Effect of phospholipase A_2 digestion on the conformation and lysine/fibrinogen binding properties of human lipoprotein[a]

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Abstract **In vitro hydrolysis of human lipoprotein[a] (Lp[a]) by phospholipase A_2 (PLA_2) decreased the phosphatidylcholine (PC) content by 85%, but increased nonesterified fatty acids 3.2-fold and lysoPC 12.9-fold. PLA_2-treated Lp[a] had a decreased molecular weight, increased density, and greater electronegativity on agarose gels. In solution, PLA_2-Lp[a] was a monomer, and when assessed by sedimentation velocity it behaved like untreated Lp[a], in that it remained compact in NaCl solutions but assumed the extended form in the presence of 6-amino hexanoic acid, which was shown previously to have an affinity for the apo[a] lysine binding site II (LBS II) comprising kringles $IV_{5–8}$. We interpreted our findings to indicate that PLA_2 digestion had no effect on the reactivity of this site. This conclusion was supported by the results obtained from lysine Sepharose and fibrinogen binding experiments, in the presence and absence of Tween 20, showing that phospholipolysis had no effect on the reactivity of the LBS-II domain. A comparable binding behavior was also exhibited by the free apo[a] derived from each of the two forms of Lp[a]. We did observe a small increase in affinity of PLA_2-Lp[a] to lysine Sepharose and attributed it to changes in reactivity of the LBS I domain (kringle IV_{10}) induced by phospholipolysis. In conclusion, the extensive modification of Lp[a] caused by PLA_2 digestion had no significant influence on the reactivity of LBS II, which is the domain involved in the binding of apo[a] to fibrinogen and apoB-100. These results also suggest that phospholipids do not play an important role in these interactions.**—Fless, G. M., E. W. Kirk, O. Klezovitch, J. Y. Santiago, C. Edelstein, J. Hoover-Plow, and A. M. Scanu. **Effect of phospholipase A_2 digestion on the conformation and lysine/fibrinogen binding properties of human lipoprotein[a].** ***J. Lipid Res.*** **1999.** 40: **583–592.**

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Lipoprotein[a] (Lp[a]) is a lipoprotein particle having as protein moiety apoB-100 linked by a single disulfide bond to apolipoprotein[a] (apo[a]), a multikringle structure with a high degree of homology with plasminogen (1–4). This correspondence in structure has functional ramifications in that apo[a] like plasminogen has kringles containing lysine binding sites (LBS). In the case of apo[a], these are grouped into two domains called LBS I and LBS II (5). The interaction of Lp[a] with lysine Sepharose appears to depend on LBS I which is located on K-IV_{10} of apo[a] (6, 7), whereas LBS II is located in the K-$IV_{5–8}$ domain and is involved in an interaction with apoB-100 (5, 8) and fibrinogen (9). LBS II is thought to interact with apoB-100 lysine and/or proline residues (5, 8, 10); however, the specific binding site(s) on apoB-100 or the amino acid residues involved in the binding have not been identified. In addition, the LBS II domain is responsible for maintaining the compact conformation of Lp[a], as suggested by the observation that an extended conformation of Lp[a] is produced when the interaction of this site with apoB-100 is disrupted by the lysine analog 6-AHA (10, 11). The lipoprotein part of Lp[a] is LDL-like (3, 12), with a surface monolayer of phospholipid that is similar in content and composition to that of authentic LDL, and consisting mainly of phosphatidylcholine (PC) and sphingomyelin (13). This monolayer is segregated into two domains, a predominant fluid bulk phase that

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; apoB, apolipoprotein B; LDL, low density lipoprotein; PLA_2, phospholipase A_2; PLA_2-Lp[a], phospholipase A_2 treated Lp[a]; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; NEFA, nonesterified fatty acid; K, kringle; K-IV 1–10, apo(a) kringle domain of subtype 1 to 10, all with homology to kringle 4 of plasminogen; LBS, lysine binding site; 6-AHA, 6-aminohexanoic acid; Na_2EDTA, sodium salt of ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, sodium salt of (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]).

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contains the majority of PC and all of sphingomyelin and a comparatively smaller rigid domain of PC that appears to interact with apo-B 100 (13–18). With Lp[a], however, the association of apo[a] with the LDL particle causes a number of subtle but significant structural changes. First, the immobilized microdomain of PC is greater in Lp[a] than in LDL without an apparent effect of apo[a] on sphingomyelin (13, 17, 19). Second, the interactions between surface and core lipids are increased (20). Third, the Lp[a] neutral lipid core is enriched in triglycerides (3, 12) causing a decrease in the reversible thermotropic order–disorder transition of the neutral lipid core (20). These differences in lipid composition between LDL and Lp[a] may be due to an effect of apo[a] on the lipid-modifying enzymes and lipid transfer processes that contribute to the lipid distribution among lipoproteins in the plasma.

The mechanism by which apo[a] increases the proportion of immobilized to mobile PC is not clear, but is thought to involve changes in the conformation of apoB-100 brought about by apo[a], rather than by the direct interaction of apo[a] with the PC molecules at the lipoprotein surface (13, 17, 19). The effect of apo[a] on the conformation of apoB-100 has also been detected by immunochemical means with a battery of monoclonal antibodies specific to apoB-100 (21). These findings illustrate the complex interdependence between the structures of apoB-100 and apo[a] and the physical state of the lipids of Lp[a]. They also raise the possibility that modifications of either core or surface lipids may have an effect on the structure and function of the two main protein components of Lp[a].

Based on the above premises, our aim was to determine whether phospholipolysis caused changes in the solution properties of Lp[a] as measured by sedimentation velocity, and also to examine the potential role of the two major lysine binding domains of apo[a], e.g., LBS-I and LBS-II, in their interaction with apoB on Lp[a], lysine Sepharose and fibrinogen. Specifically we investigated possible changes in Lp[a] conformation and the interaction of Lp[a] with fibrinogen and lysine Sepharose, all of which to some extent involve the LBS II domain of apo[a]. No structural studies have been reported previously on Lp[a] digested by PLA_2. On the other hand, there have been reports showing that phospholipolysis has only a minimal effect on the solution properties and structural stability of LDL (22), and decreases the immunoreactivity of epitopes located in the C-terminal domain of apoB (23). From the functional standpoint, recent reports have appeared showing that lipolysis by some phospholipases increases the in vitro binding of Lp[a] to lysine Sepharose (24, 25) and components of the extracellular matrix (24).

Our results indicate that the extensive remodeling of the phospholipid surface monolayer of Lp[a] by PLA_2, although causing marked changes in molecular weight and surface charge of the Lp[a] particle, had no significant effect on the capacity of apo[a] to bind to apoB-100, suggesting that phospholipids do not play a significant role in this interaction.

EXPERIMENTAL PROCEDURES

Preparation of lipoprotein[a]

The subject studied has been a major Lp[a] donor for over 15 years exhibiting a stable normal lipid profile with representative values for total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride of 152, 89, 51, and 62 mg/dl, respectively. The plasma, which contained two apo[a] polymorphs with 15 and 27 K-IV domains, at a respective relative abundance of 10 to 1, served as donor for the preparation of Lp[a]. The subject gave informed consent prior to plasmapheresis. Lp[a] (15 K-IV) was isolated from plasma by a combination of lysine Sepharose chromatography and density gradient centrifugation using previously described methods (12, 26, 27). Apo[a] was prepared from Lp[a] as described by Edelstein et al. (8).

Phospholipolysis

Chromatographically pure phospholipase A_2 from *Crotalus adamanteus* (Worthington, Freehold, NJ) (specific activity: 200 U/mg dry weight) was used to digest Lp[a]. The reaction was carried out at room temperature in the presence of 1% fatty acid-free bovine serum albumin in 7 mm $CaCl_2$, 10 mm Tris, 150 mm NaCl, and 0.01% NaN_3, pH 7.4, at a protein-to-enzyme weight ratio of 325 to 1. The progress of the reaction was usually monitored at selected time intervals for the release of NEFA using the NEFA C test kit (Wako, Richmond, VA). The reaction was stopped by addition of Na_2EDTA in excess of Ca^{2+}. In initial experiments, the loss of phosphatidylcholine and production of lysophosphatidylcholine was also determined. Lp[a] was purified from the reaction products, albumin and phospholipase A_2, by density gradient centrifugation on a linear (0–100%) D_2O gradient superimposed on 10 mm Tris, 150 mm NaCl, 0.01% NaN_3 and Na_2EDTA, pH 7.4, in the SW-40 rotor at 39,000 rpm and 20°C for 48 h. The gradient was monitored at 280 nm by pumping through an ISCO density gradient fractionator, and the fractions containing PLA_2-modified Lp[a] were pooled and dialyzed against 150 mm NaCl, 0.01% NaN_3 and Na_2EDTA, pH 7.4.

Electrophoresis

Agarose gel electrophoresis was performed using Ciba-Corning precast agarose gels (Fisher Scientific, Pittsburgh, PA) according to the manufacturers' instructions. The film was stained with Coomassie Blue R250. Migration distance was measured with a Nikon Shadowgraph Model 6C set to a magnification of 10 and equipped with an electronic readout allowing measurements of ± 10 μm. PLA_2-modified Lp[a] was examined by SDS-PAGE for potential degradation during phospholipolysis using 4–15% precast polyacrylamide and the Phast system (Pharmacia Biotech, Piscataway, NJ). Electrophoresis and Western blotting with antiapo[a] were performed as previously described (28).

Chemical analysis

Phospholipids were determined in aliquots extracted by the method of Folch, Lees, and Sloane Stanley (29) as

modified by Bligh and Dyer (30), followed by chromatography on glass plates coated with Silica Gel G, developed in chloroform–methanol–acetic acid–water 75:42:15:7.5, scraping the appropriate spots and charring with perchloric acid. Phospholipid phosphorus was determined according to Bartlett (31) and the amount of phospholipid was calculated using a conversion factor of 25.1 for PC, 16.5 for lyso-PC, and 23.6 for sphingomyelin. Minor phospholipids were separated on LHP-K TLC plates (Whatman, Hillsboro, OR) using a double solvent system. Solvent #1 consisted of ethyl acetate–1-propanol–chloroform–methanol–0.25% KCl 25:25:20:15:9, and solvent #2 contained hexane–diethyl ether–acetic acid 75:21:4. Three-quarters of the TLC plate was developed with the first solvent after which it was developed to the top with the second system. For quantification, phospholipid standards (Sigma, St. Louis, MO) were run in parallel with unknowns, visualized by spraying with 10% $CuSO_4$, 8% H_3PO_4 and charred at 130°C. The plates were imaged with an HP Scan Jet Plus scanner using the Desc Scan program (Hewlett-Packard, Palo Alto, CA) and quantified with IP Lab Gel software (Signal Analytics Corp., Vienna, VA) Free and total cholesterol were determined using enzymatic kits from Wako Chemicals (Richmond, VA); cholesteryl ester was determined by multiplying the difference between total and free cholesterol by 1.65. Triglyceride was determined using an enzymatic test kit from Sigma (St. Louis, MO). Protein was determined by the method of Lowry et al. (32) as modified by Markwell et al. (33) using bovine serum albumin as standard.

Analytical centrifugation

Sedimentation and flotation equilibrium experiments were performed with a Beckman Optima XLA ultracentrifuge interfaced to a Dell Optiplex XMT 590 personal computer, an AN-60 Ti four-place rotor and analytical cells equipped with six-channel charcoal-filled centerpieces. The molecular weight, M, and anhydrous partial specific volume, $\bar{v}$, of Lp[a] in five different solutions containing from 0 to 100% D_2O was determined by equilibrium centrifugation carried out as described previously (34, 35). The reciprocal of $\bar{v}$ is the anhydrous particle density of Lp[a].

Sedimentation velocities of Lp[a] were measured using the An-60 Ti rotor and double sector cells with aluminum-filled epon centerpieces and quartz windows (11) Rotor speed was 40,000 rpm and the temperature was controlled at 20°C. Both rotor and cells were pre-equilibrated to 20°C before starting the sedimentation velocity runs. The cells were scanned at 8-min intervals; usually 16 data sets were collected after a 16-min delay. Data from sedimentation velocity experiments were analyzed using the second moment boundary spreading method of the XLA-veloc program/Optima XL-A data analysis software (Beckman, Palo Alto, CA) in conjunction with Origin software (version 3.78) (Microcal Software, Northampton, MA). Solvent density and viscosity were measured at 20°C as previously described (10, 11).

Lp[a] was dialyzed extensively against 10 mm HEPES, 100 mm NaCl, 0.01% Na_2EDTA, and 0.01% NaN_3, pH 7.4, and was then adjusted by dilution to a protein concentration of approximately 0.2 to 0.3 mg/ml and the desired concentrations of 6-AHA or NaCl. For plots of the viscosity corrected sedimentation coefficient ($s\eta$) versus density, the latter ranged from 200 mm to 1 m. For experiments designed to determine the binding constant of 6-AHA to Lp[a], the range of ligand concentrations was extended to 1 mm. To prevent the unfolding of Lp[a] at low ionic strength, 100 mm NaCl was included in all solutions.

For each ligand examined, plots of $s\eta$ versus density were extrapolated to unity. The intercept on the ordinate was corrected to water at 20°C using the following relation

$$s_{20,w} = s_{obs} \frac{\eta_s(1 - \bar{v}\rho_w)}{\eta_w(1 - \bar{v}\rho_s)}$$

where η_s and η_w are, respectively, the viscosities of the solvent and water at 20°C; ρ_w and ρ_s are, respectively, the densities of pure water and solution at 20°C, and $\bar{v}$ is the anhydrous lipoprotein partial specific volume determined in D_2O. In the determination of binding constants, the hydrodynamic partial specific volume, $\bar{v}_h$, was used instead of $\bar{v}$ to define a differently corrected sedimentation coefficient, $s'_{20,w}$. $\bar{v}_h$ is the reciprocal of the Lp[a] particle buoyant density obtained from the x-axis intercept of the $s\eta$ versus density plot, e.g., $\bar{v}_h = 1/\rho_b$. The plots of $s'_{20,w}$ versus log 6-AHA concentration were analyzed for the dissociation constant and number of bound ligand molecules as previously described (10, 11).

Binding to lysine Sepharose

Chromatography of Lp[a] on lysine Sepharose 4B was conducted on a column (1.5 × 4 cm) equilibrated in 10 mm phosphate, 100 mm NaCl, 0.01% Na_2EDTA and NaN_3, pH 7.4 using a flow rate of 0.5 ml/min using the low pressure Bio-Rad Econo chromatography system. Aliquots of Lp[a] (250 μg protein) dialyzed against column buffer were applied to the column and the bound Lp[a] was eluted with a 50-ml linear gradient of 0–20 mm 6-AHA superimposed on the column buffer. Apo[a] was chromatographed under similar conditions except the 50-ml gradient ranged from 0 to 200 mm 6-AHA. For experiments conducted in 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma, St. Louis, MO), the Lp[a] samples were pre-equilibrated with the detergent for 1 h at room temperature before application to the column. Column buffers were identical except for the presence of the detergent and a 0–200 mm 6-AHA gradient.

Fibrinogen binding

Human fibrinogen (Sigma, St. Louis, MO) (100 μl, 10 μg/ml) in 50 mm Tris, 150 mm NaCl, pH 7.4, was coated onto microtiter plates as previously described (36). The plate was blocked with 2% BSA before adding various concentrations of Lp[a] in the above buffer, with and without 200 mm 6-AHA. The plate was incubated for 2 h at room temperature, washed, and the bound Lp[a] was detected with rabbit antiapo[a] IgG followed by goat anti-rabbit IgG-alkaline phosphatase (Sigma, St. Louis, MO). In some

cases the incubation of Lp[a] with immobilized fibrinogen was performed in the presence of 0.1% Tween 20. Color was developed with *p*-nitrophenylphosphate at 405 nm on a Biomek 100 microplate reader (Beckman, Fullerton, CA). Absorbance data were transformed into moles using standard curves constructed for both Lp[a] and PLA_2-Lp[a] after determining that phospholipolysis did not affect the immunoreactivity of Lp[a]. Lysine-mediated binding was obtained by subtracting the binding in the presence of 200 mm 6-AHA from the total binding. Binding curves were analyzed as described previously (36).

RESULTS

Chemical analysis

The time course of the hydrolysis of Lp[a] phospholipids by phospholipase A_2 was followed by the production of NEFA and by the increased mobility of the modified Lp[a] particle on agarose gel electrophoresis. The results of a typical experiment are shown in **Fig. 1**, indicating that the time course of the reaction as measured by either method was virtually identical and that hydrolysis had ceased by approximately 1 h. Phospholipid analysis of the reaction mixture by thin-layer chromatography showed that the action of phospholipase A_2 led to the disappearance of PC and to the production of lyso-PC, with the sum of PC and lyso-PC remaining virtually constant (**Fig. 2**). Despite almost complete hydrolysis of PC, the modified Lp[a] particle was stable at normal working concentrations. It was purified from BSA and phospholipase A_2 by density gradient centrifugation in the SW40 rotor (39,000 rpm, 20°C, 48 h) on a 0–100% D_2O linear gradient without any visible aggregation by keeping its concentration relatively low. Aggregation occurred only at higher concentrations of Lp[a], e.g., above 1.5 mg/ml protein, where a visible band of turbidity became evident on the density gradient, and upon flotation of the Lp[a]-containing reaction mixture in solutions of NaBr, at a density of 1.12 g/ml, in the 50.3 Ti rotor (35,000 rpm, 20 h, 20°C), which produced a clear lipoprotein gel that could not be resolubilized.

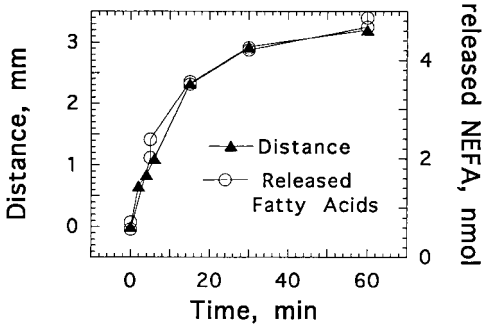

Fig. 1. Time course of hydrolysis of Lp[a] by PLA_2 followed both by the production of NEFA and by the increased mobility on agarose gels of PLA_2-Lp[a] relative to untreated Lp[a]. Lp[a] at a protein concentration of 1.27 mg/ml was hydrolyzed with PLA_2 using conditions defined in Experimental Procedures. At appropriate time intervals, 5 μl aliquots were removed for NEFA analysis.

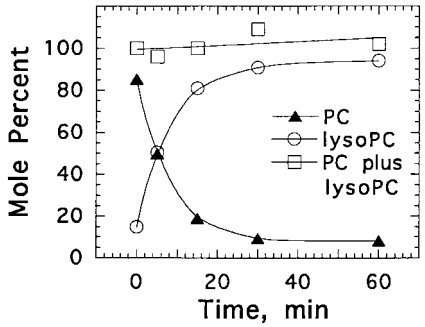

Fig. 2. Time course of the loss of PC and production of lyso-PC after the hydrolysis of Lp[a] by PLA_2. 15 K-IV Lp[a] (1 mg/ml protein) was incubated with PLA_2 at a weight ratio of 325 to 1 at room temperature in the presence of 1% fatty acid-free BSA. Aliquots of the reaction mixture were extracted; the lipids were separated by TLC and analyzed for phosphorus as described under Experimental Procedures; PC, solid triangles; lyso-PC, open circles; the sum of PC and lyso-PC is represented by the open squares.

Compared to the untreated Lp[a], digested Lp[a] exhibited a pronounced shift to higher density on the D_2O gradient (**Fig. 3**). SDS-PAGE indicated that PLA_2-Lp[a] was free of albumin and PLA_2, and had no sign of degradation upon Western blotting with antibodies specific to apo[a] (Fig. 3) and to apoB. As the enzymatic modification was carried out in the absence of EDTA, PLA_2-Lp[a] was examined for evidence of oxidation by determining its diene content. Hydrolysis of Lp[a] did not result in increased oxidation as the absorbance at 234 nm of a 50 μg/ml solution (0.22) was not significantly different from the absorbance of the control Lp[a] (0.21). The effect of phospholipase A_2 on Lp[a] was evaluated by determining the mass, anhydrous density, and chemical composition of PLA_2-Lp[a] in comparison to the unmodified control Lp[a] (**Table 1**). Phospholipolysis of Lp[a] led to a decrease in the molecular weight of Lp[a] from 3.55×10^6

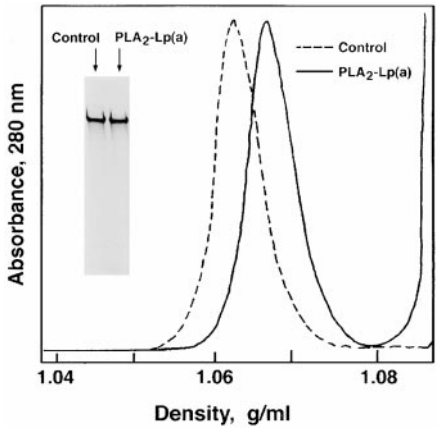

Fig. 3. Density gradient ultracentrifugation of untreated and PLA_2-Lp[a] on a linear 0–100 D_2O gradient in the SW-40 rotor at 38,000 rpm and 20°C for 48 h. PLA_2-Lp[a] was purified from PLA_2 and BSA present in the incubation mixture. The insert shows a Western blot of untreated and PLA_2-Lp[a] subjected to SDS-PAGE on a 4% acrylamide gel and visualized with anti-apo[a] serum.

TABLE 1. Molecular weight, density, and chemical composition of Lp[a] treated with PLA_2

	Control Lp[a]	PLA_2-Treated Lp[a]
Molecular weight[a]	$3.55 \pm 0.09 \times 10^6$	$3.34 \pm 0.11 \times 10^6$
Anhydrous density[a] (g/ml)	1.061 ± 0.001	1.070 ± 0.002
	weight percent[b]	
Protein	24.6 ± 0.3	27.0 ± 0.1
Carbohydrate[c]	3.5 ± 0.10	3.8 ± 0.02
Phospholipid	19.4 ± 0.5	14.3 ± 0.4
Free cholesterol	7.6 ± 0.02 (697)[e]	8.1 ± 0.04 (699)
Cholesteryl ester	40.3 ± 0.4 (2200)	41.5 ± 0.60 (2132)
Triglyceride	4.4 ± 0.3 (184)	4.8 ± 0.09 (189)
NEFA	0.14 ± 0.003 (18)	0.49 ± 0.011 (58)
PC[d]	68.6 ± 2.8 (608)	14.5 ± 1.6 (89)
Sphingomyelin[d]	24.3 ± 0.3 (229)	37.6 ± 0.60 (246)
Lyso-PC[d]	2.5 ± 0.07 (34)	46.9 ± 1.6 (438)
PI[d]	2.1 ± 0.10 (17)	0.5 ± 0.23 (3)
PE[d]	1.5 ± 0.42 (14)	0.2 ± 0.14 (1)
PS[d]	1.0 ± 0.26 (9)	0.3 ± 0.08 (2)

[a] The molecular weight and anhydrous density were obtained simultaneously by sedimentation equilibrium in several D_2O solutions of different density (see Methods).

[b] The percentage of the individual components represents the mean ± standard deviation of two analyses done in triplicate.

[c] Carbohydrate content was estimated from that of apoB (7%) and apo[a] (23%) (34).

[d] Calculated as the weight percent of the sum of total phospholipid.

[e] Numbers in parentheses represent mol lipid per mol lipoprotein.

to 3.34×10^6 as measured by sedimentation equilibrium in D_2O and to an increase in the anhydrous density from 1.061 to 1.070 g/ml. None of the equilibrium scans of PLA_2-Lp[a] in various concentrations of D_2O showed signs of aggregation as all plots of concentration versus radial position could be fitted perfectly with the equation describing a single ideal solute. These data, together with the knowledge of the mass and weight percent of each component in the Lp[a] particle, led to the conclusion that with the exception of the phospholipids and NEFA, the other components of PLA_2-Lp[a] (free cholesterol, cholesteryl ester, and triglyceride) were essentially unchanged as they remained within 3.3% of corresponding values of control Lp[a]. There was, however, a 31% loss in the mass of phospholipids, as calculated from Lp[a] mass and weight percent phospholipid of each Lp[a] particle. This loss was mainly responsible for the molecular weight difference between control Lp[a] and PLA_2-Lp[a]. In PLA_2-Lp[a] the number of molecules of NEFA increased from 18 to 58, which probably accounted for its increased mobility on agarose gels. The phospholipid composition of PLA_2-Lp[a] changed drastically in that compared to control Lp[a] the content of PC dropped 85% while lyso-PC increased 12.9-fold. Sphingomyelin remained relatively constant. Out of 519 hydrolyzed PC molecules, 404 lyso-PC and 40 NEFA molecules were retained by the PLA_2-Lp[a] particle (Table 1). Thus 22% of lyso-PC and 92% of NEFA produced by the phospholipolysis were lost and probably taken up by the defatted albumin. Moreover, in the modified Lp[a], the content of the minor phospholipids PI, PE, and PS decreased by 83%, 91%, and 79%, respectively. However, we were unable to measure their respective lyso derivatives because of their low concentrations.

Sedimentation velocity

Based on sedimentation velocity measurements, our previous studies had shown that the lysine analog, 6-AHA, induced a marked change in the overall conformation of Lp[a]. This was interpreted to be related to the binding of 6-AHA to the K-IV_{5-8} domain, e.g., the LBS II of apo[a], in contrast to the LBS-I present in K-IV_{10}. In the current study, we extended the sedimentation velocity analyses to Lp[a] completely digested by PLA_2 to determine whether phospholipolysis might induce a conformational change of Lp[a]. Four consecutive batches of Lp[a] isolated from the same individual at 2-month time intervals were used. The sedimentation coefficients of both control and PLA_2-Lp[a] were determined in solutions of NaCl (0.2 to 1.0 m) and in solutions of 0.1 m NaCl with varying amounts of 6-AHA (200–750 mm). Both sets of solutions contained 10 mm HEPES, 0.01% Na_2EDTA and NaN_3, pH 7.4. Examination of the sedimenting boundaries indicated that both the control and PLA_2-Lp[a] were homogenous and showed no signs of aggregation.

Having ascertained that aggregation or self-association of PLA_2-Lp[a] did not occur, the viscosity-corrected sedimentation coefficient was plotted as a function of solution density for both the control and PLA_2-Lp[a] in each buffer system. The conformational change was quantified by measuring the difference in the $s_{20,w}$ values of the compact and extended conformation of Lp[a] obtained in solutions of NaCl and 6-AHA, respectively (**Table 2**). Typical plots obtained with the first preparation are shown in **Fig. 4A** and **B**. The intercepts on the horizontal axis give the hydrated densities of the particles, whereas the intercepts on the ordinate, after correction for the density and viscosity of water at 20°C, yield the $s_{20,w}$ values defining the compact and extended forms of Lp[a]. As noted previously (10), different preparations of Lp[a] exhibited small differences in their sedimentation coefficient which are probably caused by slight methodological differences in Lp[a] isolation, in addition to small differences in lipid content from one preparation to the next. This observation held also for the extent of unfolding as measured by the difference in the $s_{20,w}$ values of the compact and extended forms of Lp[a]. Upon phospholipolysis, $s_{20,w}$ values of Lp[a] increased in every preparation mainly as a function of the increase in the hydrated densities of each particle. In solutions of NaCl, the hydrated density of

TABLE 2. Conformational changes in control- and PLA_2-Lp[a] determined from $s\eta$ versus ρ plots

	Experiment			
	1	2	3	4
	$s_{20,w}$ (S)			
Control				
Compact[a]	12.8 ± 0.1	13.3 ± 0.1	12.8 ± 0.2	12.1 ± 0.2
Extended	9.4 ± 0.1	11.2 ± 0.2	11.0 ± 0.4	10.5 ± 0.1
Difference[b]	3.4 ± 0.2	2.1 ± 0.3	1.8 ± 0.6	1.6 ± 0.3
PLA_2–Lp[a]				
Compact	13.6 ± 0.2	14.5 ± 0.0	14.1 ± 0.3	13.2 ± 0.4
Extended	10.2 ± 0.3	12.2 ± 0.0	11.8 ± 0.2	10.8 ± 0.2
Difference	3.4 ± 0.5	2.3 ± 0.1	2.3 ± 0.5	2.4 ± 0.6

Values represent the mean ± standard error of the mean.

[a] To obtain the s value for the compact conformation, the solution density was raised with NaCl and varied from 200 to 1000 mm. For the extended conformation of Lp[a], concentrations of 6-AHA designed to saturate the LBS II site were used, and varied from 200 to 1000 mm.

[b] The differences calculated for the control and PLA_2-Lp[a] were not significantly different when evaluated by the method of paired comparisons using Student's *t*-test ($0.05 < P < 0.1$).

Lp[a] increased on the average from 1.056 ± 0.001 to 1.063 ± 0.002 g/ml upon treatment with PLA_2 and, in solutions containing NaCl and 6-AHA, the hydrated density increased from 1.047 ± 0.004 to 1.051 ± 0.003 g/ml. However, using paired comparisons and Students *t*-test, the differences between the $s_{20,w}$ values of the compact and extended forms of PLA_2-Lp[a] were essentially the same as those determined for the untreated Lp[a] ($0.05 < P < 0.1$) indicating that no change in the conformation had occurred.

The larger $s_{20,w}$ value for PLA_2-Lp[a] was probably caused by the smaller molecular weight and increased hydrated density rather than a conformational change. For example, by using the relation $s_1/s_2 = M_1(1 - \bar{v}\rho)_1/M_2(1 - 2\bar{v}\rho)_2$ which is derived from the Svedberg equation, $s = M(1 - \bar{v}\rho)/Nf$, and by assuming no change in the frictional coefficient, one can calculate for the first preparation of Lp[a], using molecular weights and partial specific volumes listed in Table 1, that its sedimentation coefficient should increase in NaCl from 12.8 to 13.65 S upon treatment with PLA_2. This calculated value agrees well with the experimental one of PLA_2-Lp[a] in NaCl (13.6 S) (see Table 2). Had the action of PLA_2 led to the partial unfolding of Lp[a], its $s_{20,w}$ value measured in solutions of NaCl should be lower than that of untreated Lp[a], because unfolding increases the frictional coefficient and hydrodynamic radius.

Binding curves were constructed for untreated and PLA_2-Lp[a] of three different preparations. A set of curves obtained with preparation 2 are shown in **Fig. 5**. They were evaluated for the binding constant, K_d, the number of bound 6-AHA molecules, n, and the K_{50} value representing the ligand concentration at which 50% of Lp[a] is unfolded. The results presented in **Table 3** indicate that extensive digestion of Lp[a] by PLA_2 did not affect either the binding constant nor the number of bound 6-AHA molecules. The binding of 6-AHA to Lp[a] was multivalent for all preparations examined (n ranging from 2.2 to 2.8), with binding constants in the millimolar range. K_{50} values ranged from 93 to 165 mm 6-AHA and were not sig-

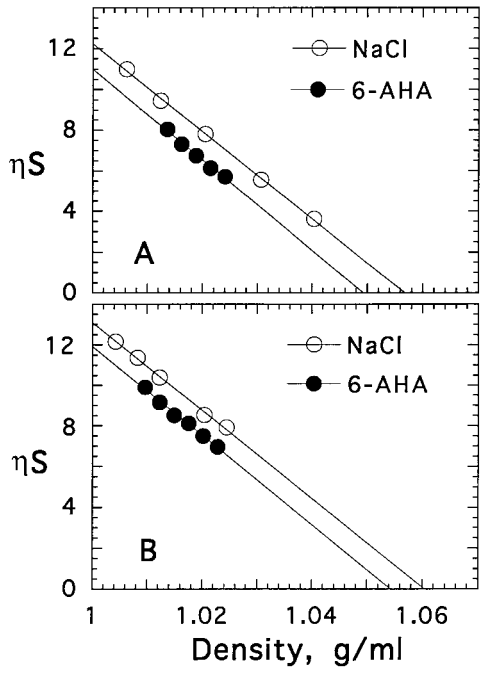

Fig. 4. Viscosity-corrected sedimentation coefficient plotted as a function of solution density. (A) Untreated Lp[a]; (B) PLA_2-Lp[a]. To obtain the sedimentation coefficient of the compact conformation, NaCl was used to change the solution density of the background buffer solution, 10 mm HEPES, 0.01% Na_2EDTA and NaN_3, pH 7.4. The s value of the extended conformation was obtained by varying the solution density of the background solution, which also contained 100 mm NaCl, with 6-AHA. The solid lines represent the least square fit of the experimental data points.

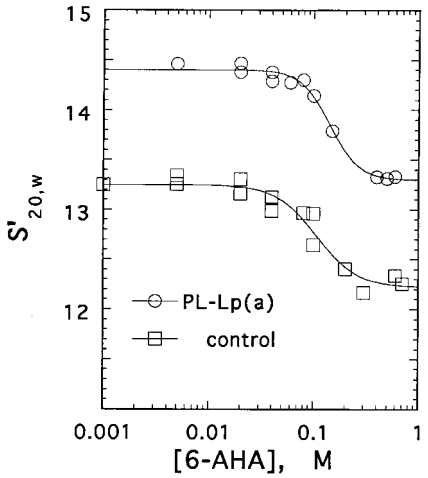

Fig. 5. Change in the conformation of control Lp[a] and PLA_2-Lp[a] with increasing concentrations of 6-AHA. Both Lp[a] samples were present in a background buffer solution containing 10 mm HEPES, 100 mm NaCl, 0.01% Na_2EDTA and NaN_3, pH 7.4. The solid lines represent the best fit theoretical curves calculated as described under Experimental Procedures. The sedimentation coefficient was corrected to standard conditions in water at 20°C using the hydrodynamic partial specific volume as defined in the text.

TABLE 3. Effect of PLA_2 treatment on the binding of 6-AHA to Lp[a]

	Experiment					
	2		3		4	
	Control	PLA_2-Lp[a]	Control	PLA_2-Lp[a]	Control	PLA_2-Lp[a]
$K_d \times 10^{3,a}$ (m)	6.76 ± 13.6	11.5 ± 17.9	1.06 ± 6.11	1.12 ± 1.77	17.4 ± 39.5	6.53 ± 8.72
n[b]	2.2 ± 0.83	2.3 ± 0.69	2.6 ± 2.2	2.8 ± 0.64	2.2 ± 1.1	2.8 ± 0.7
K_{50}[c] (mm)	110 ± 15	120 ± 15	93 ± 13	99 ± 13	147 ± 33	165 ± 25

All values represent the mean ± standard error of the mean.

[a] The dissociation constant, K_d, was calculated from the binding curves (Fig. 5) as described in Experimental Procedures.

[b] The number of bound 6-AHA molecules is represented by n.

[c] K_{50} values are equivalent to the ligand concentration at which the value of s is half-way between that of the compact and extended forms of Lp[a], and were obtained by a logit transformation of the binding curves (39).

nificantly different between untreated and PLA_2-Lp[a]. As observed with the viscosity-corrected sedimentation coefficient, there appeared to be small differences in all three binding parameters from one preparation to the next, but none reached statistical significance.

Binding to lysine Sepharose

Lipolysis of Lp[a] by PLA_2 slightly increased its affinity for lysine Sepharose. Control Lp[a] eluted at a concentration of 2.0 ± 1.0 mm 6-AHA (n = 3) whereas PLA_2-Lp[a] eluted with 4.2 ± 0.2 mm (n = 3). Exposure of both control Lp[a] and PLA_2-Lp[a] to Tween 20 increased their affinity for lysine Sepharose by an order of magnitude, reflecting the exposure of the LBS II domain which in the absence of Tween 20 is masked (5). PLA_2-Lp[a] again had the greater affinity for the lysine Sepharose by eluting at 38 ± 3.4 mm 6-AHA (n = 3) whereas control Lp[a] eluted with 27 ± 3.6 mm 6-AHA (n = 3). This dramatic increase in affinity caused by the exposure of either Lp[a] particle to Tween 20 is shown in **Fig. 6**. Finally, apo[a] was prepared from either Lp[a] particle by minimal reduction (8) and tested for any changes in affinity for lysine Sepharose as a result of the action of PLA_2 on Lp[a].

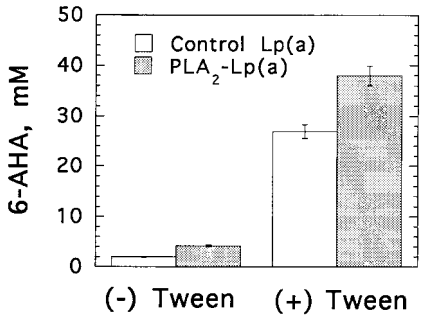

Fig. 6. Binding of control Lp[a] and PLA_2-Lp[a] to lysine Sepharose. Chromatography was conducted on a column (1.5 × 4 cm) equilibrated with 10 mm phosphate, 100 mm NaCl, 0.01% Na_2 EDTA, and NaN_3, pH 7.4. Bound Lp[a] was eluted with linear gradients of 6-AHA as described in Experimental Procedures. The concentration of 6-AHA representing the absorbance maximum of the eluted Lp[a] peak was taken as measure of the affinity of Lp[a] for lysine Sepharose. Values represent the mean ± SD of triplicate experiments.

Apo[a] derived from PLA_2-Lp[a] eluted at 123 mm 6-AHA, whereas apo[a] derived from untreated Lp[a] eluted at 122 mm 6-AHA, indicating that phospholipolysis did not affect the affinity of apo[a] for lysine Sepharose which was considerably greater than that of Lp[a] with or without Tween 20.

Binding to immobilized fibrinogen

Previous studies from this laboratory have shown that the in vitro binding of either free apo[a] or parent Lp[a] to immobilized fibrinogen involves predominantly LBS-II (9, 36), This is the same domain which by interacting presumably with lysine or proline residues of apoB-100 contributes towards maintaining the compact form of Lp[a] and when saturated with 6-AHA initiates the expanded form of Lp[a] (10, 11). In order to establish the potential relationship between these two observations, we conducted studies on the fibrinogen binding properties of PLA_2-Lp[a]. These results are summarized in **Table 4** and indicate that phospholipolysis did not significantly affect the binding of Lp[a] to fibrinogen. Thus total and lysine-mediated binding of PLA_2-Lp[a], both in the absence and presence of Tween 20, were characterized by B_{max} and K_d values that are similar to those of control Lp[a]. The addition of Tween 20 significantly increased the B_{max} of both the total and lysine-mediated binding of PLA_2- and control Lp[a], pointing to the unmasking of LBS II in the presence of the detergent. This effect of Tween 20 on lysine-mediated binding of both control and PLA_2-Lp[a] is clearly shown in **Fig. 7**, confirming the similar behavior for both types of Lp[a]. Although binding was increased, the affinity of both forms of Lp[a] to fibrinogen decreased upon the addition of Tween 20. Overall, we interpreted these results to support the conclusion derived from the ultracentrifugal studies that phospholipolysis did not perturb the ligand binding properties of apo[a] in Lp[a] at least in terms of its interaction with either apoB-100 or fibrinogen.

DISCUSSION

Our results have shown that complete in vitro hydrolysis of Lp[a] by PLA_2 caused extensive compositional remod-

TABLE 4. Binding of PLA_2-Lp[a] to immobilized fibrinogen

	Control Lp[a]		PLA_2-Lp[a]	
	K_d	B_{max}	K_d	B_{max}
	nM	*fmol/well*	*nM*	*fmol/well*
Total	1.60 ± 0.67	1.50 ± 0.04	1.45 ± 0.15	1.96 ± 0.14
Lys-mediated[a]	2.18 ± 1.22	0.86 ± 0.02	1.80 ± 0.30	0.89 ± 0.17
Total/Tween	4.90 ± 1.41	4.11 ± 1.59	4.38 ± 0.02	5.00 ± 1.30
Lys-mediated/Tween	5.63 ± 0.19	4.95 ± 0.07	6.01 ± 1.15	4.75 ± 0.35

Values represent the mean ± SD from the mean of three experiments conducted in duplicate.
[a] Lys-mediated binding represents the difference between total binding and binding obtained in the presence of 200 mm 6-AHA.

eling of the surface phospholipids, but had no significant effect on the solution properties of Lp[a]. This modification led to an 85% reduction of PC, a 3.2- and 12.9-fold increase in the content of NEFA and lyso-PC, respectively, generated during hydrolysis and also to a substantial reduction of the minor phospholipids PI, PE, and PS. The increase of approximate 40 molecules of NEFA per particle over untreated Lp[a] gave the PLA_2-Lp[a] particle an electronegative character that was expressed in an increased mobility on the agarose gels. Although extensively modified, Lp[a] remained monomeric and was optically clear.

In many respects, the behavior of Lp[a] was very similar to that previously reported for PLA_2-modified LDL, which also exhibited only modest structural changes in comparison to untreated LDL when studied by electron microscopic, circular dichroic, analytical ultracentrifugal, and small angle X-ray scattering techniques (22). Although the general structure of LDL is preserved by phospholipolysis, PLA_2-LDL has been shown to exhibit decreased immunoreactivity in the C-terminal domain of apoB-100 as detected by competitive radioimmunoassay using a battery of apoB specific monoclonal antibodies (23). However, the potential relationship between this immunological change and the conformation of apoB-100 is not apparent. In this context, based on a structural analysis of LDL particles of different sizes, McNamara et al. (37) have proposed that when the number of surface phospholipids is insufficient to provide appropriate physiological surface pressure, apoB-100 spreads out and fills in the uncovered surface area with only minimal changes of the secondary structure of the apolipoprotein. This notion is in keeping with the results by Aggerbeck, Kezdy, and Scanu (22) showing that PC depletion by the action of PLA_2 caused no change in the circular dichroic spectrum of LDL. In the case of Lp[a], the present studies using analytical centrifugation (see data in Tables 2 and 3) have provided strong evidence that the extensive phospholipid remodeling in the lipoprotein particle caused by PLA_2 digestion had no significant effect on the conformation and solution properties of Lp[a]. Our data also provide further support for the concept that the LBS-II domain of apo[a] retains its functional capacity. This conclusion is derived from several observations: *1*) phospholipolysis did not disrupt the compact conformation of Lp[a] or affect the transition to the extended conformation by the presence of 6-AHA, which is known to break the interaction between the apoB-100 and the LBS-II domain of apo[a]; *2*) in both control and PLA_2-Lp[a], the binding to both lysine Sepharose and immobilized fibrinogen was markedly enhanced in the presence of Tween 20 which is known to unmask LBS-II(5); *3*) free apo[a] isolated from either Lp[a] showed enhanced binding to lysine Sepharose compared to either Lp[a] species in the presence or absence of Tween 20, due to the unmasking of the LBS II domain as was documented previously (8, 36).

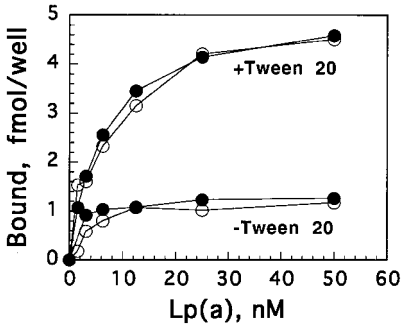

Fig. 7. Binding of control Lp[a] (filled circles) and PLA_2-Lp[a] (open circles) to fibrinogen. Lp[a] at the indicated concentrations was incubated with immobilized fibrinogen in either the absence or presence of 200 mm 6-AHA. Only the lysine-mediated binding is shown which is the difference between total binding and binding in the presence of 200 mm 6-AHA. In one set of experiments, Tween 20 (0.1%) was used to unmask the LBS II domain.

The present results indicate that treatment of Lp[a] with PLA_2 increased its affinity for lysine Sepharose over untreated Lp[a]. This finding is in general agreement with data reported by Hoover-Plow, Khaitan, and Fless (24) and Hoover-Plow and Skocir (25), showing that the amount of Lp[a] bound to lysine Sepharose beads increased upon digestion with PLA_2. However, neither study provides a clear mechanism underlying the increase in either the affinity or binding of PLA_2-Lp[a] to lysine Sepharose. As it is known that the binding of Lp[a] to lysine Sepharose involves the LBS I domain (5, 8), we would like to suggest that the increase in affinity upon phospholipolysis is caused by the removal of some steric constraints allowing for a better interaction of LBS I with lysine Sepharose. In turn, the LBS II domain may not be involved, because as discussed above, in both PLA_2- and control Lp[a] this site was unmasked only after treatment

with Tween 20, which increased the affinity of either Lp[a] particle by an order of magnitude. It is of note that LBS II also plays a dominant role in the binding of Lp[a] to fibrinogen, as this binding is present in Lp[a] species lacking a functional LBS I in K-IV_{10} (9). Hence, our results showing that PLA_2-Lp[a] had the same binding capacity for immobilized fibrinogen as untreated Lp[a], support the concept that the LBS I, rather than the LBS II domain, is affected by phospholipolysis.

In conclusion, the results of the current studies carried out with a 15 K-IV Lp[a] indicate that phospholipids do not play a key role in the interaction between apoB-100 and apo[a] of Lp[a] and support our previous in vitro data showing that apo[a] has no affinity for lipoprotein lipids (38). Thus, in spite of extensive phospholipolysis, apo[a] and more specifically its LBS-II domain retain their capacity to interact with apoB-100 and preserve the overall structural stability of Lp[a].

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