High binding affinity of electronegative LDL to human aortic proteoglycans depends on its aggregation level[S]

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Abstract **Electronegative LDL [LDL(−)] is an atherogenic subfraction of plasma LDL that has increased apolipoprotein E (apoE) and apoC-III content, high density, and increased susceptibility to aggregation. These characteristics suggest that LDL(−) could bind to proteoglycans (PGs); therefore, our aim was to evaluate its affinity to PGs. Binding of LDL(−) and native LDL [LDL(+)] to human aortic PGs was determined by precipitation of LDL-glycosaminoglycan complexes, LDL incubation in PG-coated microtiter wells, and affinity chromatography on PG column. All methods showed that LDL(−) had higher binding affinity to PGs than did LDL(+). PG capacity to bind LDL(−) was increased approximately 4-fold compared with LDL(+) in precipitation and microtiter assays. Chromatography on PG column showed LDL(−) to consist of two subpopulations, one with higher and one with lower PG binding affinity than LDL(+). Unexpectedly, the lower PG affinity subpopulation had increased apoE and apoC-III content. In contrast, the high PG affinity subpopulation presented phospholipase C (PLC)-like activity and increased aggregation. These results suggest that PLC-like activity could alter LDL lipid composition, thereby promoting particle aggregation and binding to PGs. This propensity of a subpopulation of LDL(−) to bind to PGs could facilitate its retention in the extracellular matrix of arterial intima and contribute to atherosclerosis progression.**—Bancells, C., S. Benítez, M. Jauhiainen, J. Ordóñez-Llanos, P. T. Kovanen, S. Villegas, J. L. Sánchez-Quesada, and K. Öörni. **High binding affinity of electronegative LDL to human aortic proteoglycans depends on its aggregation level. *J. Lipid Res.* 2009.** 50: **446–455.**

Supplementary key words glycosaminoglycans • lipoprotein aggregation • sphingomyelinase • phospholipase C

Human atherosclerosis is characterized by an initial accumulation of LDL-derived cholesterol in the arterial intima. Native LDL [LDL(+)] is retained by the extracellular matrix of the intima, particularly by proteoglycans (PGs) (1–3), where it can be modified and can exert inflammatory actions (4). PGs, the main component of the extracellular matrix, form a tight and negatively charged network (5). Binding of LDL(+) to PGs is via ionic interactions between positively charged residues of apolipoprotein B-100 (apoB-100) and negatively charged sulfate and carboxyl groups of glycosaminoglycan (GAG) chains of PGs (6–9). Two PG binding sites have been described in apoB-100. Site A (residues 3,148–3,158) is only exposed after degradation of phospholipids, for example by secretory phospholipase A_2 ($sPLA_2$), whereas site B is permanently exposed (residues 3,359–3,369). This $sPLA_2$ modification enables cooperation between site A and site B, increasing the affinity of apoB-100 to PGs (10). Other modifications in LDL, such as lipolysis with SMase, increase its binding affinity to PGs because they induce changes in the surface monolayer of LDL particles that may alter the conformation of apoB-100 and induce aggregation and fusion of LDL particles (10–12). Finally, other minor components of LDL, such as apoE and apoC-III can also mediate binding of lipoproteins to PGs. These apolipoproteins can mediate the binding by bridging (apoE) (13) or by facilitating PG interaction (apoC-III) (14).

Electronegative LDL [LDL(−)] is a modified LDL with atherogenic characteristics that is present in circulation. This LDL subfraction presents inflammatory, apoptotic,

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Abbreviations: apoE, apolipoprotein E; GAG, glycosaminoglycan; GGE, native polyacrylamide gradient gel electrophoresis; LDL(−), electronegative LDL; LDL(+), native LDL; lysoPLC, lysophospholipase C; PAF-AH, platelet-activating factor acetylhydrolase; PG, proteoglycan; PLC, phospholipase C; $sPLA_2$, secretory phospholipase A_2.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table and one figure.

and cytotoxic properties in vitro (15–17), and its proportion is increased in subjects with high cardiovascular risk (18, 19). Some characteristics suggest that despite their electronegative charge, LDL(−) particles could bind to PGs, owing to their increased apoE and apoC-III content compared with LDL(+) (20). Furthermore, compared with LDL(+), LDL(−) particles have higher density, smaller size (21), and increased susceptibility to aggregation (22), properties that are also related to increased affinity to PGs (10, 11).

Our results here indicate that certain LDL(−) subpopulations show increased binding to commercial GAGs and human aortic PGs, in neutral and acidic conditions. The current study also aims to clarify the mechanisms involved in such high-affinity binding.

MATERIALS AND METHODS

Isolation of LDL(+) and LDL(−)

Human LDL ($1.019 < d < 1.050$ g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation at +4°C in the presence of 1 mM EDTA. The study was approved by the institutional Ethics Committee, and subjects gave their written informed consent. LDL was subfractionated into nonmodified LDL [LDL(+)] and LDL(−) by anion-exchange chromatography, as described (22). LDL subfractions were characterized for their protein and lipid composition, and differences in electronegativity were confirmed by agarose gel electrophoresis (22).

Preparation and characterization of aortic PGs

PGs from the intima-media of human aortas were obtained at autopsy within 24 h of accidental death and were prepared essentially by the method of Hurt-Camejo et al. (23), consisting of a urea extraction and a purification by anion exchange chromatography. GAGs were quantified by the method of Bartold and Page (24), and the amounts of PGs are expressed in terms of their GAG content. Our preparation of PGs isolated from human aortas contained 53% chondroitin-6 sulfate, 27% chondroitin-4 sulfate and 20% dermatan sulfate.

Binding of LDL to PGs

Binding of LDL(+) and LDL(−) to PGs was examined by three different methods.

Precipitation of LDL-GAG complexes. The assay is based on the method described by Davidsson et al. (25). Briefly, LDL (200 μl at 0.1 g protein/l) in 5 mM HEPES, 20 mM NaCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.0, was incubated at +4°C for 2 h with heparan sulfate (Sigma H7640), dermatan sulfate (Sigma C3788), or chondroitin-6 sulfate (Sigma C4384) at 10 and 100 μg GAG/ml. Samples were centrifuged at 12,000 *g* for 10 min at +4°C, and cholesterol of LDL unbound to PGs was determined in the supernatants using an enzymatic method (Roche Diagnostics). Bound LDL is expressed as micrograms of LDL protein/well considering the following cholesterol/LDL protein ratio: 4.6 mmol of cholesterol/g of LDL protein.

Incubation of LDL in PG-coated microtiter wells. Polystyrene 96-well plates (Thermo Labsystems) were coated overnight with 100 μl of human aortic PGs (25 μg/ml) or 5% fat-free BSA in PBS by incubation at +4°C. Wells were blocked with 3% BSA and 1% fat-free milk powder in PBS for 1 h at +37°C. BSA-coated wells served as controls, and their binding was subtracted from those of wells coated with PGs. Increasing concentrations of LDL (2.5–20 μg of LDL protein) in 20 mM HEPES, 32 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4, containing 1% BSA were incubated for 1 h at +37°C. Unbound LDL was removed, and wells were washed with 20 mM HEPES, 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4. The amount of bound LDL was determined using an Amplex Red cholesterol kit (Molecular Probes) according to the manufacturer's recommendations (26).

PG affinity chromatography. Human arterial PGs (1 mg) were coupled to an *N*-hydroxysuccinimide-activated HiTrap column (1 ml) according to the manufacturer's instructions (GE Healthcare). As described by Öörni et al. (27), columns were equilibrated with buffer A (10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4), washed with buffer B (buffer A plus 250 mM NaCl), and re-equilibrated with buffer A before use. LDL was bound to the column using buffer A at a flow rate of 0.5 ml/min and eluted with a linear gradient of NaCl using buffer B. Lipoprotein elution was monitored at 280 nm, and gradient was checked by measuring the change in conductivity. Affinity of LDL to PG was also tested at acid pH using buffer C (10 mM MES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 5.5) and eluting the samples with buffer D (buffer C plus 500 mM NaCl). To determine the ionic strength of LDL(+) and LDL(−) binding to PG at neutral and acid pH, 50 μl of LDL at 0.5 g protein/l was injected on a single PG column in a Smart (Pharmacia Biotech) system (analytical method).

To isolate LDL(−) subfractions differing in PG affinity, three PG-HiTrap columns in tandem configuration enabled us to load more sample and collect sufficient amounts of LDL subfractions for further analyses (preparative method). LDL(−) (1.8 ml at 0.4 g protein/l) was applied on columns in a fast-protein liquid chromatography system (Pharmacia Biotech). Five LDL(−) subfractions eluting between 30 mM and 125 mM NaCl were collected. These fractions were concentrated by centrifugation with Centricon YM-10 (10,000 MWCO Amicon, Millipore) and used for further assays at 0.4 g protein/l (see below).

Characterization of LDL(−) subfractions

Lipid and protein composition. Total cholesterol, triglyceride, apoB-100 (Roche), phospholipids, and NEFAs (Wako) were measured in LDL(+), LDL(−), and LDL(−) subfractions by commercial methods in a Hitachi 911 autoanalyzer (22). ApoE concentration of the isolated LDL (2 μg of LDL protein) was quantified by the ELISA method using a polyclonal anti-apoE rabbit capture antibody (R107) to coat the wells and HRP-conjugated anti-apoE polyclonal rabbit antibody (DAKO) for detection as described (28). ApoC-III content was measured by an immunoturbidimetric method (Kamiya) using 35 μg of LDL protein. Peroxide content was measured by a commercial assay (Perox Say lipid quantitative assay, G-Biosciences) using 10 μg of LDL. Electronegativity was evaluated by agarose gel electrophoresis (Midigel, Biomidi).

Enzyme activity measurements. Platelet-activating factor acetylhydrolase (PAF-AH) activity was measured using 4 μg of LDL by a commercial colorimetric method using 2-thio-PAF as substrate (Cayman) essentially as described (29). Phospholipolytic activities [lysophospholipase C (lysoPLC) and SMase activities] were measured using 15 μg of LDL by a commercial fluorimetric method (Amplex Red, Molecular Probes) using lysophosphatidylcholine or sphingomyelin as substrates (30). SMase activity was also analyzed by incubation of LDL (25 μg of LDL protein) with a fluo-

rescently labeled substrate (borondipyrromethene (BODIPY)-FL-C12-SM, Molecular Probes) for 3 h at +37°C followed by lipid extraction and separation by TLC (30, 31).

Aggregation studies. LDL aggregation level was determined by measuring absorbance at 450 nm and by native polyacrylamide gradient gel electrophoresis (GGE), as described (21). The size and aggregation of LDL particles were determined by transmission electron microscopy. For this purpose, LDL preparations were negatively stained with 2% potassium phosphotungstate, pH 7.0 (32). The stained samples were viewed and photographed in a JEOL JEM-1200EX transmission electron microscope at the Institute for Biotechnology, Electron Microscopy, University of Helsinki, Helsinki, Finland. Original magnifications of electron micrographs were 60,000.

ApoB-100 secondary structure determination. Far-ultraviolet circular dichroism spectra were performed with LDL samples in PBS at 0.05 g protein/l in a JASCO J-715 spectropolarimeter, as previously described (30). Twenty spectra were averaged for each measurement, and the buffer blank was subtracted. Spectra were deconvolved by the analysis program CDSSTR from the DICHROWEB server (Birkbeck University, London) to calculate the relative contribution of α-helix, β-sheet, β-turn, and random coil secondary structures (33, 34).

Anti-apoE immunoaffinity chromatography

Polyclonal rabbit anti-human apoE IgG (R107, IgG purified by Protein G-method) was coupled to CNBr-activated Sepharose CL-4B according to the manufacturer's instructions (Amersham Pharmacia Biotech). The affinity column contained 4 mg IgG/ml of the matrix. LDL(−) (1 ml at 0.8 g protein/l) was applied on the column equilibrated with PBS, pH 7.4, at a flow rate of 0.5 ml/min using a Merck-Hitachi HPLC system. Nonbound fractions (fraction size, 1 ml) were collected, and the bound material was eluted with 0.1 M glycine, pH 2.5, into tubes containing 1 M Tris-HCl, pH 8.5, for neutralization. Both nonbound and bound fractions were dialyzed against buffer A and concentrated by centrifugation with Centricon YM-10 (10,000 MWCO Amicon, Millipore). PG binding affinity of the bound LDL(−) (apoE-enriched) and the nonbound LDL(−) (apoE-poor) was then determined by analytical PG affinity chromatography. All other details and elution conditions were as previously described (28).

Statistical analysis

Results are expressed as mean ± SD. The SPSS 15.0 statistical package was used. Differences between LDL(+) and LDL(−) groups were tested with Wilcoxon's *t*-test, except for analysis of LDL binding to PGs in microtiter wells, for which Kruskall-Wallis and Mann-Whitney tests were used. Differences among LDL(−) subfractions were tested using ANOVA. A P value of <0.05 was considered significant, except as indicated.

RESULTS

Binding of LDL to PGs

Binding of LDL(+) and LDL(−) to PGs was examined by three different methods. First, LDL binding affinity to commercial GAGs was determined by high-speed precipitation. As shown in **Table 1**, LDL(−) demonstrated higher binding affinity to GAGs than did LDL(+). The capacity of GAGs to bind to LDL(−) was 2- to 4-fold higher when compared with LDL(+). On the other hand, LDL(−) affinity was different between the GAGs used in the following order: chondroitin-6 sulfate > dermatan sulfate > heparan sulfate.

TABLE 1. Binding of LDL(+) and LDL(−) to GAGs evaluated by measuring the precipitation of LDL-GAG complexes.

	μg GAG/ml	LDL(+)	LDL(−)
		% Binding	
Heparan sulfate	0	1.7 ± 2.0	4.9 ± 3.5[a]
	10	3.7 ± 3.6	14.6 ± 5.1[a]
	100	4.4 ± 3.0	16.9 ± 6.6[a]
Dermatan sulfate	0	1.1 ± 2.0	4.6 ± 3.9[a]
	10	4.5 ± 4.7	17.8 ± 7.1[a]
	100	5.1 ± 5.5	21.3 ± 7.5[a]
Chondroitin-6 sulfate	0	0.6 ± 1.2	5.0 ± 2.2[a]
	10	11.7 ± 6.8	23.8 ± 8.2[a]
	100	13.3 ± 8.6	26.7 ± 3.3[a,b]

LDL(+), native LDL; LDL(−), electronegative LDL; GAG, glycosaminoglycan. Results are the mean ± SD of five independent experiments.
[a] $P < 0.05$ versus LDL(+).
[b] $P < 0.05$ versus heparan sulfate.

To test the binding of LDL(+) and LDL(−) under more-physiological conditions, two assays using PGs isolated from the intima-media of human aortas were performed. Microtiter well assay showed higher binding affinity of LDL(−) to human aortic PGs compared with that of LDL(+) (**Fig. 1**). The amount of LDL(−) bound to PGs was 4-fold higher than the amount of LDL(+), independently of the LDL concentration, which fully concurs with the data obtained from the precipitation assay (see above).

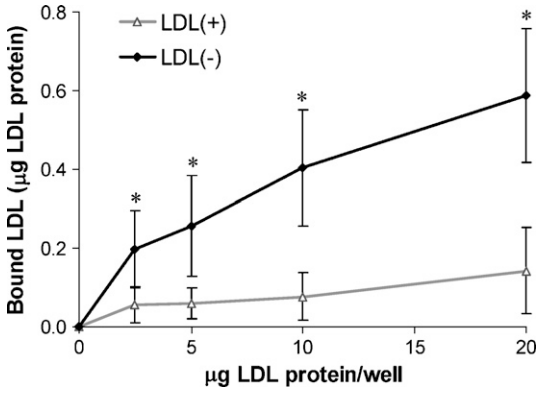

Fig. 1. Binding of native LDL [LDL(+)] and electronegative LDL [LDL(−)] to human aortic proteoglycans (PGs) measured in PG-coated microtiter wells. Increasing concentrations of both LDL fractions were incubated for 1 h at +37°C in PG- or BSA-coated wells. Unbound particles were removed, and the concentration of LDL particles bound to PGs or BSA was measured as described in Materials and Methods. Three independent experiments (each concentration assayed in triplicate) are shown. Black symbols represent LDL(−) and white symbols represent LDL(+). Kruskall-Wallis and Mann-Whitney tests were used to analyze statistical differences between LDL(+) and LDL(−). * $P = 0.005$, $P = 0.001$, $P < 0.001$, and $P < 0.001$ at the concentrations of 2.5, 5, 10 and 20 μg protein/well, respectively. Error bars indicate SD.

PG affinity chromatography was used to determine the binding strength of LDL(−) to human aortic PGs and to study the distribution of LDL particles, depending on their binding affinity. At pH 7.4, LDL(+) eluted as a single peak at 68 mM NaCl, whereas the LDL(−) elution pattern displayed two peaks at 54 and 87 mM NaCl (**Fig. 2A**). Hence, LDL(−) consisted of two subpopulations: one with higher and the other with slightly lower PG binding affinity than LDL(+). On the other hand, it has been described that a decrease in pH enhances the binding of native and aggregated LDL to PGs (26). We observed this acid pH effect in both LDL fractions (Fig. 2B). At pH 5.5, the ionic strength needed to elute LDL increased and LDL(+) could be eluted as a single peak at 114 mM NaCl and LDL(−) as two peaks at 113 and 139 mM NaCl. Interestingly, the LDL(−) subpopulation that displayed lower PG affinity than LDL(+) at neutral pH had the same affinity as LDL(+) at acid pH.

Characterization of LDL(−) subfractions

To assess why certain subpopulations of LDL(−) particles bind with increased affinity to PGs compared with LDL(+) and what the dictating factors causing differences in affinity between LDL(−) particles are, five LDL(−) subfractions with increasing PG affinity were collected using preparative affinity chromatography (**Fig. 3A**). Specifically, we collected two subfractions of the peak with low affinity (subfractions 1 and 2), one intermediate subfraction (subfraction 3), and two subfractions from the peak with high affinity (subfractions 4 and 5). These subfractions were analyzed for lipid and apolipoprotein content, enzymatic activities, aggregation level, and apoB-100 secondary structure.

Concerning the composition of LDL(−) particles (**Table 2**), subfractions with the highest PG affinity had increased triglyceride content and decreased apoB-100 content. Phospholipid content slightly decreased, but statistical significance was not reached. Despite the apoB-100 decrease, total protein did not change. No relationship between high PG affinity and NEFA or peroxide content was observed. Regarding the electronegative charge, the highest and lowest affinity subfractions presented similar electrophoretic mobility (Fig. 3B). Neither apoE nor apoC-III content was increased in the high-affinity subfractions (**Fig. 4**). In contrast, both apolipoproteins were elevated in the low-affinity subfractions, compared with those of

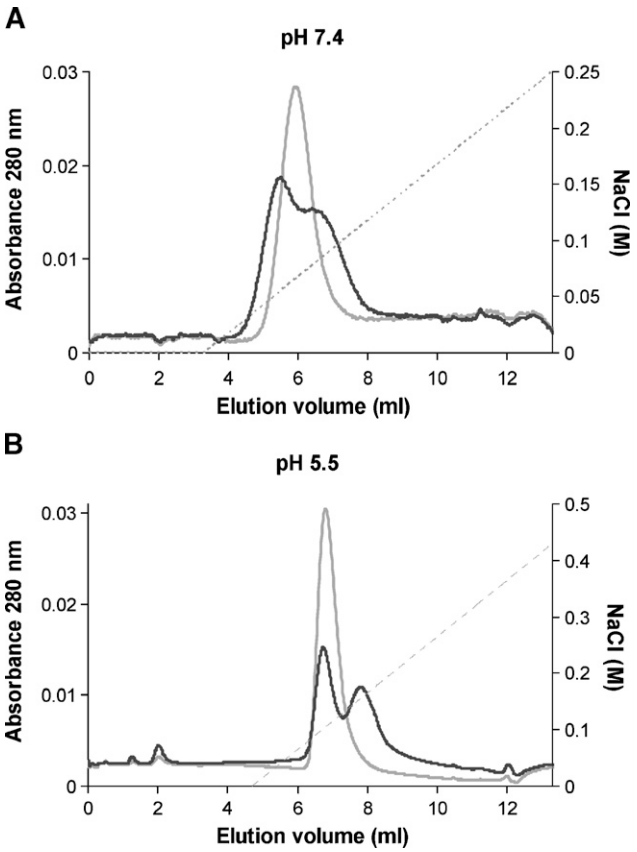

Fig. 2. Analytical affinity chromatography of LDL(+) and LDL(−) on human aortic PG column at neutral pH and acidic pH. LDL(+) (gray line) or LDL(−) (black line) (50 μl at 0.5 g/l) were analyzed on a PG-HiTrap column and eluted with a linear gradient of NaCl (dashed line). A: pH 7.4; B: pH 5.5. Figure shows two representative experiments.

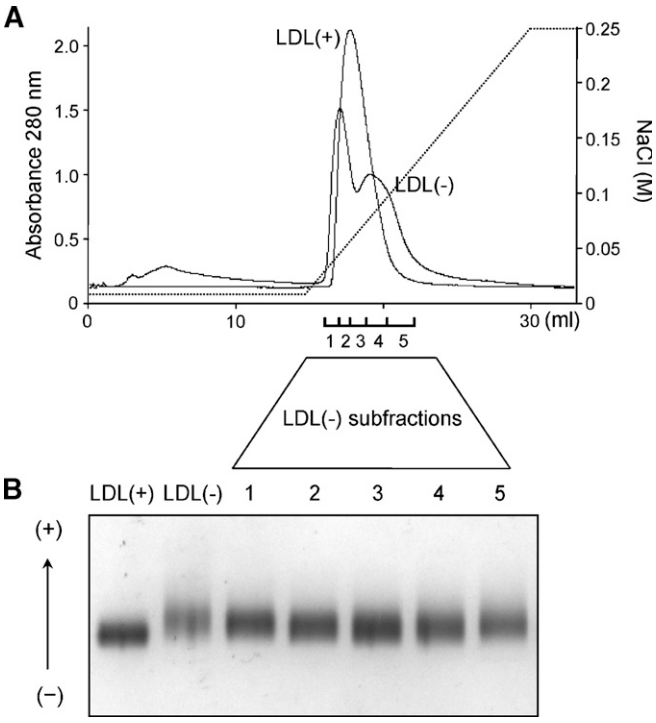

Fig. 3. A: LDL(−) subfractionation on a preparative affinity chromatography. LDL(−) (1.8 ml at 0.4 g/l) was injected on three PG-HiTrap columns in tandem and eluted with a linear gradient of NaCl (0–0.25 M). Five LDL(−) subfractions were collected between 30 mM and 125 mM NaCl with increasing affinity. Note that the volume of collected subfractions is different, because our aim was to collect two fractions of the peak with low affinity (subfractions 1 and 2), one intermediate fraction (subfraction 3), and two subfractions of the peak with high affinity (subfractions 4 and 5). Dashed line indicates NaCl gradient. B: Electronegative charge of LDL(+), total LDL(−), and LDL(−) subfractions. LDL negative charge was determined by agarose gel electrophoresis. The image is a representative experiment.

TABLE 2. Lipid and protein composition of LDL(+), total LDL(−), and LDL(−) subfractions (n = 5)

			LDL(−) subfractions				
	LDL(+)	LDL(−)	1	2	3	4	5
			mmol/g protein				
Cholesterol	4.69 ± 0.22	4.64 ± 0.06	4.59 ± 0.10	4.62 ± 0.15	4.51 ± 0.16	4.55 ± 0.11	4.51 ± 0.11
Phospholipid	1.76 ± 0.21	1.64 ± 0.24	1.63 ± 0.25	1.63 ± 0.20	1.58 ± 0.22	1.56 ± 0.22	1.47 ± 0.22
Triglyceride	0.38 ± 0.02	0.41 ± 0.02[a]	0.37 ± 0.04	0.42 ± 0.10	0.46 ± 0.08	0.51 ± 0.07[b]	0.51 ± 0.03[b]
			μmol/g protein				
NEFA,	31.7 ± 15.7	63.3 ± 20.7[a]	50.7 ± 17.6	54.2 ± 17.4	49.3 ± 19.8	48.6 ± 16.5	53.4 ± 22.0
ApoB-100	1.99 ± .0.19	1.78 ± 0.12[a]	1.74 ± 0.12	1.78 ± 0.12	1.75 ± 0.13	1.74 ± 0.12	1.55 ± 0.09[c]
Peroxides	4.7 ± 4.4	5.1 ± 4.9	6.4 ± 2.4	6.7 ± 3.5	6.8 ± 1.3	5.6 ± 3.0	5.7 ± 4.5

Results are mean ± SD. ApoB-100, apolipoprotein B-100.
[a] $P < 0.05$ versus LDL(+).
[b] $P < 0.05$ versus LDL(−) 1 subfraction.
[c] $P < 0.05$ versus LDL(−) 2 subfraction.

higher affinity. Moreover, to verify the involvement of apoE in LDL(−) PG affinity assay, LDL(−) was subfractionated to apoE-rich and apoE-poor LDL populations using anti-apoE immunoaffinity chromatography. The PG affinity of these subpopulations was analyzed, and the data demonstrated that apoE-rich LDL(−) bound with lower affinity to PG than did apoE-poor LDL(−) (**Fig. 5**). Thus, the results of both experiments support the idea that the enhanced affinity of an LDL(−) subpopulation does not depend on increased apoE content.

We also evaluated the distribution of enzymatic activities that are increased in LDL(−). No difference in PAF-AH activity was observed between LDL(−) subfractions (**Fig. 6A**). In contrast, dramatic differences were observed in LDL(−) subfractions regarding their phospholipase C (PLC)-like activities. As shown in Fig. 6B, C, the higher the PG affinity, the higher the LysoPLC and SMase activities as measured by the Amplex Red method. To corroborate these results, SMase activity was also determined by evaluating the degradation of BODIPY-SM to BODIPY-ceramide and lipid separation by TLC (Fig. 6D). In accordance with the Amplex Red data, LDL(−) subfractions with higher PG affinity demonstrated increased BODIPY-ceramide formation, indicating elevated SMase activity.

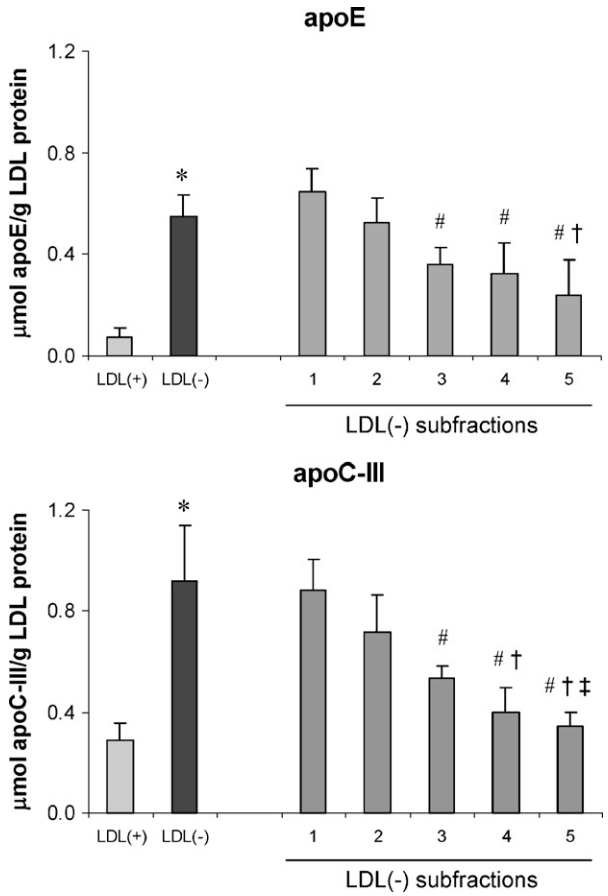

Fig. 4. Apolipoprotein E (apoE) and apoC-III content in LDL(+), total LDL(−), and LDL(−) subfractions. LDL(−) was subfractionated depending on its PG affinity (see Fig. 3A). ApoE and apoC-III were determined as described in Materials and Methods. Results are the mean ± SD of five independent experiments. * $P < 0.05$ versus LDL(+); # $P < 0.05$ versus LDL(−) 1 subfraction; † $P < 0.05$ versus LDL(−) 2 subfraction; ‡ $P < 0.05$ versus LDL(−) 3 subfraction.

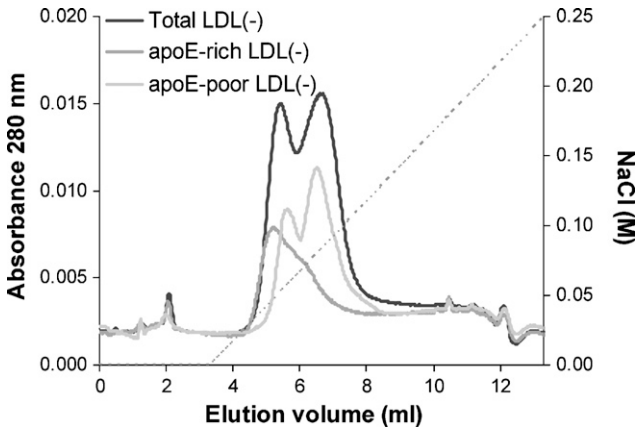

Fig. 5. Analytical PG affinity chromatography of apoE-rich LDL (−) and apoE-poor LDL(−). ApoE-rich and apoE-poor LDL(−) subfractions were obtained by anti-apoE immunoaffinity chromatography, as described in Materials and Methods. Samples were concentrated and injected in the PG analytical affinity chromatography column. Figure shows a representative experiment. Dashed line indicates NaCl gradient.

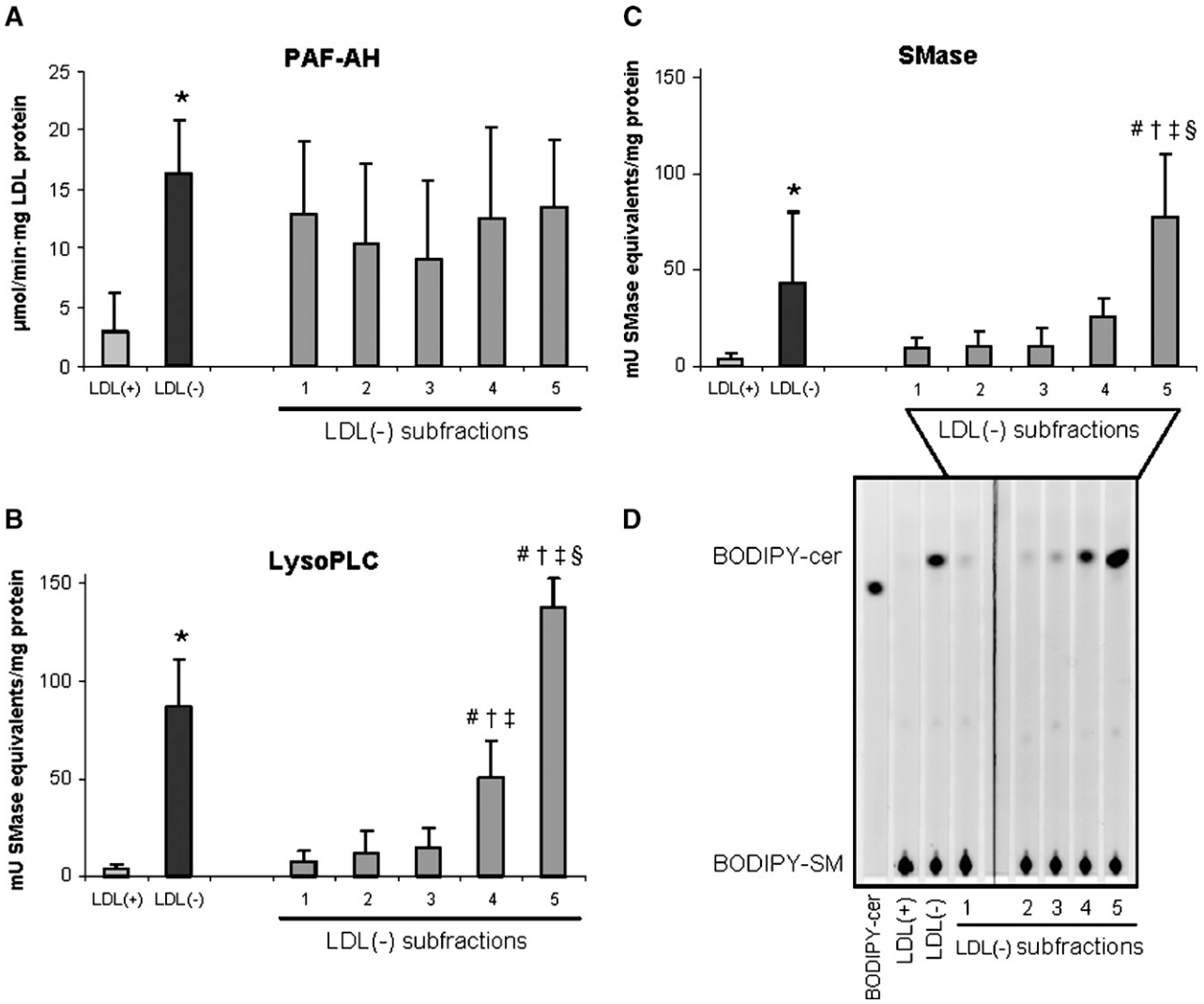

Fig. 6. Enzymatic activities of LDL(+), total LDL(−), and LDL(−) subfractions. LDL(−) was subfractionated depending on its PG affinity (see Fig. 3A). Platelet-activating factor acetylhydrolase (PAF-AH) (A), lysophospholipase C (LysoPLC) (B), and SMase (C) activities were measured by commercial colorimetric and fluorimetric methods, as described in Materials and Methods. SMase activity was also determined by degradation of BODIPY-SM to BODIPY-ceramide (BODIPY-cer) and lipid separation by TLC (D). Results are the mean ± SD of five independent experiments. The TLC image is representative of three independent experiments. * $P < 0.05$ versus LDL(+); # $P < 0.05$ versus LDL(−) 1 subfraction; † $P < 0.05$ versus LDL(−) 2 subfraction; ‡ $P < 0.05$ versus LDL(−) 3 subfraction; § $P < 0.05$ versus LDL (−) 4 subfraction.

The relationships between SMase and PLC activity and aggregation and fusion of lipoproteins were reported in earlier studies (35–38). This also motivated us to evaluate the aggregation of LDL(−) subfractions. Our results showed that, in LDL(−) subfractions, aggregation level is directly related to its PG affinity. **Figure 7A** shows that subfractions 4 and 5 displayed bands migrating to a position with higher size. This observation fully agrees with the increased absorbance measured at 450 nm (Fig. 7B). Furthermore, differences in aggregation of the particles were also seen on transmission electron microscopy (Fig. 7C). As shown in panel 1, individual LDL(+) particles can be clearly recognized. Compared with LDL(+), LDL(−) particle size was heterogeneous, and aggregated particles were observed (panel 2). LDL(−), with the lowest PG affinity (subfraction 1), contained less aggregated particles than the LDL(−) subfraction displaying the highest PG affinity (subfraction 5) (panels 3 and 4, respectively), thereby supporting the results obtained from GGE analysis and absorbance measurements at 450 nm.

The possibility that differences in PG binding affinity among LDL(−) subfractions could be due to changes in apoB-100 structure was studied by circular dichroism. Two independent experiments were performed. All subfractions presented similar proportions of secondary structures (see supplementary Fig. I and supplementary Table I).

DISCUSSION

Growing evidence supports the concept that subendothelial retention of atherogenic lipoproteins is the initi-

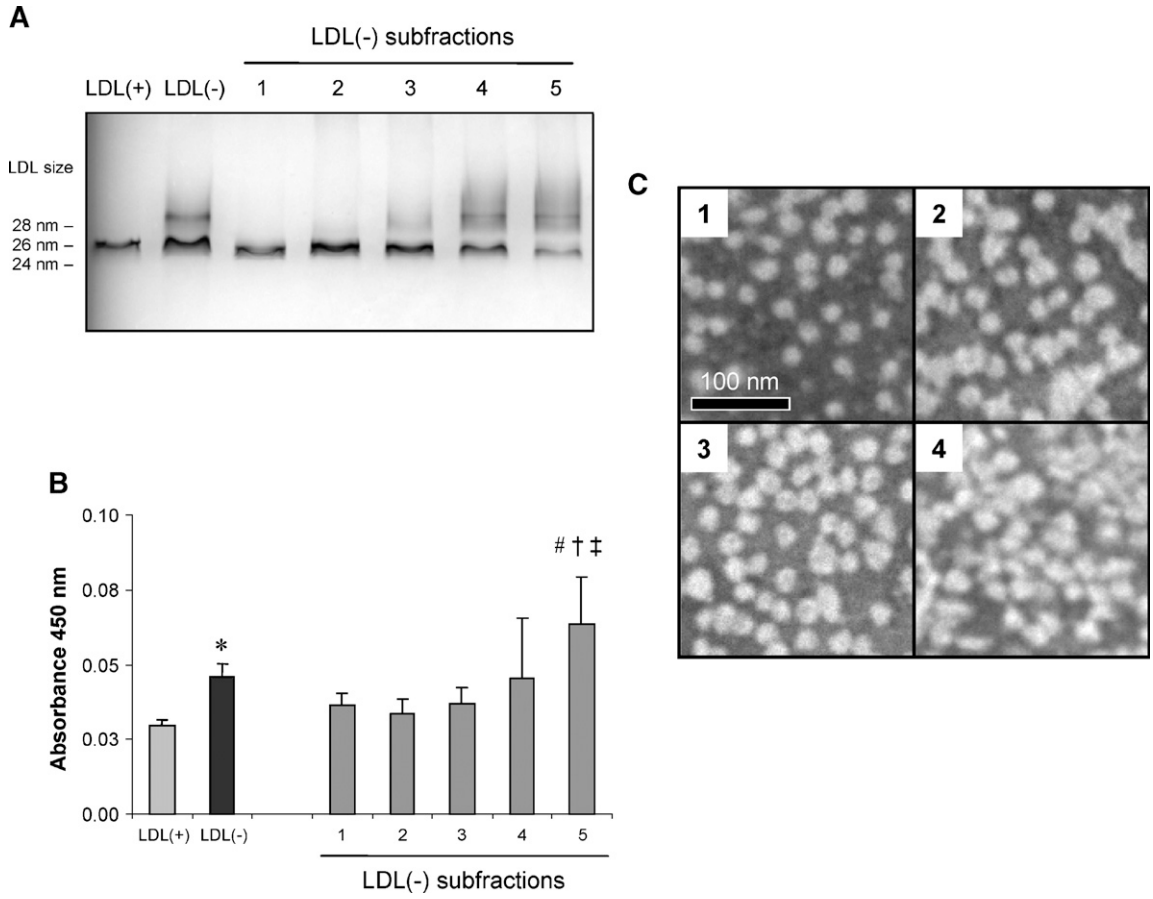

Fig. 7. Aggregation of LDL(+), total LDL(−), and LDL(−) subfractions. LDL(−) was subfractionated depending on its PG affinity (see Fig. 3A). Aggregation was determined by: nondenaturing acrylamide gradient gel electrophoresis (GGE) (A), measuring absorbance at 450 nm (B), and electron microscopy (C). A: The GGE image shows a representative experiment. B: Results are the mean ± SD of five independent experiments. C: Samples were prepared for negative-staining electron microscopy, as described in Materials and Methods. Panel 1, LDL(+); panel 2, LDL(−); panel 3, low-affinity LDL(−) subfraction (subfraction 1); panel 4, high-affinity LDL(−) subfraction (subfraction 5). * $P < 0.05$ versus LDL(+); # $P < 0.05$ versus LDL(−) 1 subfraction; † $P < 0.05$ versus LDL(−) 2 subfraction; ‡ $P < 0.05$ versus LDL(−) 3 subfraction.

ating event in atherosclerosis. As the response-to-retention hypothesis suggests, the retention of lipoproteins to extracellular matrix is followed by their oxidative or enzymatic modification, leading to inflammation and endothelial dysfunction in the vessel wall (4, 39). LDL(−) is a modified subfraction of LDL with increased atherogenicity present in blood plasma. Current results demonstrate that compared with LDL(+), LDL(−) presents increased binding affinity to arterial PGs, the main component of the subendothelial extracellular matrix. This property would favor LDL(−) retention in the superficial PG-rich layer of the arterial intima. As a consequence, the retained LDL(−) particles could contribute, even more severely than LDL (+), to the progression of atherosclerotic lesions because other atherogenic properties such as the induction in cultured endothelial cells of cytotoxicity, apoptosis, or cytokine release have been reported (15, 16).

Binding affinity of LDL(−) to GAGs and to PGs was determined. The highest LDL(−) affinity was to the GAG chondroitin-6 sulfate, which forms part of the versican-like PGs, the most abundant PGs of the intimal extracellular space. Our preparation of PGs isolated from human aortas contained more than 50% chondroitin-6 sulfate, around 25% chondroitin-4 sulfate, and 20% dermatan sulfate. Furthermore, the content of chondroitin sulfate-PGs increases with the progression of lesion size (40), which might favor LDL(−) retention. It should be noted that LDL(+) also had higher affinity to chondroitin-6 sulfate compared with dermatan sulfate or heparan sulfate. Similar results were obtained by Olsson et al. (41), who reported that LDL(+) binds preferentially to chondroitin sulfate and poorly to dermatan sulfate and heparan sulfate.

The analysis of LDL(−)-PG interaction by affinity chromatography at neutral pH demonstrated that LDL(−) consisted of two subpopulations, one with higher and one with lower PG binding affinity than LDL(+). It has been previously reported that although all LDL(−) particles have a negative charge, they are a mixture of heterogeneous particles having different sizes, density, and composition (21). This could explain why LDL(−) presents different degrees of PG affinity. At acid pH, both LDL(+) and LDL(−)

fractions bound to PGs more strongly than at neutral pH, as also reported by Sneck, Kovanen, and Oorni (26) with LDL(+). Analogously to the behavior of the particles at neutral pH, under acid conditions, a subpopulation of LDL(−) also presented greater affinity to PGs than LDL (+). These findings could be significant in more-advanced atherosclerotic lesions, which display drastic decreases in extracellular pH (42). This drop in pH is probably the result of the metabolic activity of macrophages in the lesion, because they acidify their surroundings by extruding hydrogen ions (43) or, in more advanced lesions under hypoxia, macrophages secrete lactate that locally decreases the extracellular pH (44). Interestingly, the LDL(−) subpopulation with lower PG affinity than LDL(+) at neutral pH showed the same affinity as LDL(+) at acidic pH. Thus, the extracellular acidification of atherosclerotic lesions could amplify the deleterious effects of LDL(−), which may play a role in advanced atherosclerotic lesions, with compartments having acidic extracellular fluid.

Because the main characteristic of LDL(−) is its increased electronegative charge, it was expected to have a reduced interaction with the negatively charged sulfate and carboxyl groups on PGs. However, as revealed by agarose gel electrophoresis, our results show that LDL(−) particles with the lowest affinity to PGs (subfraction 1) present electrophoretic mobility similar to those with the highest affinity (subfraction 5). Therefore, particle electronegative charge is not a limiting factor of all LDL(−) particle interaction with PGs, apparently because it can be counteracted by other particle properties that favor the PG interaction. Among the components of LDL(−) that could be involved in counteracting the electronegativity in LDL(−)-PG interaction is its increased content in two apolipoproteins, apoE and apoC-III. ApoE has two binding sites for heparin and mediates the binding of remnant lipoproteins to PGs (45, 46). However, our data indicate that the LDL (−) subfractions with the lowest affinity to PG had increased apoE content. Moreover, apoE-enriched LDL(−) also presented lower affinity to PG than apoE-poor LDL (−). These findings suggest that apoE does not play a major direct role in the increased binding of LDL(−) to arterial PGs. This apparent contradiction with the existing literature must be attributed to at least two facts. First, the content of apoE in LDL(−) is much lower (on average, less than 0.3 molecules of apoE per particle) (16, 20) than that of remnant lipoproteins (several molecules per particle) (14); and second, apoE presents high affinity for heparin and heparan sulfate, but its affinity for dermatan sulfate is moderate, and for chondroitin sulfate, very low (47). Indeed, the PGs isolated from human aorta were composed mainly of chondroitin sulfate, and had only low amounts of dermatan sulfate and no heparan sulfate. Regarding apoC-III, this apolipoprotein may modulate the binding between PGs and apoB- or apoE-containing lipoproteins (14) without binding directly to PGs. The content of apoC-III in LDL(−) is very low (on average, less than 0.3 molecules per particle), and our results indicate that its role is negligible in the binding of LDL(−) to PGs. Therefore, increased LDL(−) binding to PGs should be attributed to factors other than particle-attached apoE or apoC-III.

Current data suggest that lipoprotein aggregation may be the cause of enhanced LDL(−) binding affinity to PGs. Indeed, our results clearly show that LDL(−) subfractions with increased affinity to PGs present an increased tendency to aggregation. It is known that aggregated and fused particles bind to PGs more tightly than LDL(+) (11, 48). This increased binding of aggregated LDL can be explained by spatial arrangements of apoB-100, the existence of multiple apoB-100 molecules per LDL aggregate, or both. Lipolysis by SMase or $sPLA_2$ promotes aggregation of LDLs and increases their binding affinity to PGs. PLC-mediated lipolysis also promotes LDL aggregation (49) and enhances the binding to PGs (Öörni K., unpublished observation). Our group previously reported that intrinsic PLC and SMase activity in LDL(−) promotes self-aggregation (30). Thus, the present data suggest relationships among PLC-like enzymatic activity, aggregation level, and PG binding affinity of LDL(−) subfractions. Increased PLC-like activity can alter LDL lipid composition, thereby promoting particle aggregation and binding to PGs. PLC-like activity hydrolyzes sphingomyelin, phosphatidylcholine, and lysophosphatidylcholine molecules in LDL(−) particles, yielding phosphorylcholine, which is released, and ceramide, diacylglycerol, and monoacylglycerol that are retained in the particle. The increase in these hydrophobic molecules causes aggregation (50) because the ceramide-, diacylglycerol-, and monoacylglycerol-enriched domains act as nonpolar targets on the particle surface and lead initially to particle aggregation.

LDL(−) subfraction composition supports this assumption. LDL(−) subfractions with the highest affinity displayed slightly decreased phospholipid content, whereas triglycerides were increased. It is possible that a triglyceride increase could be related to the enzymatic method used to determine its amount. Thus, in addition to triglycerides, di- and monoacylglycerol are also measured in the assay. Therefore, increased PLC-like activity would be expected to increase the amounts of glycerides in the samples. On the other hand, the apparent apoB-100 content, based on immunodetection, slightly decreased in the highest affinity LDL(−) subpopulation, whereas total protein did not change. This suggests loss of apoB-100 immunoreactivity due to conformational changes. These results concur with the data of Flood et al. (10), who suggested that alterations in the phospholipid composition of lipolyzed LDL induce conformational changes in apoB-100. Thus, these changes may either expose some shielded PG binding regions in apoB-100 or bring positively charged regions of apoB-100 into proper or closer proximity to establish a new PG binding site, other than site B (residues 3,359–3,369), which is the functional PG binding site in LDL(+) (51). In the case of $sPLA_2$-modified LDL with increased affinity to PGs (52, 53), it has been reported that $sPLA_2$ lipolysis leads to exposure of site A (residues 3,148–3,158) in apoB-100, which in LDL(+) is a nonfunctional PG binding site, thereby enabling it to act cooperatively with site B (10). Thus, bringing site A closer to site B

would enhance the interaction with PGs (54). Whether site A is exposed and functional in LDL(−) subpopulations remains to be established in future studies.

Taken together, the present results suggest that increased PLC-like activity could alter LDL lipid composition, thereby increasing particle aggregation that causes enhanced binding to PGs. This propensity of LDL(−) to interact with PGs could facilitate its retention in the extracellular matrix of the arterial intima and contribute to the progression of atherosclerosis.

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