Isolation, quantitation, and characterization of a stable complex formed by Lp[a] binding to triglyceride-rich lipoproteins

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Abstract **Lipoprotein [a] (Lp[a]) is a cholesterol-rich lipoprotein resembling LDL to which a large polymorphic glycoprotein, apolipoprotein [a] (apo[a]), is covalently coupled. Lp[a] usually exists as a free-standing particle in normolipidemic subjects; however, it can associate noncovalently with triglyceride-rich lipoproteins in hypertriglyceridemic (HTG) subjects. In this study, 10–78% of the Lp[a] present in five HTG subjects was found in the triglyceride-rich lipoprotein (TRL) fraction. The Lp[a]-TRL complex was resistant to dissociation by ultracentrifugation (UCF) alone, but was quantitatively dissociated by UCF in the presence of 100 mM proline. Of this dissociated Lp[a], 70–88% was in the form of a lipoprotein resembling conventional Lp[a]. Incubation of Lp[a]-depleted TRL with native Lp[a] resulted in a reconstituted Lp[a]-TRL complex that closely resembled the native isolates in all examined properties. Complex formation was inhibited by several compounds in the order proline > tranexamate > ε-aminocaproate >> arginine > lysine. Neither plasminogen nor LDL inhibited binding of Lp[a] to TRL. We observed the preferential binding of Lp[a] containing higher apparent molecular weight apo[a] polymorphs to TRL both in native and reconstituted Lp[a]-TRL complexes. A disproportionate amount of Lp[a] was bound to the larger TRL particles. Although most apo[a] bound to TRL was in the form of conventional Lp[a] particles, lipid-free recombinant apo[a] was observed to bind TRL. These results provide unequivocal evidence of the existence of an Lp[a]-TRL complex under pathophysiologic conditions. The metabolic fate of the Lp[a]-TRL complex, which is more abundant in hypertriglyceridemia, may be different from that of conventional Lp[a], and may contribute uniquely to the progression or severity of cardiovascular disease.—** Gaubatz, J. W., R. C. Hoogeveen, A. S. Hoffman, K. G. Ghazzaly, H. J. Pownall, J. Guevara, Jr., M. L. Koschinsky, and J. D. Morrisett. **Isolation, quantitation, and characterization of a stable complex formed by Lp[a] binding to triglyceride-rich lipoproteins. *J. Lipid Res.* 2001.** 42: **2058–2068.**

Supplementary key words apo[a] • chylomicrons • ELISA • hypertriglyceridemia • type IV • type V • VLDL

The human lipoprotein [a] (Lp[a]) antigen, apolipoprotein [a] (apo[a]), was first discovered by Berg (1) and initially was thought to represent a variant of LDL. This specific apo[a] protein has been detected in plasma in several physical forms: *i*) lipid-free, unbound protein, *ii*) lipid-free protein disulfide linked to apoB-100, *iii*) disulfide linked to apoB-100 in an Lp[a] particle, and most recently, *iv*) associated with a triglyceride-rich lipoprotein (TRL) fraction. Apo[a] is usually present in human plasma as the cholesterol-rich Lp[a] particle but it has been detected in all of the above-mentioned forms. Lp[a] occurs ubiquitously, although the levels can vary 1,000-fold among individuals. Considerable clinical interest was first generated by qualitative tests that demonstrated a highly significant positive correlation between the presence of apo[a] in plasma and particular indicators of coronary heart disease (2, 3). Subsequent isolation, purification, and characterization of Lp[a] showed that it differed from LDL by the presence of the unique, polymorphic, high molecular weight, apo[a] protein that was disulfide linked to apoB-100 (4, 5). Further studies demonstrated a high amino acid sequence homology between apo[a] and kringle domains in plasminogen (6). A variable number of repeats of a plasminogen kringle IV-like structure in apo[a] accounts for its molecular weight polymorphism (6). These developments have broadened the search for possible pathological roles for Lp[a] to include thrombosis in addition to atherosclerosis (7, 8). Kringle structures possess specific ligand-binding properties, with documented

Abbreviations: apo[a], apolipoprotein [a]; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoLp[a], the total protein moiety of Lp[a] (mostly apo[a] and apoB-100); EACA, ε-aminocaproic acid; FPLC, fast protein liquid chromatography; HTG, hypertriglyceridemic; Lp[a], lipoprotein [a]; Lp[a]-TRL, lipoprotein [a]-triglyceride-rich lipoprotein complex; Pr-TRL, triglyceride-rich lipoprotein treated with 100 mM proline to strip off any bound Lp[a] or apo[a]; TRL, triglyceride-rich lipoprotein; UCF, ultracentrifugation.

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binding sites for L-lysine and related amino acid analogs (9, 10). Ten distinctive kringle IV types have been found in apo[a]; certain types have been identified in the specific noncovalent interaction with apoB-100, which is the first step in Lp[a] particle assembly. This ability of kringles to interact with particular macromolecules confers many important properties on the proteins in which they occur.

Several in vitro studies have indicated that Lp[a] has an affinity for apoB-containing lipoproteins (11–13), especially triglyceride-rich apoB lipoproteins. Bersot et al. (14) first reported apo[a] associated with $d < 1.006$ lipoproteins in humans after fat feeding. Additional studies of normolipidemics found as much as 16% of the apo[a] immunoreactivity distributed into TRL of postprandial subjects compared with only 2% in fasted control subjects (15, 16). In the fasting plasma of subjects with type I or III (17) or IV (18) hyperlipoproteinemias, increased proportions of apo[a] were found associated with TRL compared with normolipidemic control subjects; however, the majority of apo[a] was still distributed in the normal Lp[a] density range of 1.05–1.12. A more recent study of 12 fasting type IV hyperlipidemics detected 1.9–20.0% of apo[a] distributed into TRL compared with only 2% in normolipidemic control subjects (19). The apo[a]-TRL (apo[a]-triglyceride-rich lipoprotein complex) from several of these subjects was isolated and partially characterized. Recentrifugation or fast protein liquid chromatography (FPLC) of their isolated apo[a]-TRL fraction resulted in dissociation of most of the apo[a]; this dissociated apo[a] exhibited the electrophoretic and chromatographic properties of Lp[a], providing evidence that Lp[a]-TRL (lipoprotein [a]-triglyceride-rich lipoprotein complex) represents Lp[a] noncovalently bound to TRL. The low percentage of apo[a] immunoreactivity distributed into the TRL fraction in vivo and the ease of dissociation of Lp[a] from this complex have caused skepticism about the significance of a specific, native Lp[a]-TRL. Some alternative explanations for the observation of this complex are *i*) an ultracentrifugal artifact due to diffusion or spillover of Lp[a] from its normal density range into the range of TRL, and *ii*) nonspecific adherence of the Lp[a] to TRL, for example, via weak hydrophobic or ionic interactions.

Plasma Lp[a] levels are inversely related to TG levels in hypertriglyceridemic (HTG) patients (20–22), suggesting a possible metabolic relationship between Lp[a]-TRL and TG. One study showed that medications that lowered TG in hypertriglyceridemics produced a concomitant increase in Lp[a] levels (23). Intravenous administration of TG as Intralipid resulted in a 22% decrease in Lp[a] after 2 h (24). Oral fat loading in normolipidemic or HTG subjects produced transient increases in TG with decreases in Lp[a], which returned to baseline levels 8–12 h after ingestion (25, 26). During this period, Lp[a] partitioning into the TRL fraction increased. Estrogen in the form of oral contraceptives caused a decrease in Lp[a] of up to 30% in premenopausal women, while raising triglycerides by 34% (27).

To demonstrate the existence and stability of a native Lp[a]-TRL complex and to characterize this physical form of Lp[a], we have examined the fasting plasma of six hypertriglyceridemic subjects (28). Patients with endogenous HTG were chosen to enhance the probability of detecting, isolating, and characterizing Lp[a]-TRL. The resistance of Lp[a]-TRL to dissociation by various ultracentrifugal treatments, by size-exclusion chromatography, or by a combination of these techniques was examined. To determine the specificity of the interaction, a variety of amino acids and their analogs were tested for their ability to dissociate the Lp[a]-TRL complex. Both liquid-phase and solid-phase (microtiter plate) binding assays were developed to investigate the influence of several different factors on the formation, stability, and dissociation of the Lp[a]-TRL complex.

EXPERIMENTAL PROCEDURES

Subjects

Six HTG subjects were recruited for this study. None were taking lipid-lowering medication and only one was on a lipid-lowering diet. A healthy normolipidemic volunteer was recruited as a control subject. After a 12-h overnight fast, 500 ml of plasma was obtained from each subject by plasmapheresis at the Methodist Hospital Blood Donor Center (Houston, TX). Aprotinin [Trasylol (Mobay Chemical, Kansas City, MO), 10,000 kallikrein inactivator units (KIU)/ml] was added as a protease inhibitor to the plasma immediately after collection, to a final concentration of 100 KIU/ml. In addition, the plasma was made 0.01% in NaN_3 and 1 mM in EDTA. Study subjects signed a consent form approved by the Institutional Review Board for Human Research at Baylor College of Medicine (Houston, TX) and its affiliated hospitals.

Isolation of lipoproteins

For preparative isolation of the TRL fraction, fresh HTG plasma was diluted 2- to 5-fold with saline before ultracentrifugation (UCF) to avoid the formation of a gelatinous or viscous chylomicron/VLDL layer in the d 1.006 supernatant that would prevent aspiration and quantitative recovery of this fraction. Larger volumes of plasma were ultracentrifuged in a Beckman (Fullerton, CA) 50.2 rotor for 18 h at 30,000 rpm at 8°C; for smaller volumes, a Beckman SW 55 rotor was utilized. TRL were operationally defined as all lipoproteins floating at $d < 1.006$, including VLDL and chylomicrons. Although all subjects were fasted overnight, some had chylomicrons in their plasma. Lp[a] was isolated from plasma of donors with single apo[a] polymorphs as judged by single bands on PAGE as previously described (29).

Size-exclusion chromatography was used for fractionation of native lipoproteins and apolipoproteins from plasma or TRL, and for resolution of synthetic Lp[a]-TRL generated in vitro from mixtures of Lp[a] and plasma or TRL. Columns were eluted with 25 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 0.01% sodium azide, pH 7.4; 5 to 10 ml of plasma or TRL was chromatographed on a 2.6 × 90 cm column of Bio-Gel A-5m or A-15m agarose gel (Bio-Rad, Richmond, CA). Three-milliliter fractions were collected every 20 min at a flow rate of 9.0 ml/h. For the study of reconstituted particles, a 1.2 × 55 cm column of A-15m agarose was used; 2.5-ml fractions were collected at 5-min intervals at a flow rate of 30 ml/h. For the latter experiments, Lp[a] concentrations of 2.5, 10, 25, and 100 μg/ml were incubated with Pr-TRL (triglyceride-rich lipoprotein treated with 100 mM proline to strip off any bound Lp[a] or apo[a]) containing 0.25 mg of apoB for 3 h at room tempera-

ture. After this incubation, 1 ml of each mixture was chromatographed. Proline was included in the eluting buffer of only one experiment (Fig. 5D).

Chemical analyses

Plasma fractions were analyzed for total cholesterol (30), HDL cholesterol (31), and triglyceride (32) by the Lipid Research Laboratory (Methodist Hospital). LDL cholesterol was calculated by the Friedewald, Levy, and Fredrickson formula (33) and then corrected for the contribution of Lp[a] cholesterol (34), which is 1.05 × Lp[a] protein. Plasma Lp[a] protein levels were measured by an ELISA developed in this laboratory and described previously (35). The immunoreactivity of the assay was not significantly affected by triglyceride concentrations of <600 mg/dl. Plasma samples with higher triglyceride concentrations were diluted to this level before assaying Lp[a]. Plasma apoB levels were determined by ELISA, using clone 2-B4 (Per-Immune, Rockville, MD) as the capture antibody. This monoclonal antibody was reactive to apoB-100 in VLDL, LDL, and Lp[a] and to apoB-48 in chylomicrons.

The relative molecular weights of apo[a] polymorphs were determined by a modification of an immunoblotting method previously described (36, 37). This modified method uses high resolution 15 × 25 cm 2% agarose gels, which are capable of separating apo[a] isoforms that differ by only one kringle IV repeat. Subject apo[a] polymorphs were classified by comparison with apo[a] isoform standards containing 16,18, 22, 26, 28, 32, and 35 kringle IV repeats obtained from Intracel (Rockville, MD).

Liquid-phase method for Lp[a]-TRL complex formation

TRL that had been previously treated with 100 mM proline and ultracentrifuged to remove any endogenous, bound Lp[a] or apo[a] was added to purified native Lp[a] in PBS. The mixture was incubated for 3 h at room temperature with gentle shaking, followed by either storage or chromatography at 4°C.

Solid-phase ELISA for Lp[a]-TRL binding

Wells of an Immulon I U-bottom plate (Dynex Technologies, Chantilly, VA) were coated with 100 μl of Pr-TRL protein at a concentration of 5 μg/ml and incubated for 2 h at room temperature. BSA at a final concentration of 1% was then added as a blocking agent for 2 h at room temperature. To this Pr-TRL-coated plate, Lp[a] was observed to bind linearly up to 250-ng/ml concentration of Lp[a] protein. On the basis of this result, standard assay conditions were selected as 10–125 ng of Lp[a] per ml, incubated for 3 h with shaking at 75 rpm. A peroxidase-conjugated polyclonal antibody to Lp[a], made monospecific for apo[a] by liquid and solid-phase absorption with LDL and plasminogen, was used for detection with *o*-phenylenediamine as substrate.

RESULTS

Postplasmapheresis lipoprotein measurements

The lipid profiles of seven fasting subjects are shown in **Table 1**. The concentrations were measured in postplasmapheresis plasma, which represents the actual plasma source for these studies. There was a 20–25% dilution of the plasma due to the presence of anticoagulant solution in the collection bag at the time of plasmapheresis. The apoLp[a] [the total protein moiety of Lp[a] (mostly apo[a] and apoB-100)] concentration determined by ELISA for these subjects was inversely correlated with TG concentration ($r = -0.85$, $P = 0.012$, n = 7; Spearman correlation).

Characterization of native Lp[a]-TRL particles

Ultracentrifugal analyses. The percentage of Lp[a] distributed into the TRL fraction in these subjects ranged from only 1% in the normolipidemic subject to as high as 78% in the hypertriglyceridemic patients (Table 1). The stability of the Lp[a]-TRL complex was examined by subjecting the TRL isolated from the initial UCF to additional, sequential ultracentrifugal treatments as summarized in **Fig. 1** for five of these subjects. To normalize the values, apoLp[a] and apoB were measured in all samples by ELISA, with the results reported as the apoLp[a]/apoB ratio. Each additional cycle of UCF alone produced a gradual, incremental dissociation of Lp[a] from the complex. The TRL fraction produced by a 72-h UCF spin contained less Lp[a] than a fraction produced by a single 18-h spin, but more Lp[a] than produced by three separate 18-h spins. When 100 mM proline was used in combination with UCF, a single 18-h UCF spin resulted in specific, almost total dissociation of Lp[a] from the TRL. The proline concentration dependence of this dissociation, and the effectiveness of 100 mM ε-aminocaproate (EACA) and lysine for subject H3, is illustrated in **Fig. 2**. The percentage of total plasma Lp[a] that was partitioned into the TRL fraction decreased from 92% with 1 mM proline to 58% with 10 mM proline, and to only 3% with 100 mM proline. EACA was slightly more effective than lysine as a dissociating agent but neither equaled proline.

Because small-scale UCF of Lp[a]-TRL in the presence of 100 mM proline was found to be an effective treatment

TABLE 1. Lipid profiles and apo[a] phenotypes of study subjects

Subject	Plasma TG	Total Cholesterol	Plasma ApoLp[a]	ApoLp[a] in Fasting TRL (d < 1.006)	Apo[a][a] Polymorphs in Plasma	Apo[a][a] Polymorphs in TRL
	mg/dl	*mg/dl*	*μg/ml*	%		
N	85	175	30.0	1	24, 30	30
H1	376	530	5.0	4	20, 35	20, 35
H2	183	200	9.0	10	28, 35	35
H3	310	202	8.0	12	28, 32	32
H4	1,314	468	1.3	78	ND	36
H5	1,590	341	1.0	47	ND	28
H6	2,971	494	5.0	70	ND	26

ND, not detectable.
[a] Numbers refer to the number of kringle type IV repeats in apo[a].

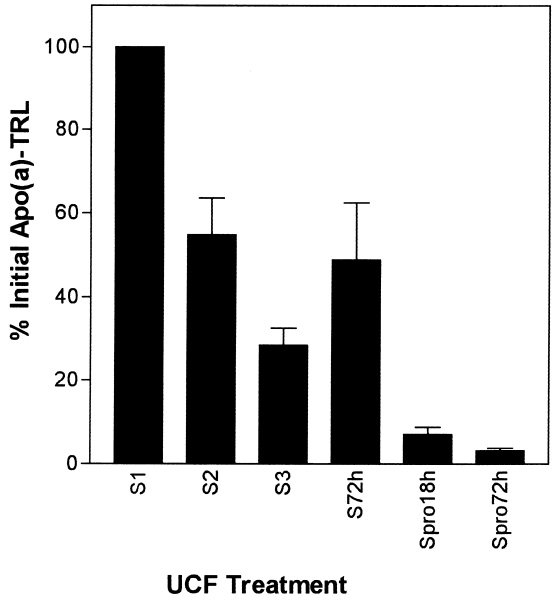


Fig. 1. Lp[a]-TRL complex stability expressed as a percentage of initial complex floating in the d < 1.006 supernatant after UCF of Lp[a]-TRL: one 18-h spin (S1), two 18-h spins (S2), three 18-h spins (S3), one 72-h spin (S72h), one 18-h spin in 100 mM proline (Spro18h), one 72-h spin in 100 mM proline (Spro72h).

for the quantitative dissociation of Lp[a], we adapted this technique to preparative-scale UCF. Larger amounts of this form of Lp[a], present in low concentrations, could thus be isolated for additional characterization. When the Lp[a]-TRL fraction isolated from an initial UCF was adjusted to 100 mM proline and recentrifuged at d 1.006, the resulting infranatant typically contained more than 95% of the Lp[a], essentially devoid of TRL. The 500 ml of plasma available from plasmapheresis provided a valuable source from which sufficient quantities of this Lp[a] could be isolated for various analyses. This was especially critical for phenotyping apo[a] from the HTG subjects because high TG levels caused anomalous band patterns when conventional SDS-agarose gel electrophoresis was used. This TG interference, coupled with the accompanying low plasma Lp[a] levels characteristic of HTG subjects, prevented adequate resolution and identification of apo[a] polymorphs by our conventional phenotyping methodology. This problem was overcome by using the Lp[a]-enriched, triglyceride-depleted fraction (d 1.006 infranatant) obtained after proline treatment and UCF. Shown in **Fig. 3** are representative SDS-PAGE patterns of TRL (d 1.006 supernatant) and Lp[a] (d 1.006 infranatant) resulting from proline treatment of Lp[a]-TRL. Immunoblotting revealed the apo[a] polymorph(s) that were bound to the Lp[a]-TRL fraction for all study subjects (**Fig. 4A**). For two subjects (H3 and N), whose plasma exhibited two apo[a] polymorphs, we compared the relative levels of these two bands in whole, unfractionated plasma with the relative amounts of these two polymorphs in the d 1.006 supernatant (Lp[a]-TRL) and d 1.006 infranatant (Lp[a]) (Fig. 4B; only N is shown). This comparison revealed a preferential partitioning of the larger apparent molecular weight apo[a] polymorph into the TRL fraction. To determine whether the apo[a] in the TRL was present as free apo[a] protein or holo Lp[a] lipoprotein, the density of this dissociated Lp[a] fraction was adjusted to 1.21 g/ml and ultracentrifuged. The resulting d 1.21 supernatant and infranatant fractions were assayed for apo[a] immunoreactivity by ELISA. By this analysis, 80 ± 8% of this apo[a] was classified as intact lipoprotein. In addition, SDS-agarose electrophoretic analysis of this apo[a]-containing fraction, without reducing agent, detected only traces of a band corresponding to free apo[a] (data not shown). Most of the detected apo[a] corresponded to higher apparent molecular weight bands consistent with the expected mobility of apo[a]-S-S-apoB-100.

SDS-PAGE analysis of various ultracentrifugal fractions

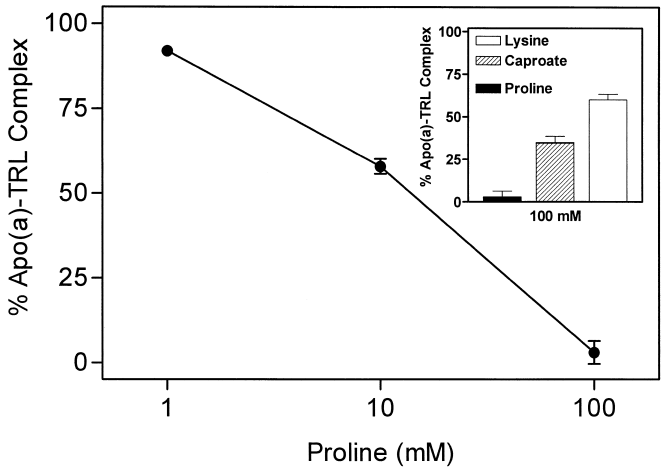


Fig. 2. Lp[a]-TRL complex stability expressed as a percentage of initial complex floating in the d < 1.006 supernatant after a single 18-h UCF in the presence of lysine, ε-aminocaproate, or proline.

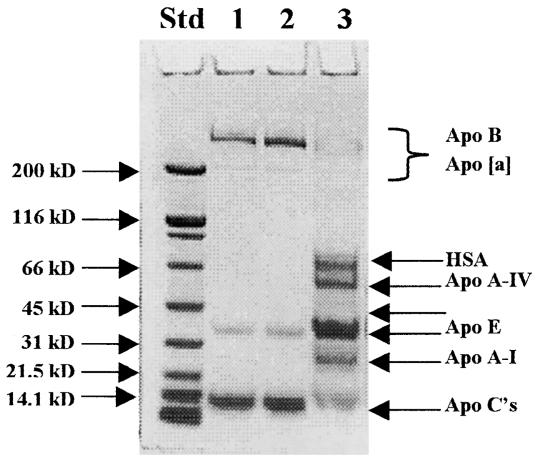


Fig. 3. SDS-PAGE detection of apolipoproteins present in TRL fractions of subject H6 isolated by UCF. Std, Protein molecular weight standards. Lane 1, TRL before proline treatment, isolated in the d 1.006 supernatant; lane 2, TRL after proline treatment, isolated in the d 1.006 supernatant; lane 3, apolipoproteins displaced from Lp[a]-TRL by proline treatment, isolated in the d 1.006 infranatant (concentrated about 10-fold).

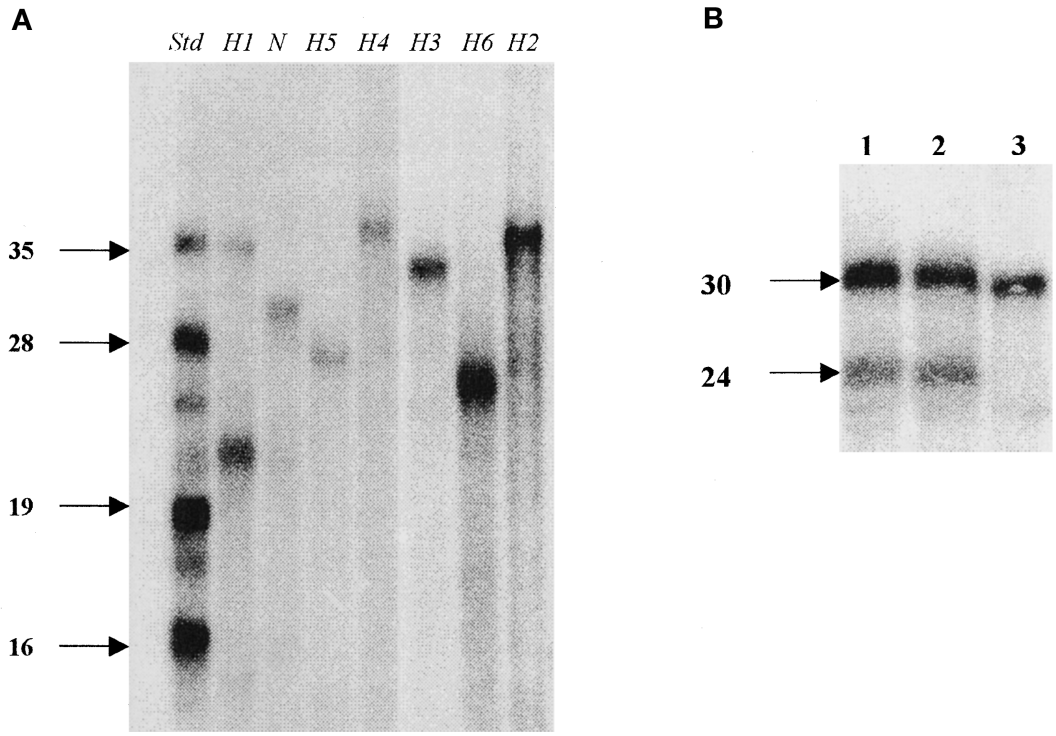


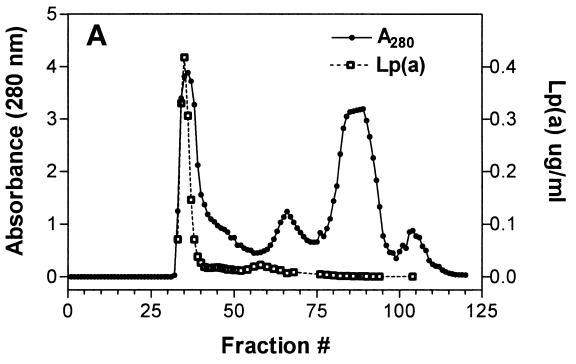
Fig. 4. Immunoblotting of apo[a] polymorphs present in Lp[a]-TRL complexes. A: Std, mixture of plasma samples containing apo[a] polymorphs of known kringle number; the other lanes represent Lp[a] dissociated by proline treatment of Lp[a]-TRL from hypertriglyceridemic subjects H1 to H6 and a normolipidemic control subject (N). B: Apo[a] polymorphs from normolipidemic subject N. Lane 1, unfractionated plasma; lane 2, d 1.006 infranatant; lane 3, d 1.006 supernatant (Lp[a]-TRL).

from hypertriglyceridemic subject H6 demonstrated a number of proteins, identified on the basis of their apparent molecular weight (Fig. 3). No differences were discerned between the SDS-PAGE patterns of TRL before and after proline treatment. ApoB-100, apoB-48, apoE, and the apoCs were present (Fig. 3, lanes 1 and 2). The proteins dissociated from the Lp[a]-TRL by UCF in the presence of 100 mM proline are shown in lane 3 (Fig. 3). The lightly stained bands near the top of the gel represent apo[a] and apoB-100. Albumin (66 kDa), apoE (33 kDa), apoA-I (28 kDa), and the low molecular weight apoCs were identified. A less prominent 53-kDa band was also observed.

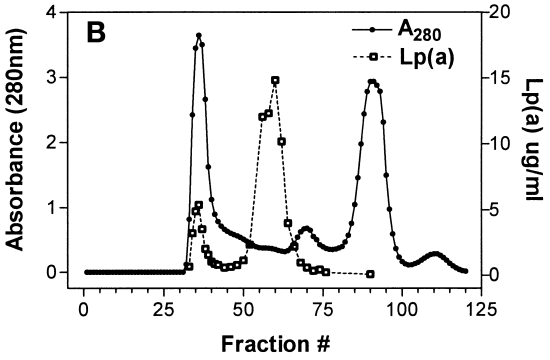
Size-exclusion chromatography. Whole plasma chromatographed over either BioGel A-5m or A-15m agarose resulted in similar elution profiles. The elution profile for plasma from patient H4 is shown in **Fig. 5A**. Four A_{280} peaks were observed: Lp[a]-TRL (peak 1) eluted in fractions 30–40; LDL (peak 2) eluted in fractions 60–75, and the plasma proteins eluted in fractions 75–95 (peak 3) and 100–110 (peak 4). HDL eluted in the region between LDL and lipid-free proteins. Lp[a] eluted immediately before LDL, overlapping slightly with its leading edge. The elution patterns were similar for all subjects. However, the relative abundance of peak 1, representing Lp[a]-TRL, was greater in the HTG subjects than in the normolipidemic control subject. Consistent with the ultracentrifugal result, most of the Lp[a] eluted with the TRL fraction. A small amount of Lp[a] eluted as free, uncomplexed lipoprotein. Essentially no apo[a] immunoreactivity eluted with plasma protein fraction 3 or 4, the expected position for free apo[a]. The greatest amount of Lp[a] eluted in fraction 35, where the Lp[a]-TRL complex would be expected to elute. When plasma was treated with 100 mM proline and then chromatographed, no dissociation of Lp[a] from the TRL was observed unless 100 mM proline was included in the eluting buffer, in which case about 67% of the Lp[a] was displaced from the complex (Fig. 5D). When the Lp[a]-TRL complex originally isolated by UCF was subsequently chromatographed in the absence of proline, the Lp[a] remained associated with the peak corresponding to the TRL, and did not elute as uncomplexed Lp[a] or free apo[a].

Reconstituted Lp[a]-TRL resembles native Lp[a]-TRL

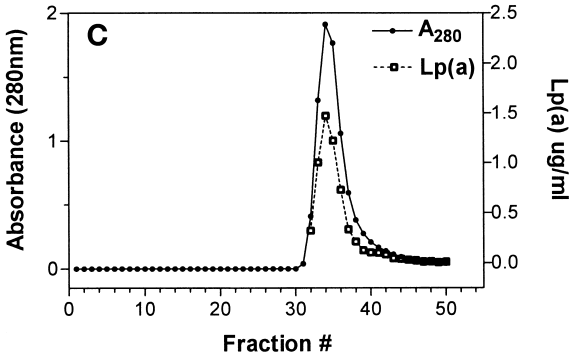
Size-exclusion chromatography. To determine whether the TRL particles could bind additional Lp[a], exogenous Lp[a] was added to either plasma or TRL as described in Experimental Procedures. When plasma treated in this way was subjected to size-exclusion chromatography (Fig. 5B), there was a >10-fold increase in the amount of Lp[a] bound to TRL, expressed either as an absolute amount (fraction 36) or as the apoLp[a]/apoB ratio. The complex formed by addition of exogenous Lp[a] to Pr-TRL eluted at a volume similar to that of native Lp[a]-TRL. An additional peak, representing uncomplexed Lp[a], eluted



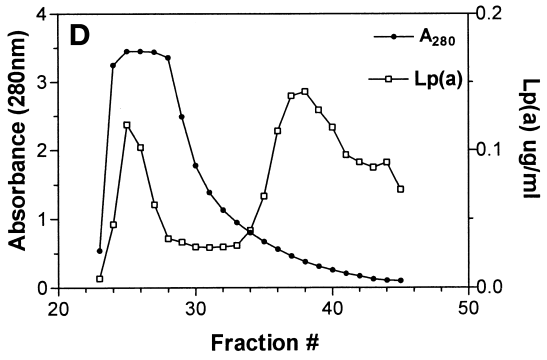
Fraction #	(ApoLp(a)/ApoB) x 10^3
34	430
35	96
36*	48
37	18
39	4
41	0.2



Fraction #	(ApoLp(a)/ApoB) x 10^3
34	6666
35	2439
36*	1408
37	808
38	476



Fraction #	(ApoLp(a)/ApoB) x 10^3
32	2000
33	4348
34*	1600
35	926
36	424



Fraction #	(ApoLp(a)/ApoB) x 10^3
23	7.8
25	10.6
26	4.6
30	1.6
34	1.9
38	6.4
43	19.0

Fig. 5. Size-exclusion chromatography over BioGel A15m of Lp[a]-TRL complex. A: Complex formed in vivo in HTG plasma. B: Complex formed in vitro by incubation of plasma with Lp[a]. C: Complex isolated from fractions 30–45 in experiment B and then rechromatographed. D: Complex partially dissociated by chromatography in the presence of 100 mM proline. Asterisk indicates the fraction with peak absorbance at 280 nm.

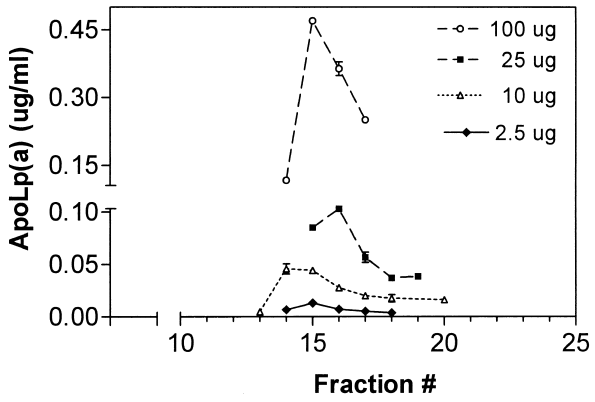


Fig. 6. Size-exclusion chromatography of Lp[a]-TRL complexes reconstituted in vitro by the addition of Lp[a] containing 2.5, 10, 25, or 100 μg of apoLp[a] to Pr-TRL containing 250 μg of apoB.

in fractions 50–70. No free apo[a] was detected. When fractions 30–45 (corresponding to the Lp[a]-TRL complex from this experiment) were combined, concentrated, and rechromatographed, essentially all of the Lp[a] remained associated with the TRL fraction (Fig. 5C).

In separate experiments, increasing amounts of Lp[a] were added to a constant amount of Pr-TRL. After incubation, the mixtures were fractionated by size-exclusion chromatography. The fractions were collected and analyzed for apoB and apoLp[a] immunoreactivity. Near linearity was obtained for binding over the range of exogenous Lp[a] tested (2.5–100 μg/ml) (**Fig. 6**).

Microplate ELISA measurement of Lp[a]-TRL complex formation. A 96-well microplate ELISA was developed as a more time-, labor-, and material-efficient method for testing the effect of a large number of variables on Lp[a]-TRL complex formation and dissociation. The optimal amount of Pr-TRL for coating the plates and the range of Lp[a] concentrations over which its binding to Pr-TRL was linear were determined. When the plates were coated at 100 μl/well with Pr-TRL at 5 μg of protein per ml, the Lp[a] binding to this substrate was linear over a 10- to 125-ng/ml concentration of Lp[a] protein. This assay was used to study the effects of a number of different amino acids and related analogs on Lp[a]-TRL complex formation. Appropriate aliquots of these test reagents were added to yield 200 mM final concentrations in the well, except where specifically prohibited by the natural solubility limit of the test substance. The pH was adjusted to the range of 7.0–7.5. The greatest inhibition (>90%) of complex formation was observed with proline (**Fig. 7**). Tranexamate and EACA produced inhibitions greater than 50%; lesser, but still significant, inhibition was produced by lysine and arginine. Surprisingly, cysteine and *N*-acetylcysteine produced nearly 2-fold increases in Lp[a] binding to TRL. Plasminogen, LDL, and Pr-TRL were tested for their ability to inhibit complex formation. When these were present at the time that Lp[a] was added to the plates, plasminogen did not inhibit binding and LDL only slightly diminished binding. Pr-TRL was the only effective competitor of Lp[a] binding to Pr-TRL.

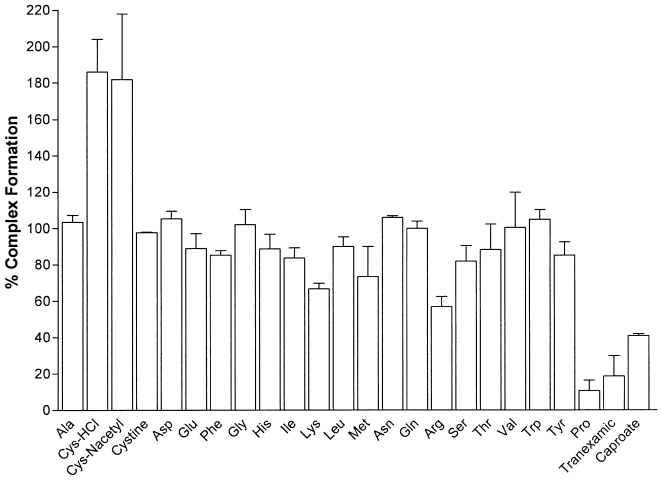


Fig. 7. Effect of different compounds on the formation of Lp[a]-TRL complexes measured by microplate ELISA. Proline, tranexamate, and ε-aminocaproate strongly inhibited complex formation, whereas cysteine-HCl and *N*-acetylcysteine considerably enhanced formation.

Binding of different apparent molecular weight polymorphs of apo[a] and recombinant apo[a] to Pr-TRL. It was desirable to determine whether apo[a] polymorphs of different molecular weights exhibit differential binding to TRL, and to determine whether lipid-free apo[a] is sufficient for complex formation. Accordingly, the binding properties of Lp[a] containing apo[a] with 20 or 30 kringles and of a recombinant apo[a] with 18 kringles were compared. The concentration-dependent binding of these high and low apparent molecular weight polymorphs to Pr-TRL was linear (**Fig. 8**). When 500 μg of Lp[a] protein was added to 5 ml of Pr-TRL, incubated, and then ultracentrifuged at d 1.006, the percentage of added Lp[a] associated with the TRL in the supernatant was determined by ELISA. About 10% of the Lp[a] containing the low apparent molecular weight apo[a] was bound compared with 22% of the

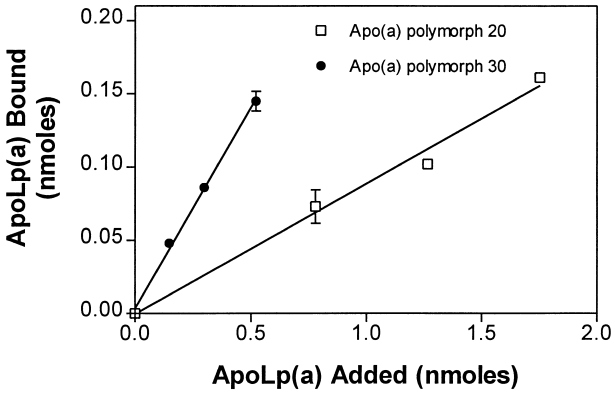


Fig. 8. Differential binding of exogenous Lp[a] containing a 20 kringle IV apo[a] polymorph (open squares) or 30 kringle IV apo[a] polymorph (solid circles) to TRL as assessed by the formation of an Lp[a]-TRL complex isolated by UCF in the d < 1.006 supernatant. More of the Lp[a] containing the 30 kringle IV apo[a] polymorph bound to TRL compared with the Lp[a] containing the 20 kringle IV apo[a] polymorph.

Lp[a] with high apparent molecular weight apo[a], and 75% of the recombinant apo[a] containing 18 kringles.

DISCUSSION

A number of investigators have reported the association of a small proportion of apo[a] with a TRL fraction after ingestion of a fatty meal or in the fasting plasma of individuals with HTG. However, the occurrence of apo[a] in the TRL fraction and its significance have remained controversial. Most investigators have found <10% of the total plasma apo[a] immunoreactivity distributed into a TRL fraction, raising doubts about whether it represents actual binding due to specific, molecular interactions between apo[a] and TRL or whether it indicates contamination of the TRL fraction with Lp[a]. The affinity of Lp[a] for apoB-containing lipoproteins, especially the TG-rich fractions, has been documented in a series of studies by one laboratory (11–13). However, these studies were based solely on in vitro experiments involving either affinity chromatography or solid-phase ligand blotting, with no corresponding in vivo evidence for a native apo[a]-TRL complex. Even when a native complex has been demonstrated, consideration of its structure has generally been limited to speculation rather than experimentation, due largely to the low natural abundance of Lp[a]-TRL in most normolipidemic subjects. Only a single study has described an isolated, native Lp[a]-TRL fraction and its partial characterization (19). That study concluded that the apo[a] associated with TRL was in the form of noncovalently bound Lp[a]. Under their study conditions of repetitive UCF or FPLC, the isolated Lp[a]-TRL complex was easily and almost completely dissociated. In the present study, we have determined the naturally occurring abundance of apo[a] in the TRL fraction, and isolated it in sufficient amounts to perform a more detailed characterization with respect to stability, physical structure, and binding properties. We have used size-exclusion chromatography and UCF to evaluate the possibility that a particular methodology used for isolation of Lp[a]-TRL might cause its artifactual formation or disintegration.

In the present study, the percentage of the total plasma Lp[a] that was associated with a TRL fraction ranged from 1% in a normolipidemic subject to 78% in a hypertriglyceridemic subject. The Lp[a]-TRL complex isolated from each subject was relatively resistant to dissociation by repeated UCF alone. Limited dissociation, occurring incrementally over three or four sequential ultracentrifugal cycles, was evidence of high affinity binding (Fig. 1). In contrast, a single UCF step in the presence of 100 mM proline resulted in nearly total dissociation of Lp[a]. Thus Lp[a] interacts with TRL by noncovalent forces, which are greatly and preferentially influenced by proline. Noncovalent interactions between specific kringles of apo[a] and apoB-100 have been demonstrated (38); these interactions can also be inhibited, in vitro, by lysine, lysine analogs, and proline (39, 40). In the present study, EACA and lysine were less effective than proline in dissociating Lp[a] from TRL, each in combination with UCF. The dissociation of Lp[a] from TRL was directly dependent on proline concentrations over the range 1–100 mM. The selectivity of proline as a dissociative agent also suggests some degree of steric specificity of binding, compatible with the model of a specific kringle-binding pocket of apo[a] interacting with a proline moiety of apoB in TRL. A number of reagents more hydrophobic than proline have negligible dissociative effects, evidence that proline does not act simply by disrupting hydrophobic interactions. Others have found that proline inhibits the binding of apo[a] to apoB-100 in vitro (41). In a study of inhibitors of the in vitro assembly of Lp[a], tranexamate and proline were also two of the most effective inhibitors, although their relative order of effectiveness was reversed from that found here for Lp[a] binding to TRL (42).

Size-exclusion chromatography of native plasma confirmed the existence of an endogenous Lp[a]-TRL complex by a method independent of UCF. Subsequent concentration and rechromatography of the Lp[a]-TRL peak fractions resulted in no dissociation of Lp[a] from the Lp[a]-TRL complex as measured by immunoassay (Fig. 5C). The Lp[a]-TRL complex was also resistant to dissociation by proline in combination with chromatography, unless 100 mM proline was included in the eluting buffer, in which case ~67% of the Lp[a] was displaced from Lp[a]-TRL. The chromatographic analyses also provided information about particle size distribution not readily available by UCF. Lp[a] was distributed disproportionately across the TRL peak fractions, as determined either by A_{280} or apoB immunoreactivity. This Lp[a] eluted with the larger TRL particles at the leading edge of the peak. These larger TRL particles may have a greater affinity and/or a larger number of binding sites for Lp[a], resulting in either a greater proportion of large particles with bound Lp[a] or more Lp[a] bound per particle. A small number of these particles represent chylomicrons, especially in the TRL fraction from hyperlipidemics. Assuming only 1 mol of apoB per particle, the apoLp[a]/apoB ratio for the column eluents suggests that the earliest eluting fractions contain particles with more than 1 mol of bound apo[a].

Because 100 mM proline treatment combined with UCF causes the quantitative dissociation of apo[a] and Lp[a] from TRL, this procedure was scaled up for preparation of these species. Analysis of dissociated material by density UCF revealed that 80% of the apo[a] immunoreactivity was in the form of Lp[a] lipoprotein, and 20% was in the form of lipid-free apo[a]. This lipid-free fraction could represent apo[a] alone, peptide fragments of apo[a] still exhibiting immunoreactivity, or an apo[a]-apoB disulfide-linked complex. SDS-agarose electrophoretic immunoblotting, without reductive treatment of the Lp[a]-TRL fraction, revealed molecular weight band(s) consistent with the presence of apo[a]-S-S-apoB, but only a trace corresponding to free apo[a]. These results support the hypothesis that most of the apo[a] bound to TRL is in the form of Lp[a] lipoprotein.

In addition to providing a method for the isolation of

Lp[a] from the Lp[a]-TRL complex, UCF with 100 mM proline also produced a TRL fraction (Pr-TRL), relatively free of Lp[a], which could be used for in vitro binding studies. SDS-PAGE of the proteins dissociated from Lp[a]-TRL by this treatment revealed that, in addition to apo[a] and apoB-100, other strong bands corresponding to albumin, apoE, apoA-I, and apoCs were present. In addition, a faint band corresponding to the apparent molecular weight of apoH was observed (Fig. 3). ApoH, also known as β_2-glycoprotein I, has been reported to have a high affinity for TRL particles (43). ApoH was found to bind Lp[a] by interaction with kringle IV type 2 of apo[a] (44). If apoH or any of the other dissociated proteins mediate the binding of apo[a] to TRL, a decrease in the capacity of the Pr-TRL for binding Lp[a] in the reconstitution experiments might be expected. Because Pr-TRL is devoid of apoH (Fig. 3) yet still avidly binds Lp[a] in both solid-phase and liquid-phase assays, it appears that apoH does not play an essential role in the formation or stabilization of the Lp[a]-TRL complex.

The preferential affinity of TRL for the higher apparent molecular weight polymorphs of apo[a] in Lp[a] has been reported (26). This observation was tested in the present study in HTG and normolipidemic subjects because two apo[a] polymorphs were detected in their plasma, and the apo[a] from both their plasma and TRL could be phenotyped. Three of these individuals clearly exhibited a distribution of apo[a] polymorphs in the TRL that differed from that in plasma and the d 1.006 infranatant (e.g., Fig. 4B). The difference in apo[a] band intensities was consistent with the preferential binding of the higher apparent molecular weight polymorph of apo[a] to the TRL.

The differential binding of high apparent molecular weight apo[a] polymorphs to TRL that was detected in these native isolates was also tested in vitro by comparing the binding of purified Lp[a] species, containing differing apparent molecular weight apo[a] polymorphs, to Pr-TRL. Similar to the previous in vivo results, Lp[a] containing the higher apparent molecular weight apo[a] polymorphs bound preferentially to the TRL. Type 2 repeats of kringle IV are responsible for the molecular weight polymorphism of apo[a]. An increased binding of the higher apparent molecular weight apo[a] suggests an involvement of this kringle type in the binding to TRL. However, recombinant apo[a], with an apparent molecular weight slightly lower than that of the naturally occurring apo[a] polymorphs contained in either of the Lp[a] species used in this experiment, bound the TRL in higher proportion. This result suggests that other kringle IV types, in addition to kringle IV type 2, may be partially masked in Lp[a] but freely accessible in the recombinant apo[a], and hence contribute to the binding. By this criterion, kringle IV types 5–9 are possible candidates (10). Our finding also establishes the sufficiency of free apo[a] for TRL binding in vitro, with no absolute requirement for the other Lp[a] protein constituents. In vivo, the apo[a] bound to TRL was predominantly in the form of the lipoprotein, Lp[a].

In vitro experiments tested the capacity of TRL to bind additional Lp[a]. When excess, exogenous Lp[a] was incubated with plasma from an HTG subject, and the mixture was then chromatographed, about 10 times more apo[a] was bound to the TRL fraction. This binding, like the native form, resulted in an Lp[a]-TRL complex that *i*) was stable to rechromatography, *ii*) was dissociated by UCF in 100 mM proline, *iii*) involved the native lipoprotein, *iv*) eluted by size-exclusion chromatography in nearly identical volumes, and *v*) exhibited a greater affinity for higher apparent molecular weight apo(a) polymorphs.

In our microplate ELISA, the binding of Lp[a] to TRL was inhibited by proline, tranexamate, and EACA in the same relative order of effectiveness as their dissociative action on the native Lp[a]-TRL complex. Results obtained by ELISA and ultracentrifugal methods were essentially equivalent. Plasminogen, which contains kringles highly homologous to those in apo[a], did not inhibit the binding of Lp[a] to Pr-TRL. In addition, no plasminogen was detected by SDS-PAGE among the proteins dissociated from Pr-TRL. Although Lp[a] has been shown to compete with plasminogen for binding to fibrin, no similar competition for binding to TRL can be attributed to plasminogen in the present study. The amino acid sequence differences between apo[a] and plasminogen are apparently crucial to TRL binding.

In contrast to the binding inhibitors described above, two sulfhydryl-containing reagents, cysteine and *N*-acetylcysteine, enhanced binding of Lp[a] to TRL. These reagents are capable of reducing disulfide bonds, action that may produce protein structural modifications that create a more energetically favorable interaction between Lp[a] and TRL (45). In a related study of the binding of Lp[a] to fibrin, it was observed that homocysteine, cysteine, glutathione, and *N*-acetylcysteine all increased the affinity of Lp[a] for fibrin (46).

If apoB is the TRL component responsible for apo[a] binding as suggested by this and other reports, the conformation of apoB on the TRL particle surface may be a critical determinant that confers the ability to bind Lp[a]. ApoB undergoes known size-related conformational changes that result in increased LDL receptor recognition as the apoB-containing TRL particles are reduced in size by metabolic processes (47). Although the affinity of Lp[a] for LDL, IDL, and VLDL has been demonstrated by solid-phase binding to nitrocellulose or by affinity chromatography, no native Lp[a]-LDL or Lp[a]-IDL complexes have been isolated as we have demonstrated for Lp[a]-VLDL (12, 48). Those in vitro studies found that as apoB particles became more TG rich, their affinity for apo[a] increased. The affinity of Lp[a] for the apoB-100 epitopes expressed at the surface of LDL or IDL is apparently so low in vivo that no stable complex is formed. Indeed, the larger particles of TRL, which elute at the leading edge of the TRL peak in size-exclusion chromatography, have the greater affinity for Lp[a] as reflected by their larger apoLp[a]/apoB ratio. On the basis of this finding, the series of particles increasing in size and triglyceride content from $VLDL_3$ to $VLDL_1$ to chylomicrons would be predicted to exhibit a corresponding increase in affinity for Lp[a]. In-

deed, we observed that chylomicrons bind more Lp[a] per particle than VLDL. Because chylomicrons contain apoB-48 but not apoB-100, this suggests that the binding epitopes are localized to the amino-terminal half of apoB-100.

The documented inverse correlation between plasma Lp[a] and TG levels and the observed decrease in Lp[a] with increased TRL implies a metabolic interrelationship. If Lp[a]-TRL is more rapidly removed than Lp[a] alone from the plasma compartment, then this mechanism could explain the observed reciprocal changes (49). However, plasma Lp[a] lowering in hypertriglyceridemics by this mechanism might not necessarily lead to a reduced risk for atherosclerosis in these patients, especially if the Lp[a]-TRL complex is avidly taken up by the arterial wall.

Argraves et al. (50) have demonstrated that fibroblasts expressing the human VLDL receptor can bind, endocytose, and degrade Lp[a], a process mediated by apo[a]. Because macrophages in human atherosclerotic lesions also express the VLDL receptor, these two cell receptor systems could provide effective pathways for Lp[a] entry into and accumulation within the arterial wall. These mechanisms could explain the high abundance of Lp[a] and/or apo[a] in stenosed coronary bypass vein grafts (51), aortic aneurysm tissue (52), and carotid endarterectomy specimens (J. D. Morrisett and S. Romboli, unpublished observations, 2001). Because the VLDL receptor accommodates both the apo[a] moiety of Lp[a] and the apoB-100 moiety of VLDL, its affinity for the Lp[a]-TRL complex may be substantially greater than for either of the individual ligands alone. Hence, hypertriglyceridemia may lead to increased arterial uptake of Lp[a] and exacerbation of atherosclerosis.

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REFERENCES

1. Berg, K. 1963. A new serum type system in man: the Lp-system. *Acta Pathol. Microbiol. Scand.* **59(Suppl.):** 369–382.
2. Dahlen, G., M. H. Frick, K. Berg, M. Valle, and M. Wiljasalo. 1975. Further studies of Lp(a) lipoprotein/pre-beta1-lipoprotein in patients with coronary heart disease. *Clin. Genet.* **8:** 183–189.
3. Kostner, G. M., P. Avogaro, G. Cazzolato, E. Marth, G. Bittolo-Bon, and G. B. Qunici. 1981. Lipoprotein Lp(a) and the risk for myocardial infarction. *Atherosclerosis.* **38:** 51–61.
4. Gaubatz, J. W., C. Heideman, A. M. Gotto, Jr., J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein(a). *J. Biol. Chem.* **258:** 4582–4589.
5. Uttermann, G., and W. Weber. 1983. Protein composition of Lp(a) lipoprotein from human plasma. *FEBS Lett.* **154:** 357–361.
6. McLean, J. W., J. E. Tomlinson, W-J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. *Nature.* **330:** 132–137.
7. Howard, G. C., and S. V. Pizzo. 1993. Lipoprotein(a) and its role in atherothrombotic disease. *Lab. Invest.* **69:** 373–386.
8. Scanu, A. M. 1993. Structural basis for the presumptive atherothrombogenic action of lipoprotein(a). Facts and speculations. *Biochem. Pharm.* **46:** 1675–1680.
9. Ernst, A., M. Helmhold, C. Brunner, A. Petho-Schramm, V. W. Armstrong, and H-J. Muller. 1996. Identification of two functionally distinct lysine-binding sites in kringle 37 and in kringles 32–36 of human apolipoprotein(a). *J. Biol. Chem.* **270:** 6227–6234.
10. Gabel, B. R., L. F. May, S. M. Marcovina, and M. L. Koschinsky. 1996. Lipoprotein(a) assembly quantitative assessment of the role of apo(a) kringle IV types 2–10 in particle formation. *Arterioscler. Thromb. Vasc. Biol.* **16:** 1559–1567.
11. Trieu, V. N., T. F. Zioncheck, R. M. Lawn, and W. J. McConathy. 1991. Interaction of apolipoprotein(a) with apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **266:** 5480–5485.
12. Trieu, V. N., and W. J. McConathy. 1990. Lipoprotein(a) binding to other apolipoprotein B containing lipoproteins. *Biochemistry.* **29:** 5919–5924.
13. Trieu, V. N., and W. J. McConathy. 1995. The binding of animal low-density lipoproteins to human apolipoprotein(a). *Biochem. J.* **309:** 899–904.
14. Bersot, T. P., T. L. Innerarity, R. E. Pitas, S. C. Rall, Jr., K. H. Weisgraber, and R. W. Mahley. 1986. Fat feeding in humans induces lipoproteins of density less than 1.006 that are enriched in apolipoprotein(a) and that cause lipid accumulation in macrophages. *J. Clin. Invest.* **77:** 622–630.
15. Cohn, J. S., J. R. McNamara, S. D. Cohn, J. M. Ordovas, and E. J. Schaefer. 1988. Plasma apolipoprotein changes in the triglyceride-rich lipoprotein fraction of human subjects fed a fat-rich meal. *J. Lipid Res.* **29:** 925–936.
16. Cohn, J. S., C. W. K. Lam, D. R. Sullivan, and W. J. Hensley. 1991. Plasma lipoprotein distribution of apolipoprotein(a) in the fed and fasted states. *Atherosclerosis.* **90:** 59–66.
17. Sandholzer, C., G. Feussner, J. Brunzell, and G. Utermann. 1992. Distribution of apolipoprotein(a) in the plasma from patients with lipoprotein lipase deficiency and with type III hyperlipoproteinemia. *J. Clin. Invest.* **90:** 1958–1965.
18. Selinger, E., J. Dallongeville, and J. Davignon. 1993. Apolipoprotein(a) is present in the triglyceride-rich fraction in type IV hypertriglyceridemia. *Clin. Biochem.* **26:** 59–64.
19. Marcoux, C., S. Lussier-Cacan, J. Davignon, and J. S. Cohn. 1997. Association of Lp(a) rather than integrally-bound apo(a) with triglyceride-rich lipoproteins of human subjects. *Biochim. Biophys. Acta.* **1346:** 261–274.
20. Bartens, W., D. J. Rader, G. Talley, and H. B. Brewer, Jr. 1994. Decreased plasma levels of lipoprotein(a) in patients with hypertriglyceridemia. *Atherosclerosis.* **108:** 149–157.
21. Walek, T., A. Von Eckardstein, H. Schulte, and G. Assmann. 1995. Effect of hypertriglyceridemia on lipoprotein(a) serum concentrations. *Eur. J. Clin. Invest.* **25:** 311–316.
22. Werba, J. P., O. Safa, G. Gianfranceschi, S. Michelagnoli, C. R. Sirtori, and G. Franceschini. 1993. Plasma triglycerides and lipoprotein(a): inverse relationship in a hyperlipidemic Italian population. *Atherosclerosis.* **101:** 203–211.
23. Hiraga, T., M. Okubo, T. Kobayashi, K. Nakanashi, T. Sugimoto, and T. Murase. 1993. Serum lipoprotein(a) levels differ in different phenotypes of primary hyperlipoproteinemia. *Metabolism.* **42:** 1327–1330.
24. Rosseneu, M., C. Labeur, N. Vinaimont, J. P. Deslypere, and E. Matthys. 1991. Plasma Lp[a] patterns after triglyceride infusion. *Atheroscler. Rev.* **22:** 137–143.
25. Kostner, G. M. 1991. Interrelation of Lp(a) with trigycerides. *Atheroscler. Rev.* **22:** 131–135.
26. Pfaffinger, D., J. Schuelke, C. Kim, G. M. Fless, and A. M. Scanu. 1991. Relationship between apo[a] isoforms and Lp[a] density in subjects with different apo[a] phenotype: a study before and after a fatty meal. *J. Lipid Res.* **32:** 679–683.
27. Morrisett, J. D., M. L. Nava, M. Reilly, and M. C. Snabes. and R. L Young. 1997. *In* Hormonal, Metabolic, and Cellular Influences on Cardiovascular Disease in Women. T. M. Forte, editor. Futura Publishing, Armonk, NY. 289–302.
28. Havel, R. J., and J. P. Kane. 1989. Metabolic Basis of Inherited Disease. C. R. Schriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, NY. 1129–1138.
29. Gaubatz, J. W., K. I. Ghanem, J. Guevara, Jr., M. L. Nava, W. Patsch, and J. D. Morrisett. 1990. Polymorphic forms of human apolipoprotein[a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein [a]. *J. Lipid Res.* **31:** 603–613.
30. Seidel, J., E. Hagele, J. Ziegenhorn, and A. W. Wahlenfeld. 1983. Reagent for the enzymatic determination of serum total cholesterol with improved lipolytic efficiency. *Clin. Chem.* **29:** 1075–1080.
31. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran sulfate-

Mg^{2+} precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin. Chem.* **28:** 1379–1388.
32. Nagele, U., E. Hagele, G. Sauer, E. Wiedermann, P. Lehmann, A. W. Wahlefeld, and W. Gruber. 1984. Reagent for the enzymatic determination of serum total triglycerides with improved lipolytic efficiency. *J. Clin. Chem. Clin. Biochem.* **22:** 165–174.
33. Friedewald, W. T., R. I. Levy, and D. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.
34. Li, K. M., D. E. Wilcken, and N. P. Dudman. 1994. Effect of serum lipoprotein(a) on estimation of low-density lipoprotein cholesterol by the Friedewald formula. *Clin. Chem.* **40:** 571–573.
35. Gaubatz, J. W., G. L. Cushing, and J. D. Morrisett. 1986. Quantitation, isolation and characterization of human lipoprotein (a). *Methods Enzymol.* **129:** 167–185.
36. Hoogeveen, R. C., J. K. Gambhir, D. S. Gambhir, K. T. Kimball, K. Ghazzaly, J. W. Gaubatz, M. Vadunathan, R. S. Rao, M. Koschinsky, and J. D. Morrisett. 2001. Evaluation of Lp[a] and other independent risk factors for CHD in Asian Indians and their USA counterparts. *J. Lipid Res.* **42:** 631–638.
37. Marcovina, S. M., Z. H. Zhang, V. P. Gaur, and J. J. Albers. 1993. Identification of 34 apolipoprotein(a) isoforms: differential expression of apolipoprotein(a) alleles between american blacks and whites. *Biochem. Biophys. Res. Commun.* **191:** 1192–1196.
38. Gabel, B. R., and M. L. Koschinsky. 1998. Sequences within apolipoprotein(a) kringle IV types 6–8 bind directly to low density lipoprotein and mediate noncovalent association of apo(a) with apoB100. *Biochemistry.* **21:** 7892–7898.
39. Gabel, B. R., R. S. McLeod, Z. Yao, and M. L. Koschinsky. 1998. Sequences within the amino terminus of apoB100 mediate its noncovalent association with apo(a). *Arterioscler. Thromb. Vasc. Biol.* **18:** 1738–1744.
40. Huby, T., C. Doucet, H. Dieplinger, J. Chapman, and J. Thillet. 1994. Structural domains of apolipoprotein(a) and its interaction with apolipoprotein B-100 in the lipoprotein(a) particle. *Biochemistry.* **33:** 3335–3341.
41. Trieu, V. N., T. F. Zioncheck, R. M. Lawn, and W. J. McConathy. 1991. Interaction of apolipoprotein(a) with apolipoprotein B-containing lipoprotins. *J. Biol. Chem.* **266:** 5480–5485.
42. Frank, S., S. Durovic, K. Kostner, and G. M. Kostner. 1995. Inhibitors for the in vitro assembly of Lp(a). *Arterioscler. Thromb. Vasc. Biol.* **15:** 1774–1780.
43. Polz, E., and G. M. Kostner. 1979. The binding of β2-glycoprotein-I to human serum lipoproteins. *FEBS Lett.* **102:** 183–186.
44. Kochl, S., F. Fresser, E. Lobentanz, G. Baier, and G. Utermann. 1997. Novel interaction of apolipoprotein(a) with β2 glycoprotein I mediated by the kringle IV domain. *Blood.* **90:** 1482–1489.
45. Gavish, D., and J. L. Breslow. 1991. Lipoprotein(a) reduction by *N*-acetyl cysteine. *Lancet.* **337:** 203–204.
46. Harpel, P. C., V. T. Chang, and W. Borth. 1992. Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proc. Natl. Acad. Sci. USA.* **89:** 10193–10197.
47. Gianturco, S. H., A. M. Gotto, Jr., S-L. Hwang, J. B. Karlin, A. H. Y. Lin, S. C. Prasad, and W. A. Bradley. 1983. Apolipoprotein E mediates uptake of S_f 100–400 hyperglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts. *J. Biol. Chem.* **258:** 4526–4533.
48. Ye, S. Q., V. N. Trieu, D. L. Stiers, and W. J. McConathy. 1988. Interactions of low density lipoprotein$_2$ and other apoB containing lipoproteins with lipoprotein(a). *J. Biol. Chem.* **283:** 6337–6343.
49. Marcovina, S. M., and J. D. Morrisett. 1995. Structure and metabolism of lipoprotein (a). *Curr. Opin. Lipidol.* **6:** 136–145.
50. Argraves, K. M., K. F. Kozarsky, J. T. Fallon, P. C. Harpel, and D. K. Strickland. 1997. The atherogenic lipoprotein Lp(a) is internalized and degraded in a process mediated by the VLDL receptor. *J. Clin. Invest.* **100:** 2170–2181.
51. Cushing, G. L., J. W. Gaubatz, M. L. Nava, B. J. Burdick, T. M. A. Bocan, J. R. Guyton, D. Weilbaecher, M. E. DeBakey, G. W. Lawrie, and J. D. Morrisett. 1989. Quantitation and localization of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at reoperation. *Arteriosclerosis.* **9:** 593–603.
52. Papagrigorakis, E., D. Iliopoulos, P. J. Asimacopoulos, H. J. Safi, D. Weilbaecher, K. G. Ghazzaly, M. L. Nava, J. W. Gaubatz, and J. D. Morrisett. 1997. Lipoprotein(a) in plasma, arterial wall, and thrombus from patients with aortic aneurysm. *Clin. Genet.* **52:** 262–271.