Interaction of lipoprotein Lp[a] with the B/E-receptor: a study using isolated bovine adrenal cortex and human fibroblast receptors

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Abstract The ability of different lipoprotein Lp[a] preparations to compete with LDL-binding to the B/E-receptor was investigated by ligand blot and filter assays. Lp[a] was purified from donors with various genetic polymorphic forms by affinity chromatography using lysine-Sepharose or specific immunoadsorbers. These preparations were free of "LDL-like" material. Part of Lp[a] was reduced and freed from specific apo[a] antigen yielding "Lpa-." ^{125}I-labeled low density lipoproteins (LDL) were incubated with B/E-receptor preparations from bovine adrenal cortex or from human skin fibroblasts, and the competition with unlabeled LDL, Lp[a], Lpa-, apo[a], and apoE-free HDL was studied by a ligand blot or filter assay technique. The following results were obtained. *1*) LDL and Lpa- were equally potent in displacing ^{125}I-labeled from B/E-receptor in the ligand blot and the filter assay. Lpa + (= Lp[a]) also displaced LDL but to a much lesser degree: 50% displacement was observed with LDL and Lpa- at a 1-fold excess, whereas a 7.5-fold excess was required of Lpa + . *2*) Apo[a], as well as apoE-free HDL, did not compete with LDL binding. *3*) Competition experiments using B/E-receptors from bovine adrenal cortex or from human skin fibroblasts were comparable. *4*) There was no difference in the behavior of Lp[a] isolated from the two affinity chromatography methods. *5*) Lp[a] of different genetic variants behaved virtually identically. The results are discussed from the point of view of the in vivo metabolism of Lp[a]. —**Steyrer, E., and G. M. Kostner.** Interaction of lipoprotein Lp[a] with the B/E-receptor: a study using isolated bovine adrenal cortex and human fibroblast receptors. *J. Lipid Res.* 1990. **31:** 1247–1253.

Supplementary key words ligand blot • filter assay • Lpa + • Lpa- • LDL • apoE-free HDL

Lp[a] has gained considerable attention because of its recently reported structural homology with plasminogen (1, 2). There is now ample evidence that Lp[a] concentrations > 20–30 mg/dl add to the risk of atherosclerotic diseases (3–7). The molecular basis of these findings, however, is still not unambiguously clear. One mechanism by which the atherogenicity may be explained is the interaction of Lp[a] with fibrinolysis (8–10) and its competition with the binding of plasminogen to specific endothelial cell receptors (11). It was also shown recently that Lp[a] forms complexes with glycosaminoglycans and proteoglycans to a higher degree than LDL, and that Lp[a]-proteoglycan complexes promote cholesteryl ester accumulation in macrophages as well as foam cell formation (12).

The physiological function of Lp[a] on the other hand is still unknown. Lp[a] is produced in the liver independently from triglyceride-rich precursors (13, 14) and exhibits a fractional catabolic rate that is somewhat lower, but still in the same order of magnitude as LDL (15). This finding has raised the question concerning the site and mode of Lp[a] catabolism, i.e., whether or not Lp[a] is catabolized via the B/E-receptor cascade. This question has been addressed by several investigators with partially divergent results (15–19). The main criticism that has been raised to all former studies lies in the possibility that the purity of Lp[a] with respect to contaminating LDL was not assessed unequivocally, and that cells in tissue culture have been used that may cause a partial dissociation of the a-protein from LDL during the incubation period.

The aim of the present study was to investigate the ability of highly purified Lp[a] to compete for the B/E-receptor in ligand blot experiments as well as in filter assays with isolated receptor preparations.

MATERIALS AND METHODS

Isolation of lipoproteins

All lipoproteins were isolated from normolipemic fasting volunteers. In order to minimize proteolytic and oxidative degradation, the following preservatives were added routinely to all sera, plasma, and buffers: 1 mg/ml for EDTA

Abbreviations: LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); HDL-E, HDL minus apoE; Lp[a], lipoprotein[a]; Lpa-, apo[a]-free Lp[a] isolated according to Armstrong (19); Lpa + , term used in relation to Lpa- (= Lp[a]); B/E receptor, LDL receptor described by Brown and Goldstein; SDS, sodium dodecyl sulfate.

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and NaN_3; 1 mmol/l for PMSF. In some cases, 0.05 mmol/l of leupeptin was added to all buffers for isolating Lp[a]. Lp[a] was isolated from persons exhibiting the Lp[a]-isoforms F, B, and S-1–S-4 according to Utermann (20) with Lp[a] concentrations ranging from 8 to 75 mg/dl; only donors who exhibited single isoforms (homozygotes) were selected. LDL and HDL_3 were isolated by sequential ultracentrifugation at density intervals of 1.020–1.050 g/ml and 1.125–1.21 g/ml, respectively. LDL was further purified by density gradient ultracentrifugation (21) in an SW-41 rotor (Beckmann). The protein moiety of the final LDL preparation consisted of ca. 96–98% apoB as checked immunochemically (22). The apoB of these LDL preparations migrated in 3.75% SDS-polyacrylamide gel electrophoresis as a single band with a molecular mass of 550 kD.

In order to obtain apoE-free HDL, the density fraction 1.125–1.21 g/ml was passed over a specific immunoadsorber (20, 22). The final HDL was apoE-free as checked by rocket electrophoresis.

Crude Lp[a] fractions were prepared by ultracentrifugation (d 1.060–1.125 g/ml), followed by column chromatography over Bio-Gel A-5m as described previously (13, 15). As these Lp[a] fractions in all cases were contaminated with substantial amounts of "LDL-like" particles (i.e., apoB-containing material not reacting with anti-apo[a] as checked by a double rocket technique), further purification was attempted by Lysine-Sepharose column chromatography (8) or by immunoadsorption using an anti-Lp[a] loaded column. The final preparation was free of "LDL-like" particles and had chemical and physico-chemical properties characteristic for Lp[a] (lipid and protein content, electrophoretic mobility, and morphology by electron microscopy).

The isolated lipoprotein fractions were stored for a maximum of 5 days (apoE-free HDL maximally 2 weeks) in 2 M NaCl, 0.01 mol/l Tris-HCl containing all the preservatives mentioned above. Immediately before use, these fractions were dialyzed exhaustively against the appropriate working buffer and checked for possible degradation by SDS–polyacrylamide gel electrophoresis.

LDL was labeled with ^{125}I by the method of McFarlane (23), yielding preparations with a specific activity of 400–640 cpm/ng protein.

Immunoaffinity chromatography

Two types of immunoadsorbers were used in this study: one specific for apoE and another for apo[a]. Both adsorbers were prepared by linking monospecific antibodies raised in rabbits to agarose 6B-CL by the CNBr method as described earlier (20, 22). The adsorbers had a capacity of ca. 1–2 mg antigen/ml.

Immunoadsorption was performed at 4°C in phosphate-buffered saline (pH 7.5) containing EDTA and NaN_3, 1 mg/ml each. The elution of the adsorbed antigen was carried out with 0.1 M glycine-HCl, pH 3.0, followed by immediate neutralization of the eluate. If storage was necessary (less than 1 week), 2 mol/l NaCl and the preservatives mentioned above were added.

Lpa– and apo[a], the two dissociation products of Lp[a], were prepared by treatment of Lp[a] with dithiothreitol (DTT) followed by heparin-Sepharose column chromatography as described by Armstrong, Walli, and Seidel (19).

Ligand blots and filter assays

The B/E-receptors for ligand blotting experiments were either solubilized in buffer A containing Triton X-100 for bovine adrenal cortex, or containing Nonidet P-40 for human skin fibroblasts, exactly as described by Schneider, Goldstein, and Brown (24). Buffer A consisted of 10 mmol/l Tris-maleic acid, 2 mM $CaCl_2$, 1% Triton X-100 or Nonidet P-40, 1 mmol/l PMSF, and 0.05 mM leupeptin, pH 6.0. The solubilized receptor preparations were subjected to SDS electrophoresis in 7% polyacrylamide gels followed by electrophoretic transfer of the proteins onto nitrocellulose in the presence of 20% methanol. Electroblotting was performed at 150 mA for 90 min at 4°C in an electrophoresis/blotting system from Bio-Rad. Ligand blots were performed as described by George, Barber, and Schneider (22). The composition of the incubation mixtures is described in the legend to Fig. 2.

For the filter assay, the detergent extracts of bovine adrenal cells or human skin fibroblasts were purified on DEAE cellulose (24). Extracts were passed over columns (7 × 1 cm) equilibrated with buffer A. The B/E-receptor, which adsorbs under these conditions on DEAE-cellulose was eluted with buffer A containing 40 mmol/l 1-O-n-octyl-β-D-glucopyranoside instead of Triton X-100, supplemented with 0.35 mol/l of NaCl. A micellar receptor/phosphatidylcholine suspension was prepared by acetone precipitation as described by Schneider et al. (24) and resuspended in 25 mmol/l Tris-HCl, 50 mmol/l NaCl, 2 mmol/l $CaCl_2$, 1 mg/ml BSA, pH 8.0. The filter assays were performed with a filtration unit from Hoefer Scientific using cellulose acetate filters (OE 67) from Schleicher & Schüll, West Germany. The composition of the assay mixtures is given in the legend to Fig. 3.

Laurell electrophoresis, polyacrylamide gel electrophoresis, and chemical analytical methods were the same as described previously (21, 25).

Chemicals

n-Octyl-D-glycopyranoside, phenylmethyl sulfonyl fluoride, and Nonidet P-40 were obtained from Sigma. ^{125}I was purchased from Amersham Radiochemicals, egg phosphatidylcholine from United States Biochemical Corp., bovine serum albumin from Janssen and lysine and heparin-Sepharose from Pharmacia, Uppsala. All other chemicals were from E. Merck, Darmstadt.

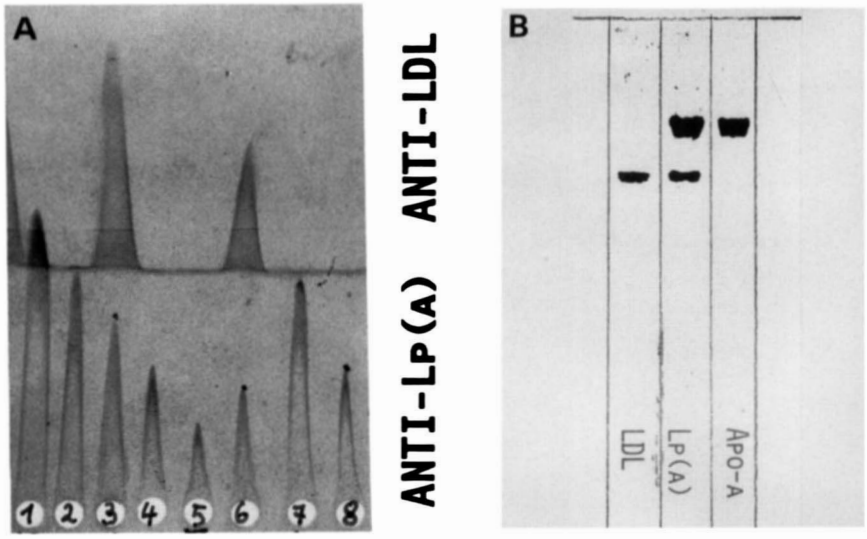

Fig. 1. Double-antibody Laurell electrophoresis of different Lp[a] samples. The lower gel contained 1% monospecific anti-Lp[a] from rabbit and the upper gel contained 0.3% anti-LDL from rabbit. The following samples were applied: 1, 2, 4, and 5: Lp[a] purified by immunoadsorption (50, 40, 20, and 10 mg/100 ml); 7, 8: Lp[a] purified by affinity chromatography using Lys-Sepharose (40 and 20 mg/100 ml); 3 and 6: "crude Lp[a]" after column chromatography over Bio-Gel A-5m (30 and 15 mg/dl). B: SDS-polyacrylamide gel electrophoresis in 3.5% gels of freshly purified LDL, Lp[a]-isoform S-3 and the corresponding apo[a] in the presence of 1% mercaptoethanol. Staining was performed with Coomassie blue R-250.

RESULTS

Isolation and characterization of lipoproteins

The Lp[a] used for most experiments (except those described in Fig. 6) was derived from two donors with an apo[a] pattern of S-1 subtype, according to the nomenclature of Utermann et al. (20). In several control experiments Lp[a] of subtypes "F, B, and S-1–S-4" were also used. **Fig. 1A** displays a double antibody rocket electrophoresis of "crude" Lp[a] prepared by ultracentrifugation followed by steric exclusion column chromatography, a method that has been used in most of the previous experiments (4, 8, 15). As can be seen, there are substantial amounts of "LDL-like" particles present that are not adsorbed by the first anti-Lp[a]-containing gel. Using this double antibody rocket electrophoresis technique, we found that conventional Lp[a] preparations were contaminated by 5–12% apo[a]-free material. After further purification of crude Lp[a] by immune affinity or Lys-Sepharose chromatography, we obtained Lp[a] that was virtually free of "LDL-like" material (Fig. 1).

Lys-Sepharose is known to adsorb only ca. 50–70% of the Lp[a] present in plasma. The nonadsorbed material, which cannot be recovered even upon rechromatography, is indistinguishable chemically and physico-chemically from adsorbed Lp[a] (V. Armstrong, personal communication, as well as G. M. Kostner, unpublished data). For the present experiments, only the fraction adsorbed by Lys-Sepharose was used.

The purified Lp[a] was further checked by Laurell electrophoresis for the presence of other non-apoB proteins (apoA-I/II, apoC, apoE) using specific antibodies. It was found to be >98% pure; apoE was absent as demonstrated by Western blot experiments. As it is of crucial importance for these experiments that the purified Lp[a] is mostly intact and not degraded, all preparations were investigated prior to use by SDS-polyacrylamide gel electrophoresis under reducing conditions. Only preparations free of apo[a] or apoB fragments as shown in Fig. 1B were used for subsequent experiments.

Ligand blots

Fig. 2 shows the ligand blots where ^{125}I-labeled LDL was bound at a concentration of 2–4 μg/ml to the blotted B/E-receptors from bovine adrenal gland in the presence of increasing amounts of competitor. As competitors, we used LDL, Lpa+, Lpa–, apo[a], and apoE-free HDL_3 up to a 40-fold excess. In these experiments, LDL, Lpa–, and Lpa+ displaced the radiolabeled ligand to a comparable degree. The blots suggest that LDL and Lpa– were markedly more reactive than Lpa+. Apo[a] and HDL_3 had no effect. Because Lp[a] may be altered physico-chemically by immunoadsorption, we performed control experiments where Lp[a] was purified by Lys-Sepharose adsorption. There was virtually no difference between these two Lp[a] preparations (**Fig. 3**).

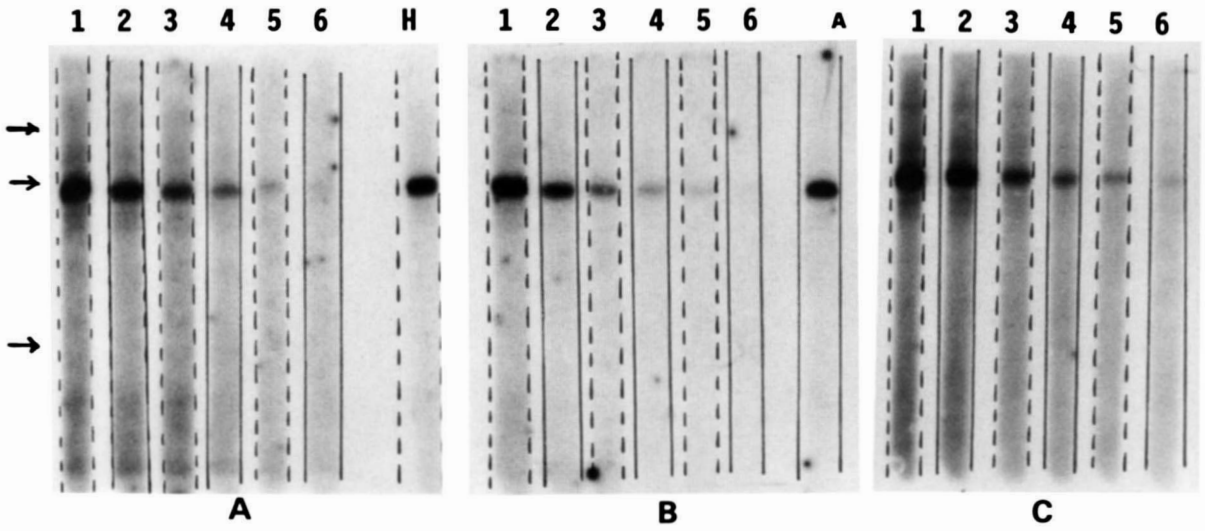

Fig. 2. Ligand blotting of B/E-receptor from bovine adrenal cortex. Triton x-100 extracts (30 μg/lane) were electrophoretically separated on a 7% polyacrylamide SDS-containing gel under nonreducing conditions, followed by electroblotting. Ligand blot was performed according to George et al. (22). Each incubation mixture contained 2–4 μg/ml ^{125}I-labeled LDL (sp act 620 cpm/ng apoB in the presence or absence of unlabeled competitors). Exposure of nitrocellulose strips was performed at −70°C for 24 h on a high performance autoradiograph film (Amersham Hyperfilm MP). Molecular mass standards from Pharmacia are indicated with arrows at the outside lanes: top, middle, bottom: 232, 140, 67 kD, respectively. A, Competition with Lpa−; B, competition with LDL; C, competition with Lpa+. The lanes contain: 1, reference, no competitor; 2–6, 1-fold, 5-fold, 10-fold, 20-fold, and 40-fold excess of unlabeled lipoproteins, respectively; H, 40-fold excess of apoE-free HDL; A, 40-fold excess of apo[a].

Filter assays

In order to obtain quantitative data, further experiments were carried out using the filter assay technique. In **Fig. 4A and B**, the displacement of ^{125}I-labeled LDL with LDL, Lpa−, Lpa+, and HDL from B/E-receptor preparations isolated from bovine adrenal cortex and from human skin fibroblasts, respectively, are shown. In both cases, LDL and Lpa− were equally efficient in competing for the B/E-receptor binding, whereas Lpa+ competed to a lesser degree. In the assay using receptors from human skin fibroblasts, a somewhat lower concentration of all three lipoproteins was necessary to obtain 50% displacement as compared to B/E-receptors from bovine adrenal gland. With the fibroblast receptor, 50% displacement was observed at 1-fold excess of LDL and Lpa−, and at a 7.5-fold excess of Lpa+. ApoE-free HDL had no signifi-

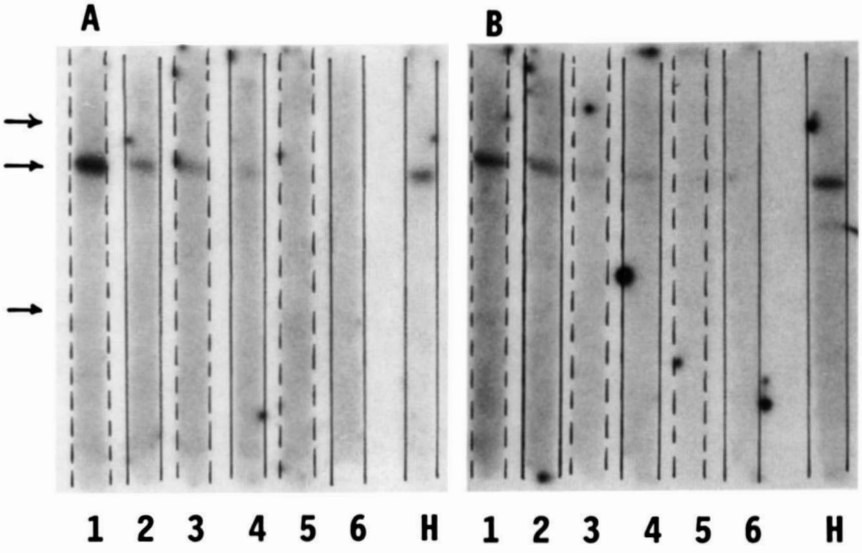

Fig. 3. Ligand blotting of B/E-receptors from bovine adrenal cortex. The conditions are the same as in Fig. 2. A: Competition with Lp[a] isolated by immunoadsorption; B: competition with Lp[a] isolated by Lys-Sepharose. 1, Reference, no competitor; 2–6, 1-fold, 5-fold, 10-fold, 20-fold, and 40-fold excess of Lp[a], respectively; H, 40-fold excess of apoE-free HDL. The arrows indicate the position of molecular weight standards, the same as in Fig. 2.

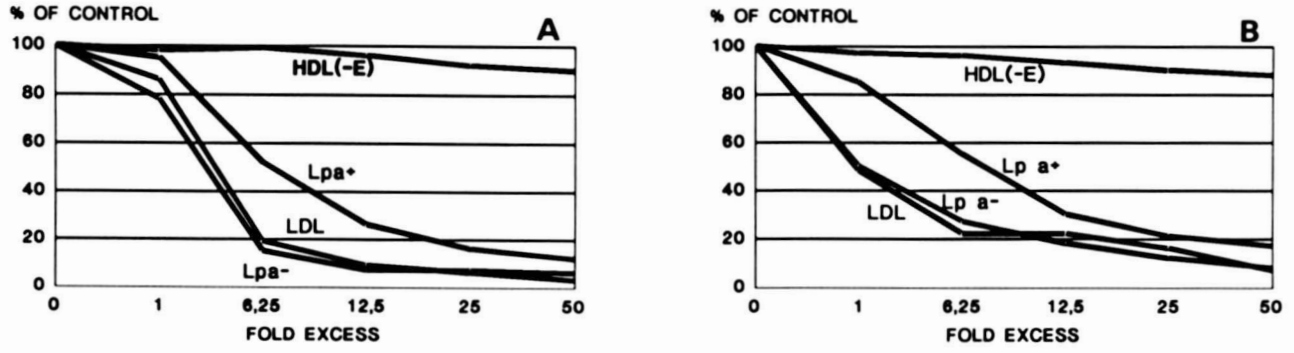

Fig. 4. Filter assays: ability of ligands to compete with ^{125}I-labeled LDL for binding to the B/E-receptor isolated from bovine adrenal cortex (A) or from human skin fibroblasts (B). The 100% value is the binding of ^{125}I-labeled LDL in the absence of unlabeled ligands. Filter assays were performed as described in Materials and Methods. Each tube contained 20–30 μg partially purified and resuspended B/E-receptor protein (after DEAE-cellulose and phosphatidylcholine/acetone precipitation, ref. 22), 2 μg/ml ^{125}I-labeled LDL-protein (sp act 640 cpm/ng apoB) and the indicated amounts of unlabeled lipoproteins. The total assay volumes were 100 μl each. All assays were performed in duplicate. Standard deviations were calculated from three separate experiments and were <12% (not shown in the graph for clarity).

cant effect in displacement of LDL from B/E-receptors (Fig. 4).

As it is known from published work of Utermann et al. (20) that apo[a] has a minimum of six isoforms differing considerably in molecular weight, it was of interest to investigate possible differences in the ability of these isoforms to displace LDL from its receptor. We thus isolated Lp[a] from donors homozygous for the six major isoforms by immunoadsorption and tested their behavior in the filter assay. **Fig. 5** shows the apo[a] pattern of these samples as assessed by Western blotting using specific antisera against apo[a].

The ability of different Lp[a] isoforms to displace ^{125}I-labeled LDL from the B/E-receptor using the filter assay method is displayed in **Fig. 6**. It was found that the interaction was virtually identical and independent of the type and the molecular weight of the apo[a] isoform.

DISCUSSION

From experiments with cultured human skin fibroblasts, we postulated in the past that Lp[a] binds to the B/E-receptor and suppresses HMG-CoA reductase activity, but is some 30% less reactive compared to LDL (15). In those, as well as in all subsequent studies, however, no attempts were made to demonstrate unequivocally the purity of Lp[a] with respect to the absence of LDL-like particles (15–19). Such particles are co-isolated by many of the conventional methods as shown by the double antibody rocket electrophoresis. Therefore, we purified Lp[a] in this study further by affinity chromatography and could demonstrate that it was virtually free of "non-apo[a]-containing" material (Fig. 1A). Most importantly in addition, the Lp[a] preparations isolated by the described procedures were free of any detectable degradation products (Fig. 1B).

Even by using such pure Lp[a] in a system with living cells one could be mislead by the possibility that apo[a] may be dissociated by the action of enzymes or metabolites during the incubation period. In addition, in such assays it is impossible to demonstrate unequivocally by which receptors the cell interaction occurs.

To overcome these potential drawbacks, we used ligand blot experiments that have been applied successfully in previous experiments for studying B/E-receptor interactions (22, 24). Thereby we clearly demonstrated that Lpa+ does interfere with the B/E-receptor. Lpa+ was less effective as compared to LDL or Lpa- (Fig. 2). In the

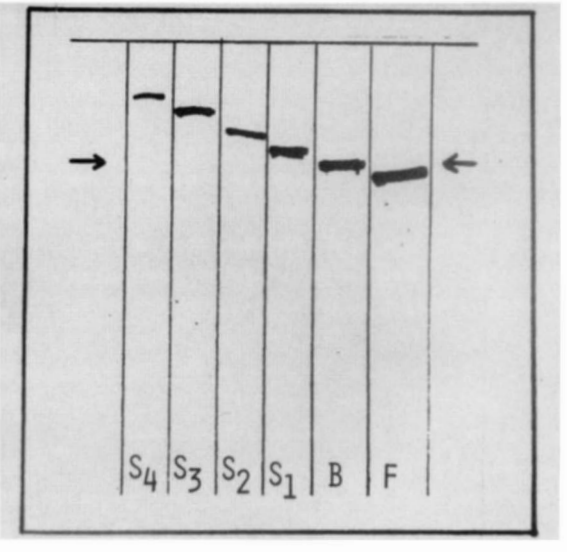

Fig. 5. Western blot analysis of plasma samples from the six different donors used in this study. Whole plasma samples (0.2–1 μl each depending on the Lp[a] content) were electrophoresed in 3.5% polyacrylamide gels, transblotted to nitrocellulose, and developed with an antiserum from rabbit, monospecific for apo[a], as described in ref. 8. As a second antibody, peroxidase-labeled goat anti-rabbit IgG was used. The arrow indicates the position of apoB-100.

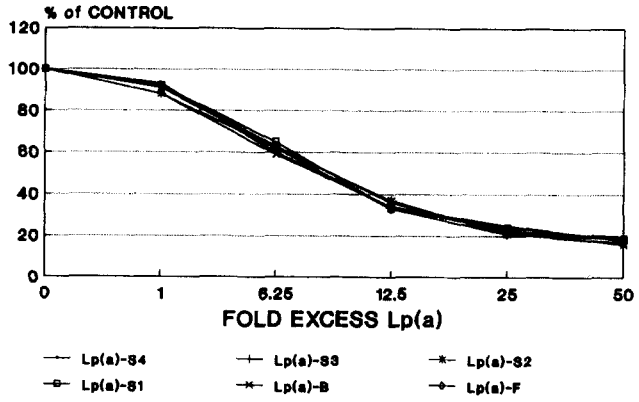

Fig. 6. Competition filter assay of bovine adrenal cortex receptors using ^{125}I-labeled LDL and unlabeled Lp[a] of various isoforms from six different donors. The experimental conditions were the same as indicated in Fig. 4. Lp[a] was isolated by immunoadsorption from six donors, homozygous for different Lp[a] isoforms as displayed in Fig. 5, and added in increasing amounts to the B/E-receptor/^{125}I-labeled mixture. Mean values from four (S-4 and S-3) and from two (other isoforms) experiments carried out in triplicate are displayed. The coefficient of variation of the assay was <8%.

filter assay, Lpa+ was significantly less reactive than LDL, whereas Lpa- and LDL were virtually indistinguishable. Comparable results were obtained with receptors derived from bovine adrenal glands or from human skin fibroblasts (Fig. 4A and B). We are aware of the fact that a true evaluation of quantitative differences with respect to binding affinities is only possible by Scatchard analysis by measuring the amount of free and bound ligands at increasing concentrations. We have not performed such experiments since we *i*) tried to avoid the direct labeling of Lp[a] because of its instability , and *ii*) we were aware of the fact that we were dealing here with an artificial system that could not be directly translated to the in vivo situation.

It should be mentioned at this point that we have compared LDL, Lpa-, and Lpa+ in these experiments on a protein basis; considering the fact that the protein moiety of Lp[a] consists of apoB and apo[a] in a molar ratio of 1, and further that both proteins have comparable molecular weights, Lp[a] might be even more reactive in displacing LDL from B/E-receptors based on molar concentrations of apoB. Because of the variability of molecular weights of individual LDL and Lp[a] preparations, we have not done these calculations.

It is also noteworthy that it is essential to use fresh Lp[a] preparations for such experiments. In earlier work, material stored for 2-3 weeks was used with grossly unaltered physico-chemical properties. This "old" Lp[a], however, was much less reactive especially in the filter assay. We therefore prepared Lp[a] fresh for each experiment, and stored it for less than 1 week.

Because of the instability of Lp[a], we also avoided labeling Lp[a] directly and studied its displacement by LDL. This was done in previous experiments using cultured human skin fibroblasts, where we found little difference whether LDL was bound and competed with Lp[a] or vice versa (15).

Apo[a] exhibits a striking size heterogeneity that is genetically determined (20). As the large isoforms (S-3 and S-4) are associated with Lp[a] concentrations of <20 mg/dl, values that are not considered to increase the risk of atherosclerosis, it was of interest to investigate potential individual differences of various apo[a] isoforms. In the ligand blot, S-3 and S-1 isoforms were virtually indistinguishable (data not shown). In addition, filter assays were carried out with all known Lp[a] isoforms. For this particular study, the isoforms were isolated by immunoadsorption and assayed simultaneously in two consecutive experiments. Within the experimental error (± 8%) the six Lp[a] isoforms behaved similarly if not identically (Fig. 6).

The idea that Lp[a] might be catabolized by the B/E-receptor has been challenged not only by other research groups working with tissue cultures (18, 19), but also by more recent findings demonstrating that lipid-lowering drugs, which are thought to exert their mode of action by increasing the B/E-receptor activity (26) are ineffective in reducing Lp[a] plasma concentrations (27). We have also performed such studies along this line, treating hypercholesterolemic subjects with either cholestyramine (28) or the HMG-CoA reductase inhibitors mevinolin and synvinolin (29). In these studies, LDL-cholesterol and apoB could be lowered by 25-45%, but no reduction of Lp[a] was observed whatsoever.

On the basis of the present results it is not possible to provide any plausible explanation for the apparent discrepancies mentioned above. There are, of course, several speculative theories, e.g., that binding of Lp[a] to the B/E-receptor occurs, though with somewhat reduced affinity, but that internalization and/or degradation of Lp[a] might be impaired due to the presence of apo[a]. An alternative theory might be that lipid-lowering drugs that increase the B/E-receptor number simultaneously increase Lp[a] biosynthesis. These and other considerations are currently under investigation in our laboratory.

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