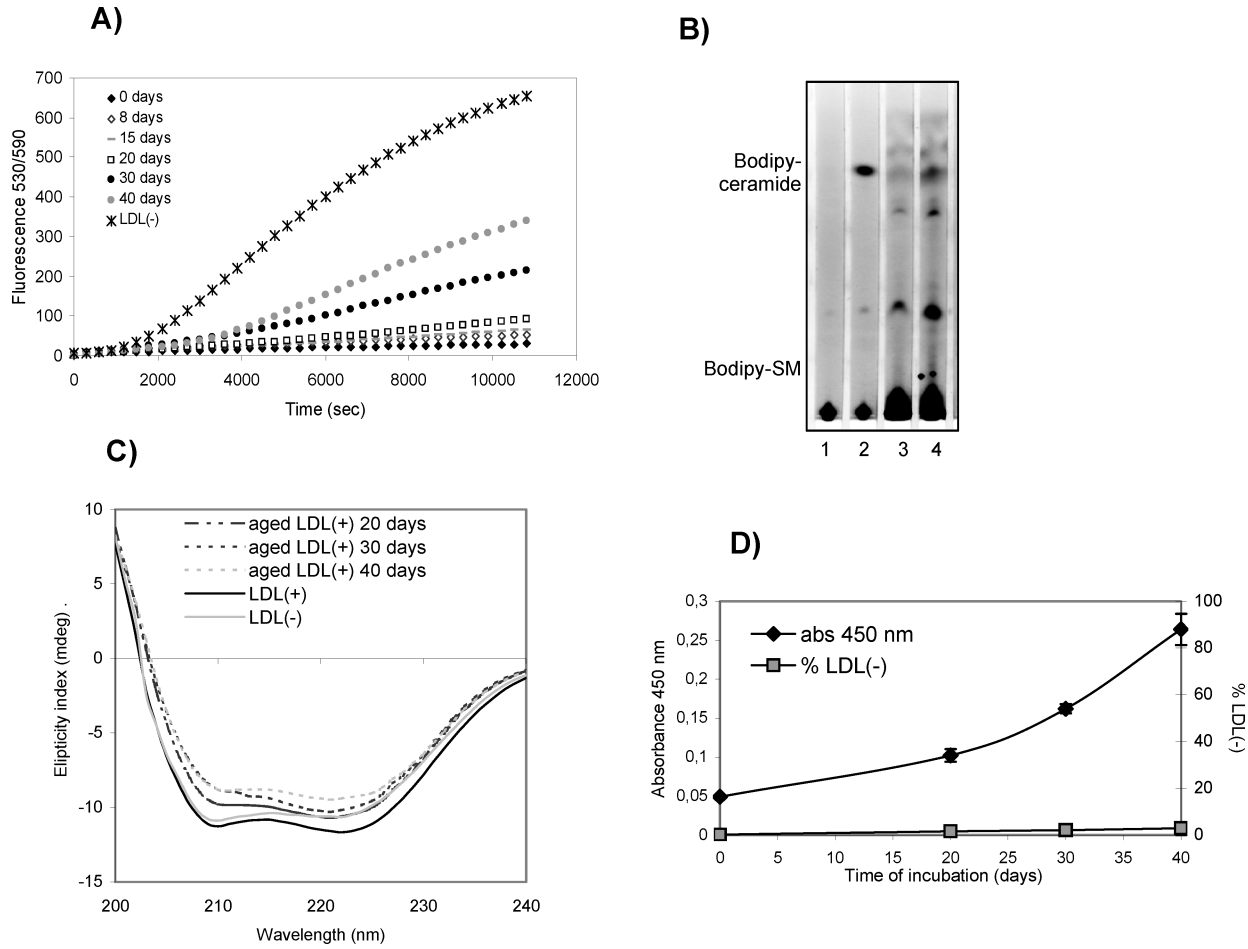


FIGURE 5: Phospholipolytic activity of "in vitro"-aged LDL. LDL(+) (0.5 g/L) was aged by incubation at 4 °C in PBS supplemented with 2 μM BHT for up to 40 days. (A) Amplex Red assay. LDLs (100 μL at 0.3 g/L) were incubated with LPC (0.25 mmol/L, final concentration), and Amplex Red assay was performed as described in Materials and Methods. The increment in fluorescence was monitored for 3 h at 37 °C. (B) Bodipy-SM/TLC. LDLs (0.3 g/L) were incubated with Bodipy-SM (0.025 mmol/L for LDL(+) and LDL(−), 0.05 mmol/L for aged-LDL, final concentration) for 8 h at 37 °C. After lipid extraction, Bodipy-SM was separated from its degradation product (Bodipy-ceramide) by thin-layer chromatography (TLC) and visualized in a Chemi-Doc densitometer (Bio-Rad), as described in Materials and Methods (inverted image). Lanes: 1, LDL(+); 2, LDL(−); 3, LDL(+) aged 30 days; 4, LDL(+) aged 40 days. (C) Far-UV CD spectra of aged LDL(+). Spectra of LDL(−) and LDL(+) aged for 0, 20, 30, and 40 days of incubation in PBS with 2 μM BHT at 4 °C. Samples were measured at 0.05 g/L of apoB using a 0.2 mm quartz cuvette at 25 °C. Represented spectra are those deconvolved after analysis by the K2D software (DICHROWEB server). (D) Self-aggregation and self-generation of LDL(−) in aged LDLs. Self-aggregation was determined by measuring absorbance at 450 nm of LDLs (0.5 g/L of apoB) after increasing incubation time, as described in Materials and Methods. LDL(−) proportion was determined by analytical anion-exchange chromatography in a MonoQ column.

Since phospholipolytic activity in VLDL and HDL was almost zero (*15*), the involvement of these apoproteins in the phospholipolytic activity of LDL(−) is unlikely.

Regarding apoB, Holopainen and colleagues hypothesized that the SMase activity of LDL is due to apoB, since protein sequence homology studies revealed a high homology between apoB and three regions of *Staphylococcus* sp. SMase. Those authors suggested that a catalytically active His-Ser-Asp triad common to a wide range of lipolytic enzymes is also present in apoB-100 (*15, 29*). In agreement with this hypothesis, the observation that "in vitro" aging of LDL(+) progressively increases its phospholipolytic activity suggests that structural changes in apoB could underlie the appearance of such activity. This is supported by the observation that progressive loss of elipticity signal concurs with an increased aggregation, as measured by absorbance at 450 nm. The loss of regular secondary elements in favor of random coil conformation generates a more flexible apoB, which would account for enzymatic activity by the His-Ser-Asp triad. These data concur with results reported by de Spirito et al. (*34*), who observed that LDL aged in plasma increased aggregation in parallel to the loss of secondary structure as measured by circular dichroism.

Since the apoB amino acid sequence is the same in LDL(+) and LDL(−) from the same subject, the hypothesis of Holopainen and Kinnunen (*15, 29*) would imply that structural differences in apoB within LDL(+) and LDL(−) could be responsible for phospholipolytic activity. The CD spectrum of LDL(−) was slightly different from that of LDL(+) with a 10% loss of signal at the 215−225 nm region; this would indicate a loss of regular secondary structures, according to previous reports (*35, 36*). However, the LDL(−) spectrum was also different from that of aged LDLs. This observation indicates that whatever changes occur in LDL(+) during "in vitro" aging, the process should differ from that occurring "in vivo" with LDL(−). Thus, although aged LDLs point to a role for apoB, data from LDL(−) overall secondary structure do not unequivocally confirm this hypothesis. The role of apoB should be confirmed in further studies focused on ascertaining the

putative regions that could be involved in the generation of phospholipolytic activity in LDL(−).

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