ASBMB JOURNAL OF LIPID RESEARCH

Isolation, characterization, and uptake in human fibroblasts of an apo(a)-free lipoprotein obtained on reduction of lipoprotein(a)[1]

Victor W. Armstrong, Autar K. Walli, and Dietrich Seidel

Abteilung Klinische Chemie, Universitätskliniken Göttingen, Robert-Koch-Str. 40, 3400 Göttingen, FRG

Abstract Treatment of native human Lp(a) under non-denaturing conditions with dithiothreitol yielded both a lipoprotein particle and a lipid-free protein component that could be separated by either ultracentrifugation at d 1.063 g/ml or heparin-Sepharose chromatography. The protein component only showed antigenicity against anti-Lp(a) but not against anti-B. It was heterogeneous according to SDS polyacrylamide gel electrophoresis (PAGE) consisting of two bands, a major band with molecular weight similar to apoB and a minor band with slightly lower molecular weight. The lipoprotein particle was similar to LDL with regard to its electrophoretic mobility, lipid-protein composition, its apparent molecular weight according to gel-exclusion chromatography, and its apoprotein content; only apoB was found to be present by SDS-PAGE and immunochemical analysis. This lipoprotein also proved to be identical to LDL in its uptake by the receptor-mediated LDL-pathway in cultured human fibroblasts as shown by the similarity of the concentration-dependent binding, internalization, and degradation curves at 37°C of the ^{125}I-labeled lipoproteins. Normal Lp(a) was not taken up as readily as either its reduced lipoprotein component or LDL in the various steps of the receptor-mediated pathway. The maximal capacity for Lp(a) in the degradation assay was only 25% of that of LDL and it had a fourfold higher K_m. It is therefore probable that the LDL-receptor-mediated pathway is not a major route for the clearance of Lp(a) in vivo. These studies suggest that Lp(a) is, in essence, an LDL-particle to which the protein (a) is attached through disulfide bonds to apoB. — **Armstrong, V. W., A. K. Walli, and D. Seidel.** Isolation, characterization, and uptake in human fibroblasts of an apo(a)-free lipoprotein obtained on reduction of lipoprotein(a). *J. Lipid Res.* 1985. **26:** 1314–1323.

Supplementary key words low density lipoproteins • LDL-receptor • apoB

Human Lp(a), first described by Berg (1), is similar to LDL in many of its physical and chemical properties, but is unique in that it contains a carbohydrate-rich protein carrying the (a) epitopes. This apo(a) is attached to apoB by disulfide linkages (2, 3). On account of the additional protein moiety, Lp(a) migrates with preβ-mobility on agarose gel electrophoresis and has a density distribution ranging from 1.05 to 1.12 g/ml. More recent evidence has shown that Lp(a) exhibits heterogeneity with regard to its protein and lipid composition (4). Contradictory results have been reported as to whether Lp(a) can be cleared by the LDL-receptor-mediated pathway. Whereas in one study (5) utilizing fibroblasts from normal subjects and from subjects with autosomal dominant hypercholesterolemia the conclusion was reached that Lp(a) entered fibroblasts independently of the LDL-receptor, other investigators (6–8) have concluded that Lp(a) can bind to and be taken up by the same receptor site as LDL. Clinical interest in Lp(a) has been aroused by the suggestion that it may be an independent risk factor in the development of atherosclerosis (9, 10).

Previous investigators have only studied the nature of the disulfide linkage between apoB and apo(a) under denaturing conditions (2–4). In this present work we have been able to show that the disulfide bonds between apoB and apo(a) can be cleaved under physiological conditions to yield a lipid-free protein component and a lipoprotein particle. The latter has been characterized and its uptake by human skin fibroblasts compared with the uptake of normal unreduced Lp(a) and LDL.

MATERIAL AND METHODS

Isolation of plasma lipoproteins

Normal blood donors at the University Blood Donor Center were screened for Lp(a) immunoreactivity by counter electrophoresis (ME Radiophor, Immuno AG,

Abbreviations: Lp(a), lipoprotein(a); LDL, low density lipoproteins containing apoB as the sole apoprotein; VLDL, very low density lipoproteins; apo(a), a major apoprotein found in no other lipoprotein than Lp(a); DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, disodium salt of ethylenediamine tetraacetic acid; FPLC, fast protein liquid chromatography.

[1]This work is dedicated to Professor Fritz Scheler, Department of Internal Medicine, University Hospital, Göttingen, on his 60th birthday.

Downloaded from www.jlr.org by guest, on June 18, 2012

Austria). Plasma freshly obtained from Lp(a)-positive donors was collected in 0.02% sodium azide, 1 mM EDTA, and 10 units/ml kallikrein inactivator, and immediately subjected to differential ultracentrifugation. The plasma was adjusted with solid KBr to d 1.06 g/ml and centrifuged at 150,000 *g* for 20 hr at 10°C. The lipoproteins that floated were removed by tube slicing and the infranatant was adjusted with solid KBr to d 1.12 g/ml and recentrifuged. The d 1.06–1.12 g/ml fraction was then dialyzed against 20 mM Tris (pH 7.4), 1 M NaCl, 1 mM EDTA, 0.02% NaN_3, and subjected to gel filtration chromatography on a Sephacryl-S 400 (Pharmacia, Sweden) column (2.6 × 90 cm) equilibrated with the same buffer. Fractions from the first UV-absorbing peak that eluted from the column were monitored by fast protein liquid chromatography (FPLC). Those Lp(a) fractions that did not contain LDL (<1%) were combined and used for further analysis.

For comparison purposes LDL were isolated from the d 1.02–1.055 g/ml fraction. The purity of this fraction was checked by FPLC, SDS-PAGE, and immunochemical reaction against antisera to apoB, Lp(a), apoA-I, apoA-II, apoC and apoE. An antigenic reaction was only observed against anti-B.

Reductive cleavage of Lp(a)

Reduction of native Lp(a) was routinely performed at lipoprotein concentrations of 1–2 mg/ml total apoprotein in 20 mM Tris (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 0.02% NaN_3. Dithiothreitol (DTT) was freshly added to a final concentration of 10 mM (5–10 μmol of DTT/mg of protein) and the solution was incubated at 37°C for 3 hr. For characterization of the product lipoprotein by agarose gel electrophoresis (Lipidophor, Immuno AG, Austria) and immunoelectrophoresis according to Grabar and Williams (11), the solution was dialyzed overnight at 4°C against the above buffer, but omitting DTT.

Ultracentrifugation of reduced Lp(a)

Lp(a) was reduced with DTT as above and the solution was then adjusted to d 1.063 g/ml with solid KBr. After centrifugation at 150,000 *g* for 20 hr at 10°C, the lipoproteins that floated were recovered by tube slicing. The infranatant was also retained for analytical purposes. A pellet that had sedimented at the base of the tube could only be solubilized in 20 mM Tris (pH 8.0), 1 mM EDTA, 10 mM DTT, containing 25 g/l SDS.

Heparin-Sepharose chromatography of reduced Lp(a)

Lp(a) at a concentration of 2 mg/ml total apoprotein was reduced in 10 ml of 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.02% NaN_3, for 3 hr at 37°C. The solution containing 20 mg of total protein was then eluted over a heparin-Sepharose-6 CL-B (Pharmacia, Sweden) column (2.5 × 11 cm) equilibrated with the same buffer, but omitting DTT. The flow rate was 15 ml/hr and the fractions were monitored for UV absorption at 280 nm. When no further UV-absorbing material eluted from the column, the salt concentration was raised to 0.5 M NaCl and elution was continued to release bound lipoprotein.

Fast protein liquid chromatography (FPLC)

FPLC was performed using the Pharmacia FPLC system and a Mono Q HR 5/5 strong anion exchanger column. The FPLC system consisted of an LCC-500 liquid chromatography controller controlling two P-500 reciprocating pumps, each pump delivering one buffer into a dynamic mixing chamber. The resulting buffer mixture was then passed into a manually operated V-7 valve for the introduction of the sample via a 0.5-ml sample loop, and then onto the Mono Q ion exchanger column. From the column, the eluate was monitored by a single-path ultraviolet monitor at 280 nm and the chromatogram was recorded on an REC-482 two-channel recorder. Peak area was evaluated with the aid of the LCC-500.

Electrophoretic techniques

SDS-PAGE was performed in 2–16% slabs gels (Pharmacia) using an electrophoresis buffer consisting of 40 mM Tris (pH 7.4), 20 mM sodium acetate, 2 mM EDTA, and 2 g/l SDS. Samples were diluted in a sample buffer consisting of 10 mM Tris (pH 8.8), 1 mM EDTA, 50 g/l SDS and, where indicated in the legends to the figures, 10 mM DTT. They were incubated at 95°C for 10 min before addition of glycerol and tracking dye (bromophenol blue) to aid application to the gels. Electrophoresis was performed at a constant voltage of 150 V for 4 to 5 hr. The gels were fixed for 1 hr in 45% ethanol, 10% acetic acid, 45% H_2O and stained overnight in 0.04% Coomassie Blue R 250 in 10% acetic acid. Destaining was performed in 10% acetic acid.

Agarose gel lipoprotein electrophoresis was carried out according to a previously published procedure (12).

Lipid and protein analyses

Total cholesterol, free cholesterol, phospholipids, and triglycerides were determined using standard enzymatic test kits (13) and protein was measured by the method of Lowry et al. (14).

Antisera

Antisera to apoB were raised in goats using LDL obtained from the density fraction 1.02–1.055 g/ml. Antisera to Lp(a) were raised in rabbits using Lp(a) preparations purified by ultracentrifugation and gel chromatography over Sephacryl S.400. The antisera were absorbed with LDL (d 1.02–1.05 g/ml) to precipitate immunoglobulins specific for apoB (15).

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012

Labeled lipoproteins

Lipoproteins were labeled with ^{125}I using the iodine monochloride method (16). The radioactive preparations of Lp(a), reduced Lp(a), and LDL had specific activities ranging from 0.38 to 1.16 × 10^3 cpm/ng of protein. The TCA-precipitable radioactivity was 96–97%, of which 10% was found in the lipid moiety.

Cultured human fibroblasts

Fibroblasts were derived from skin biopsies of normal subjects. Cultures were maintained in a humidified incubator (95% air + 5% CO_2) at 37°C in Dulbecco's minimal essential medium (DME) which contained 25 mM $NaHCO_3$, 20 mM HEPES buffer (pH 7.4) with 100 U of penicillin/ml and 100 μg of streptomycin/ml.

Binding, internalization, and degradation assays

After 3 days in culture in DME containing 10% fetal calf serum, the medium was changed to DME containing 10% human lipoprotein-deficient serum (LDS) and incubated for 48 hr. On the day of assay the medium was replaced with 1 ml of fresh DME + 10% LDS and labeled lipoproteins were added with or without 25-fold excess of unlabeled lipoproteins. The cells were incubated for 5 hr at 37°C. The medium was removed and the cells were placed on ice. The culture dishes were then washed five times with a cold buffer containing 0.15 mM NaCl, 50 mM Tris (pH 7.4), and 2 mg/ml bovine serum albumin as described by Goldstein et al. (17). The cells were then washed twice with 0.15 M NaCl, 50 mM Tris (pH 7.4). Cell surface-bound lipoproteins were released by addition of 2 ml of a heparin solution (50 mM NaCl, 10 mM HEPES (pH 7.4), 0.4% heparin). The cells were incubated at 4°C for 60 min. The medium was then removed and the pellets were washed twice with 0.15 M NaCl, 50 mM Tris (pH 7.4). The pooled washes were counted for radioactivity in a Beckman Gamma 4000 Spectrometer (Beckman Instruments, Irvine, CA). The releasable activity represents the binding at 37°C. The cell pellets were dissolved in 0.1 M NaOH and radioactivity and protein content were measured. The radioactivity in the pellet was used as a measure for internalization. Non-iodide TCA-soluble radioactivity served as a measure of lipoprotein degradation. TCA was added to 0.9 ml of culture medium to give a final concentration of 5%. After centrifugation, 0.9 ml of TCA supernate was mixed with 0.45 ml of 5% silver nitrate and shaken for 10 min. After centrifugation, 1 ml of supernate was assayed for radioactivity (18). This method was compared with the hydrogen peroxide–chloroform extraction procedure and was found to give identical results (19).

For each concentration of labeled lipoproteins, blank dishes without fibroblasts were used as controls and these values were subtracted for binding, internalization, and degradation. The specificity of binding was on average 80% for LDL, 76% for the reduced Lp(a), and 51% for unreduced normal Lp(a).

RESULTS

Reductive cleavage of lipoprotein(a)

After treatment of Lp(a) with DTT at pH 7.4 for 3 hr at 37°C and removal of the DTT by dialysis, the resultant lipoprotein migrated with β-mobility on agarose gel electrophoresis (**Fig. 1A**). Immunoelectrophoresis showed that, in contrast to untreated Lp(a), only apoB was clearly associated with the new lipoprotein (Fig. 1B); the (a) antigen, though still present, migrated with slightly faster mobility. On ultracentrifugation of the DTT-treated Lp(a) at d 1.063 g/ml, over 80% of the lipids (cholesterol, triglycerides, and phospholipids) were recovered in the upper fraction (**Table 1**). The infranatant contained less

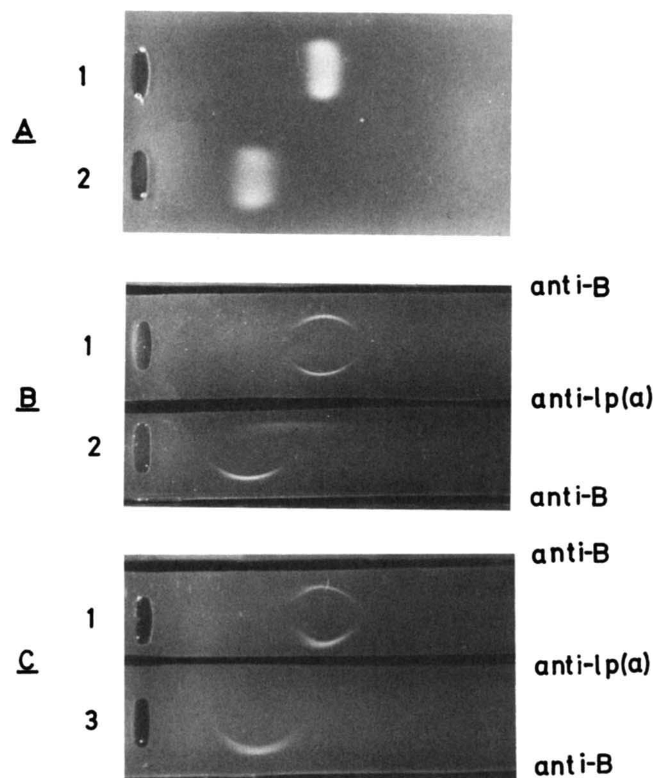


Fig. 1. Agarose gel lipoprotein electrophoresis and immunoelectrophoresis of Lp(a) before and after reduction with DTT. A) Lipoprotein electrophoresis of (1) unreduced Lp(a) and (2) reduced Lp(a). Reduction was performed with 10 mM DTT as described in Materials and Methods and the DTT was removed by dialysis prior to electrophoresis. B) Immunoelectrophoresis of (1) unreduced Lp(a) and (2) reduced Lp(a). Reduction as in (A). C) Immunoelectrophoresis of (1) unreduced Lp(a) and (3) the lipoprotein component from reduced Lp(a) after purification by ultracentrifugation as described in Materials and Methods.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 1. Recovery of lipid and protein after ultracentrifugation at d 1.063 g/ml of the DTT-reduced Lp(a)

Fraction	Percent Recovery[a]			
	Total Cholesterol	Phospholipid	Triacylglycerol	Protein
d < 1.063 g/ml	86.5	81.5	86.5	52.0
d > 1.063 g/ml	3.5	3.0	3.5	0.6
Pellet	<1	<1	1.8	32.0

[a]Values are the mean of three different experiments.

than 5% of the original lipids. Some loss of lipid may be attributed to the tube-slicing technique. In a control experiment using normal LDL, 84% of the cholesterol was recovered in the upper fraction. The protein recovery in the upper fraction of the reduced Lp(a), however, only amounted to 52% (average from three experiments) of the original protein. Analysis of the lipid-containing fraction by agarose gel electrophoresis revealed a lipoprotein with β-mobility that showed an antigenic reaction against anti-B but not against anti-Lp(a) (Fig. 1C). A further 32% of the original protein was found in a pellet that had sedimented during ultracentrifugation. This pellet could only be solubilized in the presence of SDS.

Heparin-Sepharose chromatography of reduced Lp(a)

When reduced Lp(a) was eluted over a heparin-Sepharose column at low salt concentration (0.05 M NaCl), all of the applied lipid remained bound to the column. Two peaks of UV-absorbing material eluted at this salt concentration (**Fig. 2**). The second peak was due to DTT, whereas the first peak contained protein but no lipid. Immunochemical analysis by double diffusion revealed the presence of the (a) antigen but no apoB (**Fig. 3**). The bound material could be eluted by raising the NaCl concentration in the buffer to 0.5 M. It contained over 90% of the applied lipids. This lipoprotein migrated with β-mobility on agarose gel electrophoresis and immunochemical analysis showed the presence of apoB but no apo(a) (Fig. 3).

Lipid and protein analysis of reduced and unreduced Lp(a)

The lipid and protein composition of the lipoprotein component of reduced Lp(a), whether purified by ultracentrifugation or heparin-Sepharose chromatography, was similar to that of normal LDL (**Table 2**). Unreduced Lp(a) differed in its higher protein content and its slightly lower content of cholesteryl esters.

Gel-exclusion chromatography of reduced and unreduced Lp(a)

Unreduced Lp(a) eluted from a Sephacryl-S400 column (2.6 × 90 cm) at an average volume of 256 ml (peak maximum). The lipoprotein component of reduced Lp(a), purified by either ultracentrifugation or heparin-Sepharose chromatography, eluted from the gel column at 286 ml. This was similar to the elution volume of 290 ml for normal LDL.

Fast protein liquid chromatography (FPLC)

FPLC proved to be a rapid analytical procedure for assessing the possible contamination with LDL of native unreduced Lp(a) preparations purified by gel-exclusion chromatography. This was of particular importance for the cellular studies. Using a Tris buffer of pH 8.2 and a linear gradient of 0–0.4 M NaCl at a flow rate of 2 ml/min, a good resolution of LDL and unreduced Lp(a) was obtained on a strong anion exchanger column (**Fig. 4**). LDL eluted at the lower salt concentration of 0.24 M NaCl, while unreduced Lp(a) eluted at 0.34 M NaCl. After reduction of Lp(a) with DTT and purification by

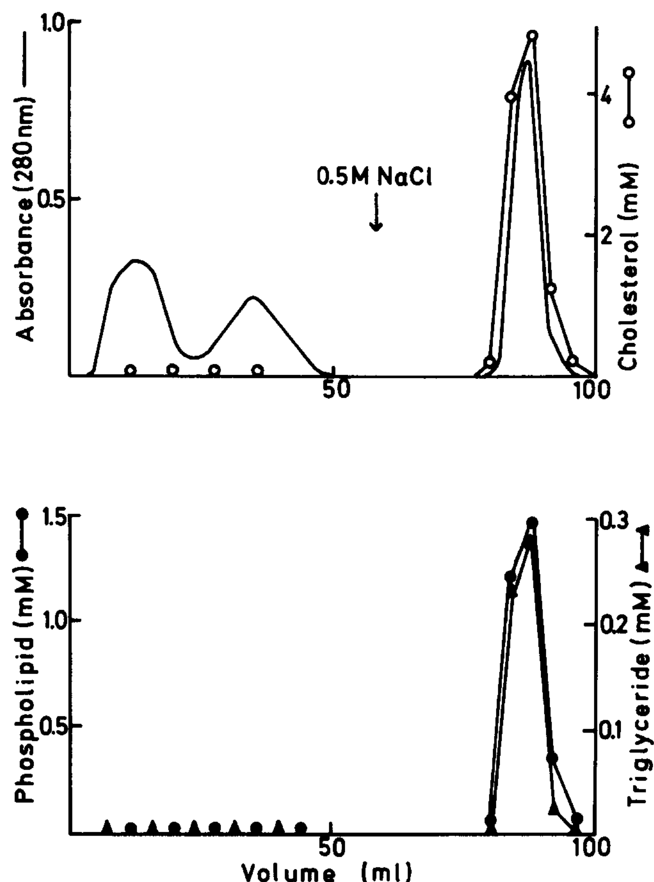


Fig. 2. Chromatography of reduced Lp(a) over heparin-Sepharose. Reduction was performed with DTT as described in Materials and Methods. Elution was initially performed with 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM EDTA, 0.2% NaN_3, and the salt concentration was then raised to 0.5 M NaCl where indicated. Fractions eluting from the column were monitored for: (—) UV absorbance at 280 nm; (○—○) cholesterol concentration; (●—●) phospholipid concentration; (▲—▲) triacylglycerol concentration.

Downloaded from www.jlr.org by guest, on June 18, 2012

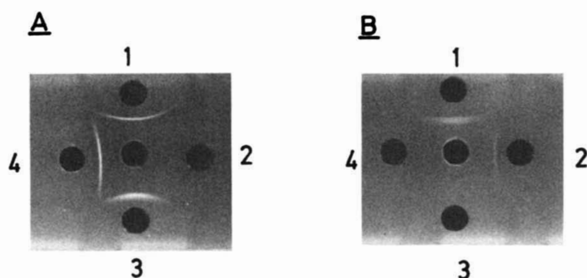


Fig. 3. Double diffusion of lipoprotein(a) and its reduced components obtained after heparin-Sepharose chromatography. The center wells contained (A) anti-B, and (B) anti-Lp(a). The outer wells contained (1) unreduced Lp(a); (2) first peak eluting from heparin-Sepharose column (Fig. 2); (3) third peak eluting from heparin-Sepharose column (Fig. 2); (4) normal LDL.

ultracentrifugation or heparin-Sepharose chromatography, the reduced apo(a)-free lipoprotein component was found to elute at the same salt concentration of 0.24 M NaCl as LDL. For analysis of the different lipoprotein preparations, 50-100 μg of protein was generally applied to the column. Contamination of unreduced Lp(a) with as little as 1% LDL (expressed as total peak area of the chromatogram at 280 nm) could be detected. The column could, however, be loaded with up to 500 μg of total protein and still give good resolution (Fig. 4) of LDL and unreduced Lp(a). Recovery of lipoprotein from such experiments as measured by recovery of total cholesterol and total protein was found to range between 85 and 95%.

SDS-PAGE analysis

Unreduced Lp(a) showed a major, high molecular weight band on SDS-PAGE (**Fig. 5**, lane 1). After reduction with DTT under denaturing conditions, this band disappeared and was replaced by a band (Fig. 5, lane 2) with a molecular weight similar to that of apoB from normal LDL (Fig. 5, lane 4). A minor band of slightly lower molecular weight could also be observed. The lipoprotein from reduced Lp(a), that had been purified by ultracentrifugation on heparin-Sepharose chromatography only showed one band (Fig. 5, lane 3) with a molecular weight similar to that of apoB. The protein component of reduced Lp(a) possessing the (a) antigen that did not bind to heparin-Sepharose had two bands on SDS-PAGE (Fig. 5, lane 5), one of molecular weight similar to apoB and the other of slightly lower molecular weight. The apo(a) bands did not stain as intensively as the apoB bands, an observation that has been reported by others (2, 3). The pellet obtained from ultracentrifugation of reduced Lp(a) also showed the same two bands (data not shown).

Uptake of Lp(a) and reduced Lp(a) by the LDL-receptor pathway

In control experiments we first ascertained that the degradation of LDL by the LDL-receptor pathway in cultured human fibroblasts was not affected by prior treatment of the LDL with DTT. Both native Lp(a) and reduced Lp(a) were then compared with LDL in the various steps of the receptor pathway (20). The results described in this article were obtained with the lipoprotein particle from reduced Lp(a) that had been purified by heparin-Sepharose chromatography. Similar findings were also made with the lipoprotein particle purified by ultracentrifugation. Experiments were performed in the presence and absence of a 25-fold excess of the corresponding unlabeled lipoprotein to determine the amount of unspecific uptake and the results are expressed in terms of receptor specific binding, internalization, and degradation of the lipoprotein (**Fig. 6, A, B, C**).

In all three steps, reduced Lp(a) showed saturation curves identical to LDL. Although native Lp(a) also showed saturation curves, indicative of a specific uptake, the responses were much reduced compared to those of

TABLE 2. Comparison of lipid and lipoprotein compositions of Lp(a), its reduced lipoprotein component, and LDL

Fraction	Percent of Total Lipid and Protein[a]				
	Unesterified Cholesterol	Cholesteryl Ester	Phospholipid	Triacylglycerol	Protein
Lp(a)	7.9 ± 1.8	37.1 ± 1.6	19.0 ± 0.2	5.0 ± 1.2	30.9 ± 0.8
Reduced Lp(a)-uc[b]	8.3 ± 1.4	41.7 ± 1.3	19.6 ± 1.9	5.7 ± 1.4	24.5 ± 1.1
Reduced Lp(a)-hs[c]	9.5 ± 1.1	42.8 ± 1.4	21.2 ± 0.7	4.6 ± 1.1	21.8 ± 0.9
LDL	8.5 ± 1.2	40.7 ± 1.5	21.3 ± 1.3	7.1 ± 1.3	22.4 ± 0.9

[a]Values (mean ± SD) were obtained from three different experiments.
[b]Lp(a) was reduced with DTT and the lipoprotein component was recovered by ultracentrifugation at d 1.063 g/ml.
[c]Lp(a) was reduced with DTT and the lipoprotein component was purified by chromatography on heparin-Sepharose.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

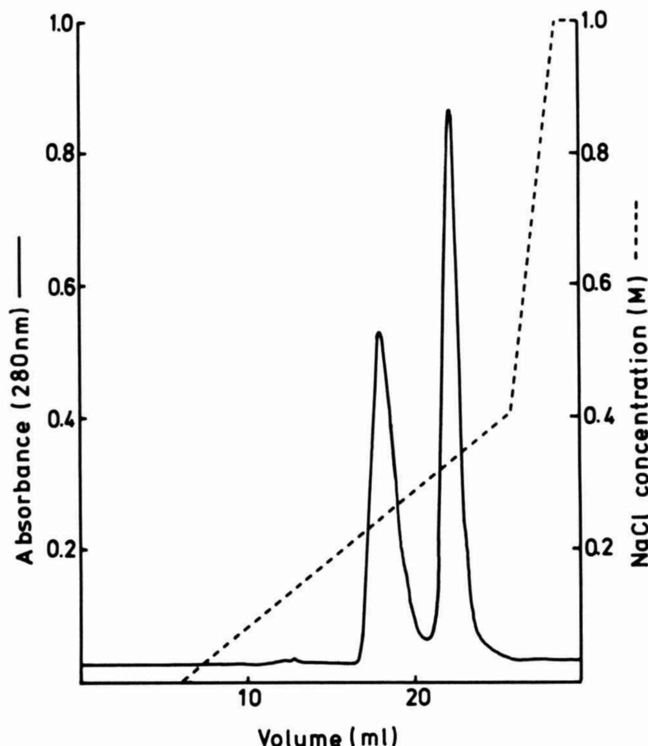


Fig. 4. Elution profile at 280 nm of a mixture of LDL (257 μg of protein) and unreduced Lp(a) (255 μg of protein) from a Mono Q strong anion exchanger column eluted at a flow rate of 2 ml/min. Formation of the linear salt gradient was controlled by the LCC-500 using the two buffers A) 0.02 M Tris, pH 8.2, and B) 0.02 M Tris, pH 8.2, 1.0 M NaCl. The first peak eluting at 0.24 M NaCl is that of LDL and the second peak at 0.34 M NaCl is that of Lp(a).

LDL and reduced Lp(a). Thus, at a protein concentration of 100 μg, binding of Lp(a) was 15% of LDL, internalization was 20% of LDL, and degradation was 18% of LDL. On the assumption that the degradation saturation curves obey Michaelis-Menten kinetics, the data were analyzed by means of a Scatchard plot (21). The slope of the line is then $-1/K_m$, K_m being the concentration of lipoprotein at which half maximum degradation capacity is observed, and the abcissa intercept is the maximum degradation capacity. Reduced Lp(a) and LDL both had similar K_ms and maximum degradation capacities according to this analysis (**Table 3**), whereas Lp(a) had an almost fourfold higher K_m and a much reduced maximum degradation capacity. Competition experiments were also performed in which an excess of each of the unlabeled lipoproteins was allowed to compete with 10 μg of labeled lipoprotein in the binding, internalization, and degradation assays. In accordance with the above findings, reduced Lp(a) and LDL were able to effectively compete against each other in the different assay systems (**Table 4**). These two lipoproteins were also able to suppress the uptake of normal Lp(a) by the receptor pathway. Although Lp(a) could also suppress the uptake of both reduced Lp(a) and LDL, it was less effective than either of the other two lipoproteins, in keeping with its higher K_m.

DISCUSSION

Treatment of Lp(a) with DTT under physiological conditions yielded both a lipoprotein particle and a lipid-free protein. The two components could be separated by ultracentrifugation at d 1.063 g/ml or by chromatography over heparin-Sepharose. The lipoprotein proved to be virtually identical to LDL in many of its physicochemical properties. Thus it had β-mobility on agarose gel electrophoresis, its molecular weight was similar to that of LDL by gel-exclusion chromatography, and it eluted at the same salt concentration as LDL on ion-exchange chromatography by FPLC. The lipid and protein composition of reduced Lp(a) was also similar to that of LDL, and differed from normal Lp(a) in that it had a lower amount of protein on a percentage basis. The results of SDS-PAGE and the immunochemical findings show that the only apoprotein associated with the lipoprotein component from reduced Lp(a) is apoB.

Further evidence for the similarity of this lipoprotein to LDL is provided by the studies on its uptake through the apoB-mediated receptor pathway in cultured human fibroblasts (20). The lipoprotein particle from reduced Lp(a) was specifically bound, internalized, and degraded as efficiently as LDL in the various steps of the LDL path-

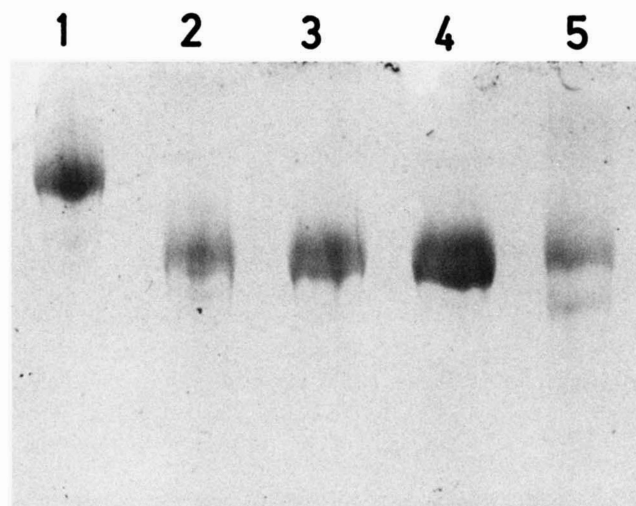


Fig. 5. SDS-PAGE in 2–16% gels of Lp(a) and its reduced components: (1) unreduced Lp(a); (2) Lp(a) after reduction with DTT in presence of SDS; (3) reduced Lp(a) after purification by heparin-Sepharose chromatography (peak 3, Fig. 2); (4) normal LDL; (5) lipid-free protein component from reduced Lp(a) after purification by heparin-Sepharose chromatography (peak 1, Fig. 2). Ten μg of protein was applied in the lanes 1, 2, 3, and 5, and 18 μg of protein in lane 4.

JOURNAL OF LIPID RESEARCH
ASBMB
Downloaded from www.jlr.org by guest, on June 18, 2012

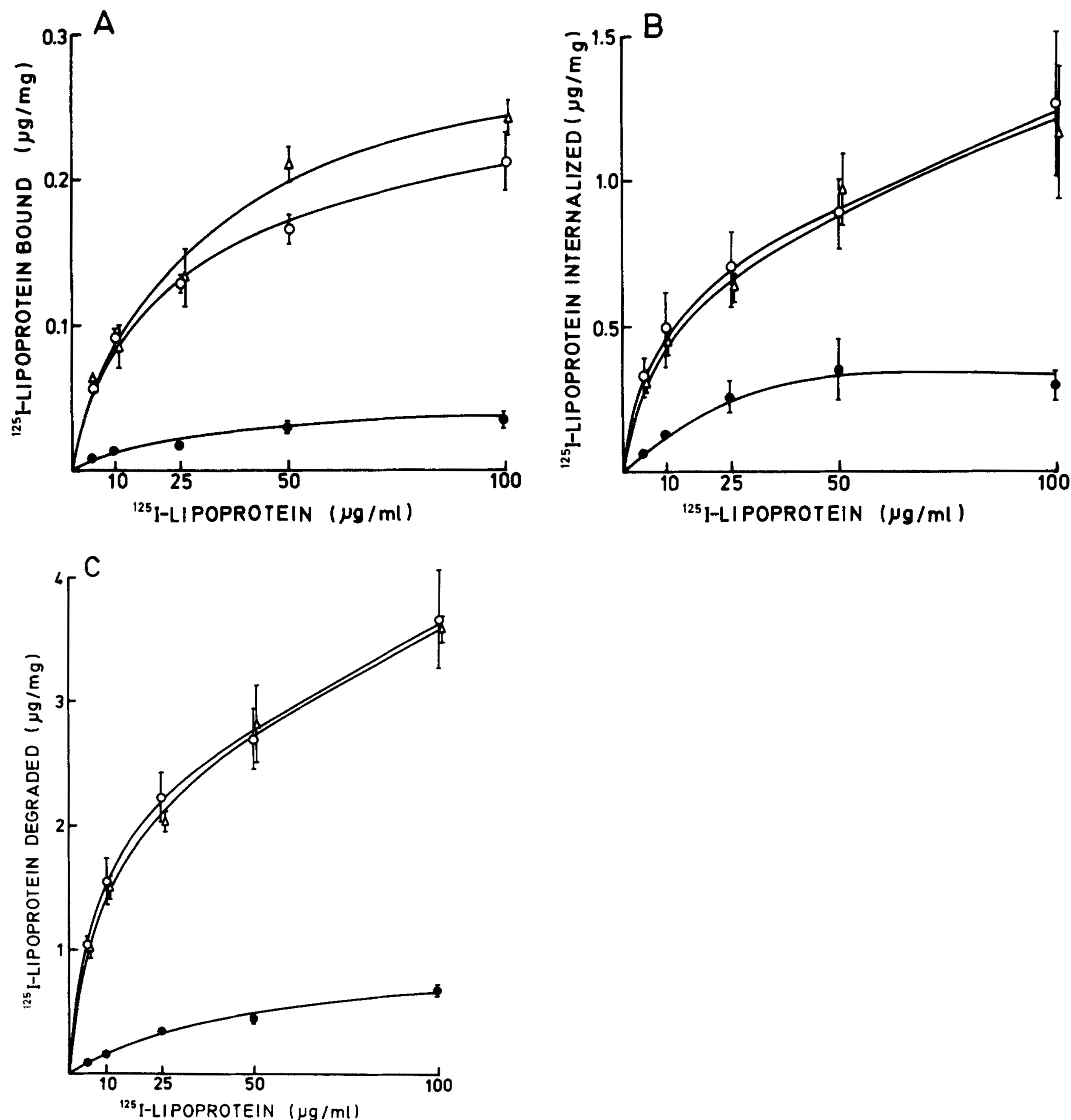


Fig. 6. Concentration-dependent uptake of (○—○) normal LDL; (△—△) lipoprotein component from reduced Lp(a) after heparin-Sepharose chromatography; and (●—●) unreduced Lp(a) by the apoB receptor-mediated pathway in human fibroblasts. A, Receptor-specific, heparin-releasable binding at 37°C; B, receptor-specific internalization at 37°C; and C, receptor-specific degradation at 37°C.

way (Fig. 6). It was also able to inhibit competitively the uptake of labeled LDL just as effectively as LDL itself. Normal unreduced Lp(a) showed poorer specificity for the LDL-receptor since a 25-fold excess of unlabeled lipoprotein could only suppress about 50% of the binding as compared with 75-80% suppression in the case of reduced Lp(a) and LDL. It was also bound, internalized, and degraded far less efficiently than the other two lipoproteins. The difference between normal Lp(a) and its reduced derivative and LDL is best illustrated by comparing the kinetic constants derived from the degradation curves. Thus normal Lp(a) had a fourfold higher K_m

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 3. K_m and maximum degradation capacities for Lp(a), reduced Lp(a), and LDL[a]

Fraction	K_m (µg protein/ml medium)	Maximum Capacity (ng/mg protein per 5 hr)
LDL	13.7	3779
Reduced Lp(a)[b]	13.0	3509
Lp(a)	48.0	922

[a]Derived from Fig. 6c.
[b]Lp(a) was reduced with DTT and the lipoprotein component was purified over heparin-Sepharose.

than the other two lipoproteins and the maximum degradation capacity for Lp(a) was only 25% that of LDL and unreduced Lp(a).

Previously published findings on the binding and uptake of Lp(a) by the LDL-receptor-mediated pathway have been contradictory. Martmann-Moe and Berg (5) compared total uptake (specific and unspecific) of LDL and Lp(a) in cells from normal persons and persons with both heterozygous and homozygous familial hypercholesterolemia (FH). Only slightly higher uptake of Lp(a) was obtained in normal and heterozygous cell strains as compared to homozygotes and the authors therefore concluded that Lp(a) enters cultured fibroblasts independently of the LDL-receptor.

Havekes et al. (6), however, observed a specific binding, internalization, and degradation of Lp(a) by normal human fibroblasts but they made no comparison with LDL, while Floren, Albers, and Bierman (7) reported that Lp(a) was specifically degraded by the LDL-receptor but to a lesser extent than LDL. Krempler et al. (8) found that Lp(a) bound almost as effectively as LDL to the LDL-receptor in human fibroblasts at 4°C; furthermore, they showed that cellular HMG-CoA-reductase activity was suppressed by Lp(a) at 37°C. Our own findings indicate that, although Lp(a) can be taken up by LDL-receptor-mediated pathway in vitro, it is a much poorer ligand for the LDL-receptor than LDL itself. Considering the fact that, in plasma, concentrations of LDL are normally far greater than those of Lp(a), it is probable that the LDL-receptor pathway only provides an insignificant route for the degradation of Lp(a) in vivo as compared to the unspecific pathway. Indeed, Krempler et al. (8) reported that the fractional catabolic rate for Lp(a) in homozygous FH was only slightly reduced compared with that in normals, in contrast to the large reduction seen in the fractional catabolic rate of LDL. If Lp(a) is not metabolized to any great extent in vivo by the LDL-receptor-mediated pathway, then it may potentially be a more atherogenic lipoprotein than LDL itself since it will not induce the same regulation on the cellular synthesis of cholesterol.

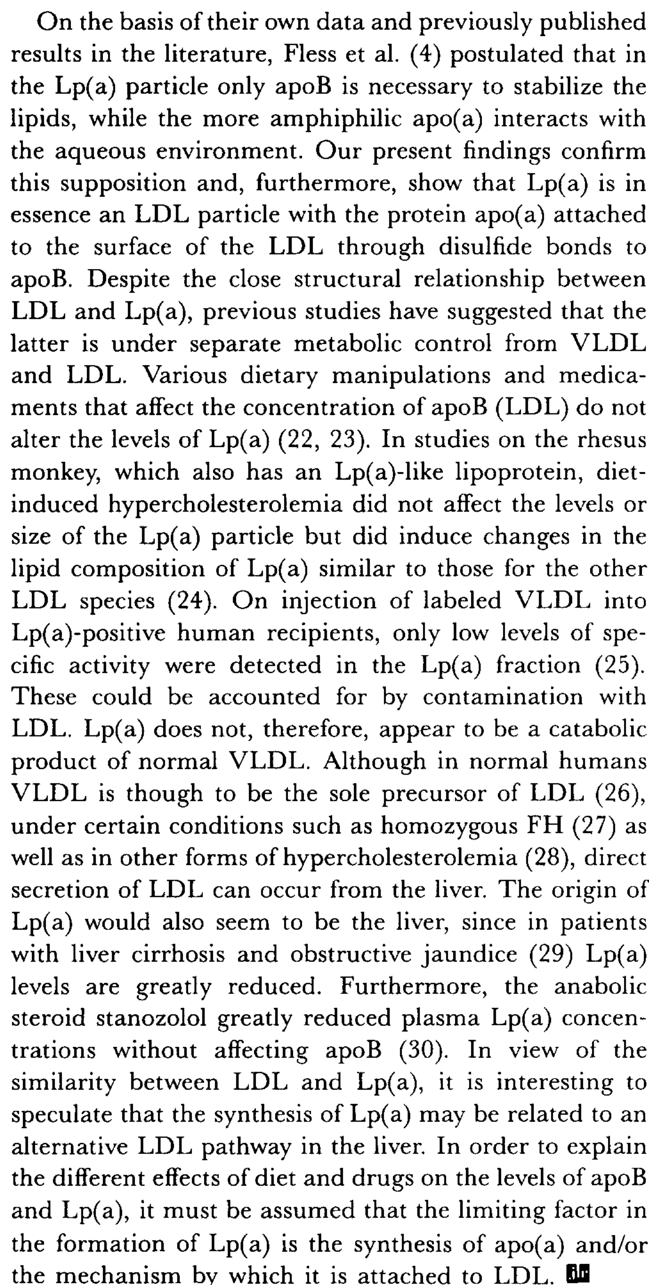
The lipid-free protein isolated from reduced Lp(a) under non-denaturing conditions contained the antigenic determinants of apo(a). This apoprotein was heterogeneous according to SDS-PAGE, consisting of a major band with molecular weight similar to apoB and a minor band of lower molecular weight. Fless, Rolih, and Scanu (4) have shown that Lp(a) exists as a series of heterogeneous particles partly due to different sized apo(a) proteins. In their investigations they observed apo(a) of three different sizes, larger than apoB, similar to apoB, and smaller than apoB. Gaubatz et al. (3) and Uterman and Weber (2) also observed an apo(a) with molecular weight larger than that of apoB. Despite having investigated Lp(a) from several different subjects, we have not as yet observed the larger molecular weight apo(a). The present purification procedure for apo(a), however, will allow a more detailed analysis of its structure and composition.

TABLE 4. Ability of an excess of unlabeled lipoprotein to compete with labeled lipoprotein in the binding, internalization, and degradation assays on cultured human fibroblasts

Labeled Lipoprotein[a]	25-fold Excess Unlabeled Lipoprotein	Heparin-Releasable Surface Binding	Internalization	Degradation
		ng/mg protein (%)		
Lp(a)		20.1 ± 3.9 (100)	37.8 ± 22.9 (100)	259.7 ± 14.6 (100)
Lp(a)	Lp(a)	11.9 ± 4.0 (59)	14.9 ± 3.5 (39)	114.3 ± 23.1 (86)
Lp(a)	Reduced Lp(a)	10.7 ± 2.7 (53)	14.1 ± 8.1 (37)	78.7 ± 2.3 (26)
Lp(a)	LDL	10.1 ± 4.3 (50)	20.8 ± 7.1 (55)	80.3 ± 12.9 (27)
Reduced Lp(a)		133.4 ± 11.7 (100)	294.5 ± 7.6 (100)	1766.0 ± 37.2 (100)
Reduced Lp(a)	Lp(a)	125.8 ± 0.7 (94)	219.8 ± 6.7 (75)	1220.0 ± 7.8 (69)
Reduced Lp(a)	Reduced Lp(a)	25.9 ± 4.4 (19)	71.1 ± 2.8 (24)	737.7 ± 28.9 (42)
Reduced Lp(a)	LDL	55.2 ± 6.6 (41)	104.0 ± 7.4 (35)	494.7 ± 40.4 (28)
LDL		147.8 ± 10.1 (100)	302.2 ± 42.0 (100)	1830 ± 136.6 (100)
LDL	Lp(a)	101.7 ± 7.9 (58)	175.2 ± 19 (58)	943.3 ± 51.3 (52)
LDL	Reduced Lp(a)	17.2 ± 5.2 (10)	35.6 ± 2.6 (12)	348.7 ± 17.8 (19)
LDL	LDL	43.6 ± 9.6 (25)	61.6 ± 5.8 (20)	431.3 ± 32.0 (24)

[a]^{125}I-Labeled lipoprotein was employed at a concentration of 10 µg/ml.

Downloaded from www.jlr.org by guest, on June 18, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR



On the basis of their own data and previously published results in the literature, Fless et al. (4) postulated that in the Lp(a) particle only apoB is necessary to stabilize the lipids, while the more amphiphilic apo(a) interacts with the aqueous environment. Our present findings confirm this supposition and, furthermore, show that Lp(a) is in essence an LDL particle with the protein apo(a) attached to the surface of the LDL through disulfide bonds to apoB. Despite the close structural relationship between LDL and Lp(a), previous studies have suggested that the latter is under separate metabolic control from VLDL and LDL. Various dietary manipulations and medicaments that affect the concentration of apoB (LDL) do not alter the levels of Lp(a) (22, 23). In studies on the rhesus monkey, which also has an Lp(a)-like lipoprotein, diet-induced hypercholesterolemia did not affect the levels or size of the Lp(a) particle but did induce changes in the lipid composition of Lp(a) similar to those for the other LDL species (24). On injection of labeled VLDL into Lp(a)-positive human recipients, only low levels of specific activity were detected in the Lp(a) fraction (25). These could be accounted for by contamination with LDL. Lp(a) does not, therefore, appear to be a catabolic product of normal VLDL. Although in normal humans VLDL is though to be the sole precursor of LDL (26), under certain conditions such as homozygous FH (27) as well as in other forms of hypercholesterolemia (28), direct secretion of LDL can occur from the liver. The origin of Lp(a) would also seem to be the liver, since in patients with liver cirrhosis and obstructive jaundice (29) Lp(a) levels are greatly reduced. Furthermore, the anabolic steroid stanozolol greatly reduced plasma Lp(a) concentrations without affecting apoB (30). In view of the similarity between LDL and Lp(a), it is interesting to speculate that the synthesis of Lp(a) may be related to an alternative LDL pathway in the liver. In order to explain the different effects of diet and drugs on the levels of apoB and Lp(a), it must be assumed that the limiting factor in the formation of Lp(a) is the synthesis of apo(a) and/or the mechanism by which it is attached to LDL. ■

We gratefully acknowledge the excellent technical assistance of Roswitha Niedmann, Margit Stix, and Christina Wiese. We also thank Christine Berwanger for secretarial assistance in the preparation of the manuscript. This research was supported by the 'Deutsche Forschungsgemeinschaft' (Sonderforschungsbereich 89, Cardiology, D-3400 Göttingen, Federal Republic of Germany).

Manuscript received 26 December 1984.

REFERENCES

1. Berg, K. 1963. A new serum type system in man, the Lp-system. *Acta Pathol. Microbiol. Scand.* **59:** 369–382.
2. Utermann, G., and W. Weber. 1983. Protein composition of lp(a) lipoprotein from human plasma. *FEBS Lett.* **154:** 357–361.
3. Gaubatz, J. W., C. Heideman, A. M. Gotto, J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein(a): structural properties. *J. Biol. Chem.* **258:** 4582–4589.
4. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of human plasma lipoprotein(a). *J. Biol. Chem.* **259:** 11470–11478.
5. Martmann-Moe, K., and K. Berg. 1981. Lp(a) lipoprotein enters cultured fibroblasts independently of the plasma membrane low density lipoprotein receptor. *Clin. Genet.* **20:** 352–362.
6. Havekes, L., B. J. Vermeer, T. Brugman, and J. Emeis. 1981. Binding of lp(a) to the low density lipoprotein receptor of human fibroblasts. *FEBS Lett.* **132:** 169–173.
7. Floren, C. H., J. J. Albers, and E. L. Bierman. 1981. Uptake of lp(a) lipoprotein by cultured fibroblasts. *Biochem. Biophys. Res. Commun.* **102:** 636–639.
8. Krempler, F., G. M. Kostner, A. Roscher, F. Haslauer, K. Bolzano, and F. Sandhofer. 1983. Studies on the role of specific cell surface receptors in the removal of lipoprotein(a) in man. *J. Clin. Invest.* **71:** 1431–1441.
9. Dahlen, G., M. H. Frick, K. Berg, M. Valle, and M. Wilzasolo. 1975. Further studies of Lp(a) lipoprotein/pre-β_1-lipoprotein in patients with coronary heart disease. *Clin. Genet.* **8:** 183–189.
10. Kostner, G. M., P. Avogaro, G. Cazzolato, E. Marth, G. Bittolo-Bon, and G. B. Quinci. 1981. Lipoprotein(a) and the risk for myocardial infarction. *Atherosclerosis.* **38:** 51–61.
11. Grabar, P., and C. A. Williams. 1955. Methode immonoelectrophoretique d'analyse de melanges de substances antigeniques. *Biochim. Biophys. Acta.* **17:** 67–74.
12. Wieland, H., and D. Seidel. 1983. Quantitative lipoprotein electrophoresis. *In* Handbook of Electrophoresis. Volume III. L. A. Lewis, editor. CRC Press, Boca Raton, FL. 83–102.
13. Walli, A. K., and D. Seidel. 1984. Role of lipoprotein-X in the pathogenesis of cholestatic hypercholesterolemia. *J. Clin. Invest.* **74:** 867–879.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
15. Albers, J. J., and W. R. Hazzard. 1974. Immunochemical quantification of human plasma lp(a) lipoprotein. *Lipids.* **9:** 15–26.
16. Bilheimer, D. W., J. L. Goldstein, S. M. Grundy, and M. S. Brown. 1975. Reduction in cholesterol and low density lipoprotein synthesis after portacaval shunt surgery in a patient with homozygous familial hypercholesterolemia. *J. Clin. Invest.* **56:** 1420–1430.
17. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
18. Drevon, C. A., A. D. Attie, S. H. Pangburn, and D. Steinberg. 1981. Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J. Lipid Res.* **22:** 37–46.
19. Bierman, E. L., and J. J. Albers. 1977. Regulation of low density lipoprotein receptors by cultured human arterial smooth muscle cells. *Biochim. Biophys. Acta.* **488:** 152–160.
20. Goldstein, J. L., and M. S. Brown. 1976. The LDL pathway in human fibroblasts: a receptor-mediated mechanism for the regulation of cholesterol metabolism. *In* Current

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012

Topics in Cellular Regulation. Vol. 11. B. L. Horecker and E. R. Stadtman, editors. Academic Press, New York. 147–181.
21. Ostlund, R. E., R. A. Levy, J. L. Witztum, and G. Schonfeld. 1982. Familial hypercholesterolemia: evidence for a newly recognized mutation determining increased fibroblast receptor affinity but decreased capacity for low density lipoproteins in two siblings. *J. Clin. Invest.* **70:** 823–831.
22. Albers, J. J., V. G. Cabana, G. R. Warnick, and W. R. Hazzard. 1975. Lp(a) lipoprotein: relationship to sinking pre-β-lipoprotein, hyperlipoproteinemia, and apolipoprotein B. *Metabolism.* **24:** 1047–1054.
23. Vessby, B., G. M. Kostner, H. Lithell, and J. Thomis. 1982. Diverging effects of cholestyramine on apolipoprotein B and lipoprotein Lp(a). A dose-response study of the effects of cholestyramine in hypercholesterolaemia. *Atherosclerosis.* **44:** 61–71.
24. Fless, G. M., K. Fischer-Dzoga, D. J. Juhn, S. Bates, and A. M. Scanu. 1982. Structural and functional changes of rhesus serum low density lipoproteins during cycles of diet-induced hypercholesterolemia. *Arteriosclerosis.* **2:** 475–486.
25. Krempler, F., G. M. Kostner, K. Bolzano, and F. Sandhofer. 1980. Turnover of lipoprotein(a) in man. *J. Clin. Invest.* **65:** 1483–1490.
26. Sigurdsson, G., A. Nicoll, and B. Lewis. 1975. Conversion of very low density lipoprotein to low density lipoprotein: a metabolic study of apolipoprotein B kinetics in human subjects. *J. Clin. Invest.* **56:** 1481–1490.
27. Soutar, A. K., N. B. Myant, and G. R. Thompson. 1977. Simultaneous measurement of apolipoprotein B turnover in very-low- and low-density lipoproteins in familial hypercholesterolemia. *Atherosclerosis.* **28:** 247–256.
28. Janus, E. D., A. Nicoll, R. Wootton, P. R. Turner, P. J. Magill, and B. Lewis. 1980. Quantitative studies of very low density lipoprotein to low density lipoprotein in normal controls and primary hyperlipidemic states and the role of direct secretion of low density lipoprotein in heterozygous familial hypercholesterolemia. *Eur. J. Clin. Invest.* **10:** 149–159.
29. Kostner, G. M. 1983. Apolipoproteins and lipoproteins of human plasma. *Adv. Lipid Res.* **20:** 1–43.
30. Albers, J. J., H. M. Taggart, D. Applebaum-Bowden, S. Haffner, C. H. Chesnut, and W. R. Hazzard. 1984. Reduction of lecithin-cholesterol acyltransferase, apolipoprotein D and the lp(a) lipoprotein with the anabolic steroid stanozolol. *Biochim. Biophys. Acta.* **759:** 293–296.

ASBMB
JOURNAL OF LIPID RESEARCH
JLR
Downloaded from www.jlr.org by guest, on June 18, 2012