Lipoprotein Lipase–Enhanced Binding of Lipoprotein(a) [Lp(a)] to Heparan Sulfate Is Improved by Apolipoprotein E (apoE) Saturation: Secretion-Capture Process of ApoE Is a Possible Route for the Catabolism of Lp(a)

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Recently, it has been recognized that cell-bound heparan sulfate (HS) proteoglycans (HSPG) are able to bind and subsequently initiate degradation of lipoproteins. Two mediators of lipoprotein catabolism, both with HS binding capacity, lipoprotein lipase (LPL) and apolipoprotein E (apoE), are involved in this process. This mechanism is known as the secretion-capture process of apoE. Lipoprotein(a) [Lp(a)] was shown to have a strong binding capacity to cell-associated HSPG. This binding capacity was increased by LPL addition. We investigated the effects of recombinant apoE (r-apoE) enrichment of Lp(a) on the binding to HS. Lp(a), isolated by ultracentrifugation and gel filtration, was incubated with r-apoE and reisolated by ultracentrifugation, resulting in r-apoE–enriched Lp(a). ApoE-enriched Lp(a) and control Lp(a) were coated to microtiter plates. The capacity to bind biotin-conjugated HS (b-HS) in the presence or absence of inactivated bovine LPL was studied. R-apoE–enriched Lp(a) showed increased b-HS binding capacity versus control Lp(a). Addition of LPL resulted in an increased b-HS binding capacity of both control and r-apoE–enriched Lp(a). To investigate whether binding of Lp(a) to endothelial cell HSPG occurred in vivo, 39 volunteers were injected with heparin (50 U/kg) and plasma lipid and Lp(a) levels were determined before and 20 minutes after heparin injection. No significant increase in plasma Lp(a) concentrations was found. The results showed that Lp(a) can be enriched with apoE and that this resulted in increased LPL-enhanced binding to HSPG. From the in vitro studies, it can be concluded that the secretion-capture process of apoE is a possible catabolic route for Lp(a). However, whether this also occurs in vivo remains to be confirmed.

FIRST DESCRIBED BY BERG,[1] lipoprotein(a) [Lp(a)] is an independent risk factor for coronary heart disease.[2] Lp(a) is a low-density lipoprotein (LDL)-like particle characterized by a heavily glycosylated protein called apolipoprotein(a) (apo(a)), which is covalently linked to apoB-100 by a disulfide bridge.[3] Apo(a) shows strong structural homology with plasminogen.[4] This homology is the basis for the presumed antifibrinolytic action of Lp(a).[5,6] Another explanation for the association of Lp(a) with vascular disease is its accumulation within the vascular wall, apparently by traversing the endothelium and binding to interstitial proteoglycans.[7] Plasma Lp(a) concentrations are remarkably stable over time and are relatively uninfluenced by either diet or pharmacologic treatment. Generally, it is accepted that the apo(a) gene is the major determinant of plasma Lp(a) concentrations.[8,9] Little is known about the catabolism of Lp(a). Lp(a) binds weakly to the LDL receptor, and it is therefore unlikely that the LDL receptor is the major determinant of Lp(a) degradation.[10-12]

Many reports indicate that the cellular catabolism of apoB-containing lipoproteins is increased by lipoprotein lipase (LPL),[13-16] the rate-limiting enzyme in the hydrolysis of triglyceride (TG).[17] The LPL protein has a bridge function between the cell surface and lipoproteins independent of its enzymatic action. LPL increases cell association and cellular degradation of Lp(a).[18] The LPL-enhanced degradation of Lp(a) showed LDL receptor–dependent and –independent components. LDL receptor–independent degradation involves mainly binding to heparan sulfate (HS) proteoglycans (HSPG). Recently, it was postulated that uptake of lipoprotein remnants in the hepatic sinusoidal space involves apoE enrichment and interaction with HSPG.[19-21] Free apoE, secreted by hepatocytes, binds to lipoprotein remnants in the Disse space, and consequently increases lipoprotein binding to the cell surface. This mechanism is known as the secretion-capture process of apoE.[20,22,23]

The existence of apoE-containing Lp(a) has been reported.[24] This represents a minor subpopulation of Lp(a) that differs from normal (ie, apoE-poor) Lp(a) by a relative enrichment in apoC, apoE, TG, and phospholipids and a density less than 1.019 g/mL. ApoE-containing Lp(a) was found to bind significantly better to the LDL receptor of HeLa cells than normal Lp(a).[24] Since there is no evidence for a TG-rich precursor of Lp(a) and greater than 75% of Lp(a) can be found in the characteristic density range of 1.050 to 1.125 g/mL,[25] apoE-containing Lp(a) should be considered a distinct subpopulation.

Our aim was to study whether Lp(a) isolated at the density range of 1.060 to 1.120 g/mL could be enriched with apoE, and whether this resulted in increased HSPG binding. Furthermore, the effect of LPL on HSPG binding to Lp(a) was studied.

SUBJECTS AND METHODS

Lp(a) Isolation

Lp(a) was isolated from plasma obtained from a single healthy blood donor, as described by Leerink et al.[26] Briefly, after collection, EDTA (3 mmol/L), NaN_3 (0.02%), phenylmethylsulfonyl fluoride (0.015%), and aprotinin (0.4 mg/mL) were added to plasma. A lipoprotein density fraction (1.06 to 1.12 g/mL) was prepared by sequential ultracentrifugation in a Beckman (Mydrecht, The Netherlands) 60 Ti rotor (50,000 rpm for 20 hours at 8°C). Lp(a) was separated from LDL and high-density lipoprotein (HDL) particles using Sephacryl S-400HR column (Pharmacia, Uppsala, Sweden) as described previously.[26] According to the

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nomenclature of Utermann et al,[25] the Lp(a) phenotype was F/S2. No apoE was detected in the Lp(a) preparation by immunoblotting procedures.[27]

Apolipoprotein Composition

To identify apolipoproteins, samples were delipidated with chloroform: methanol (1:1) and diethylether.[27] The pellets were resuspended in sample buffer containing 1.25% sodium dodecyl sulfate (SDS), 10% glycerol, 0.03 mol/L Tris, pH 6.8, 0.003% bromophenol blue, and 2.5% β-mercaptoethanol. Apolipoproteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) using precast 4% to 15% gels (Phastsystem; Pharmacia). Following electrophoresis, the gels were stained with silver according to the manufacturer's instructions. Apolipoproteins were identified by immunoblotting[27] using specific monoclonal antibodies against apoE (1D7; a kind gift from Dr Y.L. Marcel, Ottawa, Ontario, Canada) and apo(a) (1A2; a kind gift from Dr G. Utermann, Innsbruck, Austria).

Modification of Lp(a)

Lp(a) was dialyzed extensively against phosphate-buffered saline ([PBS] 1.5 mmol/L KH_2PO_4, 8.1 mmol/L Na_2HPO_4, 140 mmol/L NaCl, and 2.7 mmol/L KCl), pH 7.4, at 4°C. Lp(a) suspensions were incubated at a molar ratio of 1:5 with recombinant apoE ([r-apoE] isoform E-3; kindly provided by Dr T. Vogel, Bio-Technology General, Rehovot, Israel) for 1 hour in a 37°C water bath. Following the incubations, Lp(a) was reisolated by ultracentrifugation using a discontinuous gradient according to the method of Redgrave et al[28]: 3.5 mL incubation solution was brought to a density of 1.25 g/mL with solid KBr, and overlayered with 2.8 mL KBr solutions with densities 1.21, 1.125, and 1.063 g/mL, respectively. The density gradients were ultracentrifuged for 22 hours at 36,000 rpm and 4°C in a Beckman SW 40.1 rotor. Fractions of 0.5 mL were aspirated. Lp(a) was isolated as densities less than 1.12 g/mL. Fractions containing Lp(a) were dialyzed extensively against PBS. An identical Lp(a) preparation underwent similar treatment but without apoE addition, and served as a control.

Apolipoprotein and Lipid Analysis

The concentration of apoB in Lp(a) fractions was measured by a sandwich enzyme-linked immunosorbent assay, as previously described.[29] Plasma concentrations of Lp(a) were determined by a commercial immunoradiometric assay (Pharmacia). The reported within-assay variation is 4.0%.[27] Concentrations of cholesterol and TG in total plasma were measured enzymatically using commercial kits (#237574 and #701912, respectively; Boehringer, Mannheim, Germany). Plasma HDL cholesterol was determined following precipitation of apoB-containing lipoproteins with phosphotungstate and magnesium chloride. LDL cholesterol was calculated according to the method of Friedewald et al.[30]

Preparation of Biotin-Conjugated HS

HS (Sigma, St Louis, MO) was mixed with *N*-hydroxysuccinimide-biotinate (Bio-Rad, Richmond, CA) on a 1:10 molar basis. The mixture was incubated for 4 hours at 4°C with continuous stirring. The reaction mixture was dialyzed against 20 mmol/L Tris buffer, pH 7.4, to block unbound *N*-hydroxysuccinimide-biotin. Free biotin was removed by passing the mixture over a PD-10 column (Pharmacia). Biotin-conjugated HS (b-HS) was stored at 4°C until used. HS concentration was determined with HS (dry weight per milliliter) as standard.[31]

LPL Preparation

LPL was isolated from bovine milk according to the method of Posner et al[32] as previously described.[33] Isolated LPL was enzymatically inactivated by incubation at 50°C for 4 hours and stored at −70°C. Heat inactivation did not alter LPL protein, but blocked its enzymatic activity completely.[29] Protein concentrations were determined by the SDS Lowry assay.[34]

Assay for Binding of b-HS to Lp(a)

A modification of the method of Baker and Christner[35] was used as previously described.[29] In brief, Lp(a) was immobilized by passive adsorption to the wells of flat-bottom microtiter plates. After washing with PBS, aspecific binding was blocked by incubation with 5% bovine serum albumin (BSA) in PBS. After again washing with PBS, interaction buffer containing 1% BSA with or without LPL (1.0 μg/mL) was added. After incubation, LPL was removed by washing the wells with PBS, and b-HS (10 μg/mL) in interaction buffer containing 0.1% BSA was added. Unbound b-HS was removed by washing the wells with PBS. Streptavidin peroxidase (Bio-Rad), 1:5,000 diluted in 50 mmol/L Tris buffer containing 1% BSA was added to the wells. The wells were washed with PBS, and freshly prepared staining solution was added to the wells. The reaction was stopped after 15 minutes by addition of H_2SO_4. Absorbance at 450 nm was measured on a microtiter plate reader (SLT Labinstruments, Salzburg, Austria). All samples were corrected with a blank reading (wells not coated with Lp(a)). All experiments were performed at least in duplicate.

Competition of HSPG Binding by Heparin Injection

Normolipidemic healthy volunteers (24 females and 15 males) were injected with unfractionated heparin 50 IU/kg body weight after an overnight fast. Blood samples were obtained before and 20 minutes after heparin injection. All samples were stored at −20°C and analyzed within 3 months, on the same day. LPL and hepatic lipase activities in plasma were determined in vitro by measuring the release of free fatty acids from [^{14}C]-labeled trioleoyl emulsion according to the method of Huttunen et al.[36] Lipolytic activity is expressed as nanomoles of free fatty acids per minute (milliunits) per milliliter of plasma. LPL mass was determined by commercial assay (markit-FLPL kit; Dainippon, Osaka, Japan). In our hands, within-assay and between-assay variance is 6% and 5%, respectively, for LPL mass determination. This study was approved by the Ethics Committee of Utrecht Academic Hospital. Subjects provided informed consent.

Statistical Analysis

Lp(a) and TG showed non-Gaussian distribution, and therefore a logarithmic transformation of the data was applied. A nonparametric test (Wilcoxon test) was used to evaluate the differences. The paired *t* test was used to test differences in normally distributed parameters. Comparisons of binding curves were made by multivariant ANOVA with contrast polynomial, using the SPSS/PC+ program.[37]

RESULTS

Assessment of LPL Binding to Lp(a)-Coated Wells

LPL binding to both Lp(a) and b-HS was studied. Lp(a) with an apoB concentration of 8 mg/L had been applied to the wells. Addition of LPL protein in a concentration range of 0.01 to 4 μg/mL (Fig 1) resulted in increased binding of b-HS; saturation was reached at LPL 1 μg/mL.

Effects of Modification on Lp(a) Composition

Incubation of Lp(a) with r-apoE resulted in binding of r-apoE to the Lp(a) particle as detected by colocalization of apo(a) and apoE in the first seven density gradient fractions, with a density less than 1.12 g/mL (Figs 2 and 3). Unbound r-apoE was found in fractions 22 to 26 with a density less than 1.21 g/mL. No protein or lipid was detectable in intermediate fraction 13

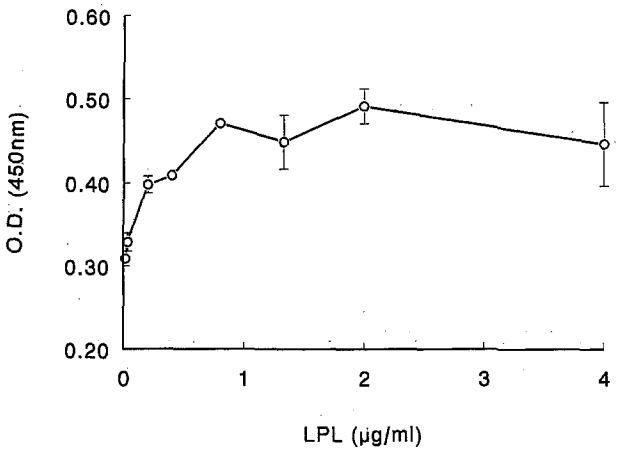

Fig 1. Characterization of LPL-mediated b-HS binding to Lp(a). Wells were coated with Lp(a) at an apoB concentration of 8 mg/L. After the wells were washed with PBS, immobilized Lp(a) was incubated with heat-inactivated LPL at a range of 0.01 to 4 μg/mL. b-HS was then added to measure binding. Results represent the mean ± SD for wells measured in triplicate.

(d = 1.17 g/mL) (Fig 3). The control Lp(a) showed no protein and lipid besides the first seven fractions. Silver staining and immunoblots of SDS-PAGE showed no apo(a) and apoB in density fractions greater than 1.11 g/mL (Fig 3).

Effects of Modified Lp(a) on (LPL-enhanced) b-HS Binding

Binding experiments with apoE-saturated Lp(a) showed that addition of r-apoE increased the capacity of Lp(a) to bind b-HS by 68% compared with control Lp(a) ($P < .01$; Fig 4). Associating the coated Lp(a) or apoE-enriched Lp(a) with LPL increased the binding capacity even further, by 21% ($P < .005$; Fig 4), but the relative contribution of apoE enrichment remained constant. The latter is indicative of the independent action of apoE and LPL on the HSPG binding of Lp(a).

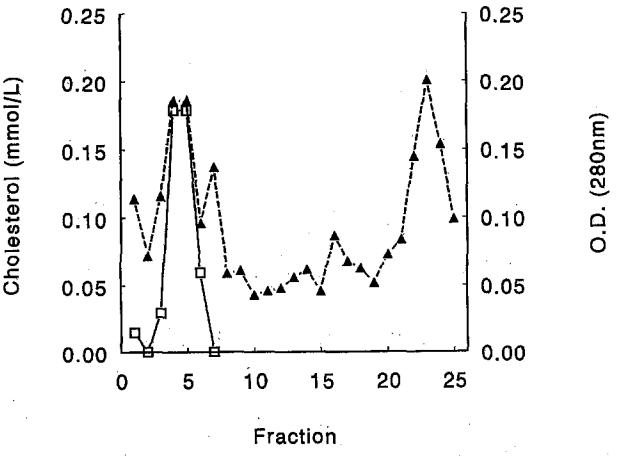

Fig 2. Distribution of cholesterol and protein in discontinuous density gradient fractions. Lp(a) incubated with r-apoE was reisolated by means of discontinuous density gradient ultracentrifugation. The resulting gradient was collected in fractions of 0.5 mL. (□) Cholesterol concentration; (▲) O.D. 280 nm, an indicator of protein concentration. The first 7 fractions have a density <1.12 g/mL, fraction 13 has a density of 1.17 g/mL, and fractions 22 to 26 have a density >1.21 g/mL.

In Vivo Binding of Lp(a) to HSPG

To investigate if the binding of Lp(a) to HSPG occurred as an in vivo phenomenon, 39 volunteers were injected with heparin 50 IU/kg body weight. Heparin is a strong competitive inhibitor for protein binding to cell-bound HSPG,[38] and is commonly used to release LPL and hepatic lipase from their HSPG binding sites on the endothelial wall.[39] Lp(a), lipid, and LPL mass and activity were determined before and 20 minutes after heparin injection. Heparin-liberated LPL, hepatic lipase, or fatty acids did not influence measurements of Lp(a) and lipids, as described previously.[40] No significant difference in Lp(a), cholesterol, LDL cholesterol, and HDL cholesterol before and after heparin injection was found for individual values (Table 1). Furthermore, no significant correlation was found between apoE isoforms as determined by genotyping[41] and Lp(a) concentrations measured before and after heparin injection. Neither were correlations present between apoE isoforms and the difference in Lp(a) concentration measured in preheparin and postheparin plasma samples (results not shown). A significant decrease in TG levels was found ($P < .01$), due to increased lipolytic activity of released LPL.

DISCUSSION

The route for catabolism of Lp(a) is still an enigma. Although Lp(a) does not participate in classic very–low-density lipoprotein (VLDL)-LDL catabolism, involvement of the LDL receptor in the catabolism has been established, but to a lesser extent compared with the catabolism of LDL.[11,42,43] No specific receptor for Lp(a) has been detected, although it has been suggested that other receptors with known affinity for lipoproteins could be involved. The macrophage scavenger receptor has been shown to bind modified Lp(a), and the LDL receptor–related protein was shown to have binding affinity for Lp(a).[44-46]

In our cell-free test system, immobilized Lp(a) was shown to bind b-HS. Furthermore, association with LPL increased the capacity to bind b-HS, although to a lesser extent than seen for LDL.[29] These data confirmed the results obtained by Williams et al,[18] who found that LPL promotes binding of Lp(a) to cell-surface HSPG. The findings suggested the possibility for Lp(a) to be cleared by the liver in a nonreceptor pathway involving HSPG, mediated by LPL and potentially apoE.[19]

Therefore, we investigated if apoE-free Lp(a) isolated in the density gradient 1.050 to 1.120 g/mL could be enriched with r-apoE and if this could subsequently increase the binding to HSPG in the presence or absence of LPL. Furthermore, we investigated if Lp(a) binding to HSPG occurred in vivo.

Our results clearly establish that lipid-free apoE can associate with Lp(a), resulting in an apoE-Lp(a) complex resistant to high-salt and ultracentrifugation conditions. This apoE-enriched Lp(a) showed a higher binding capacity with b-HS. Incubation of immobilized apoE-Lp(a) with LPL increased the binding of b-HS further. The additional effect of LPL on b-HS binding to Lp(a) or apoE-Lp(a) was small. It was shown that apoE saturation increased the capacity of Lp(a) to bind HSPG without affecting LPL-mediated HSPG binding. These results indicate independent actions of LPL and apoE in terms of an increase in the b-HS binding capacity of Lp(a), a finding recently observed for TG-rich lipoproteins.[29] The present findings suggest that Lp(a) may be catabolized by non–receptor-

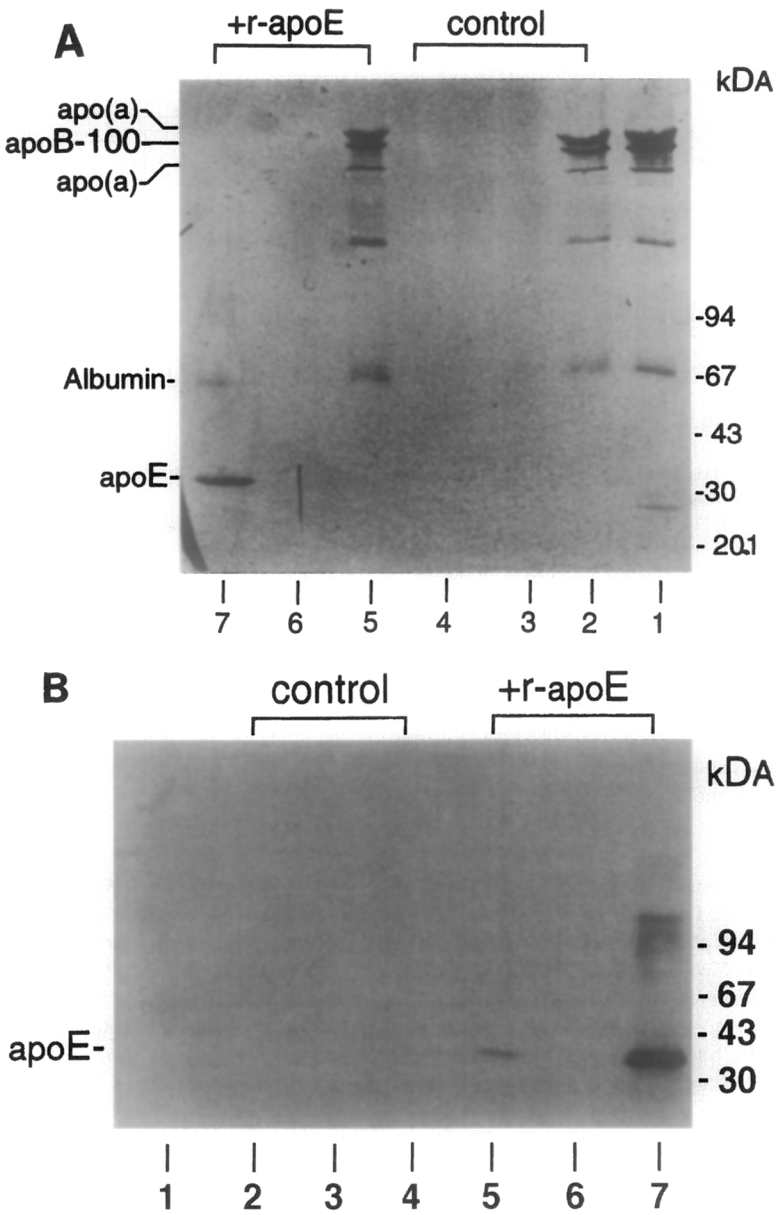

Fig 3. Analysis of control and r-apoE–enriched Lp(a). Lp(a) protein was separated on 4% to 15% SDS-PAGE gradient gels. The proteins were stained with silver (A), or the gels were immunoblotted with anti-apoE (B). Lane 1, Lp(a) preparation before modification; lanes 2 to 4, density gradient fractions of control Lp(a): lane 2, pooled fractions 1 to 7; lane 3, fraction 13; lane 4, pooled fractions 22 to 26; lanes 5 to 7, density gradient fractions of Lp(a) incubated with r-apoE: lane 5, pooled fractions 1 to 7; lane 6, fraction 13; lane 7, pooled fractions 22 to 26. The position of low–molecular-weight SDS-PAGE standards (Pharmacia) is indicated at right. The position of apolipoproteins is indicated at left.

mediated pathway(s) involving HSPG binding and potentially apoE enrichment, in a way similar to that postulated for other apoB-containing lipoproteins.[19,20]

We investigated if Lp(a) binding to endothelial HSPG occurred in vivo. For this purpose, volunteers were injected with heparin, a strong competitor for protein binding to cellular HSPG. Plasma Lp(a) concentrations were monitored before and after heparin injections. We could not detect a significant release of Lp(a) into the circulation due to heparin. This is in agreement with a report from Shimano et al.[20] This group could not detect increased uptake of hepatic lipoproteins in human apoE transgenic mice, and therefore postulated that the secretion-recapture process of apoE is not functional for hepatic-derived lipoproteins. In contrast, heparinase infusion in mice to hydrolyze liver HSPG inhibited plasma clearance and liver uptake of chylomicrons and β-VLDL, but not of LDL. No additional effect of apoE enrichment on β-VLDL catabolism was seen in contrast to chylomicron remnant catabolism.[21] Together, this suggests a mechanism whereby both intestinal- and hepatic-derived lipoproteins can bind to liver HSPG, but the additional effect of apoE enrichment is only functional for intestinal lipoproteins. We studied our subjects in a fasting state, excluding the competition of Lp(a) with intestinal-derived lipoproteins for both apoE enrichment and HSPG binding. Another possibility is

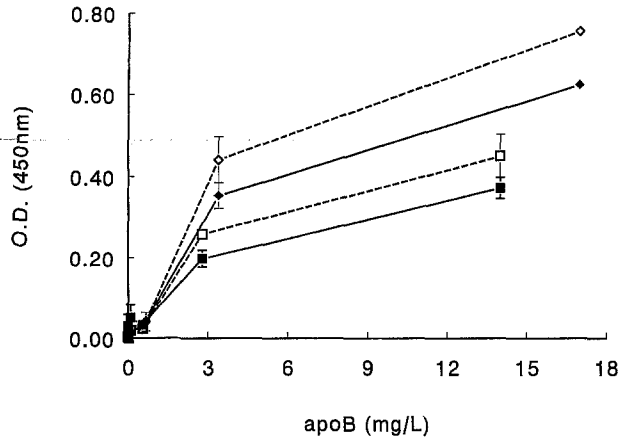

Fig 4. b-HS binding to modified and control Lp(a). Wells were coated with control Lp(a) (■) and Lp(a) enriched with r-apoE (◆) at an apoB concentration of 0.001 to 18 mg/L (100 μl/well). Unbound Lp(a) was removed by washing. Experiments were performed in the absence of LPL (■, ◆) or the presence of 1 μg/ml LPL (□, ◇). Finally, 10 μg/mL b-HS was added and used as the detection step. Results represent the mean ± SD for wells measured in triplicate.

Table 1. Lp(a) and Lipid Concentrations Before and After Heparin Injection

	Before	After	Difference (%)	P
Lp(a) (mg/L)	277 ± 296	283 ± 309	9	NS
TG (mmol/L)	1.38 ± 0.42	1.08 ± 0.67	−30	<.01
Cholesterol (mmol/L)	5.40 ± 0.64	5.53 ± 0.97	3	NS
HDL cholesterol (mmol/L)	0.94 ± 0.33	1.04 ± 0.69	0	NS
LDL cholesterol (mmol/L)	3.74 ± 0.89	4.10 ± 0.89	6	NS
LPL activity (mU)	ND	133 ± 48	—	—
LPL mass (ng/mL)	18 ± 20	332 ± 133	2,896	<.001

NOTE. Values are expressed as the mean ± SD. *P* values were determined by *t* test for normally distributed paired samples. Lp(a) and TG *P* values were determined by Wilcoxon matched-pair signed-rank test.

Abbreviation: ND, not determined.

that HSPG binding sites in vivo are occupied by lipases and therefore are not available for binding detectable amounts of Lp(a).

A small but nonsignificant increase in Lp(a) plasma levels was detected; therefore, we cannot exclude the possibility of in vivo Lp(a) binding to HSPG, mediated by LPL and/or apoE enrichment, as a route of catabolism. More conclusive evidence about the in vivo importance of this process for Lp(a) has yet to be obtained.

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