Relationship between apo[a] isoforms and Lp[a] density in subjects with different apo[a] phenotype: a study before and after a fatty meal

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Abstract Plasma Lp[a] levels and apo[a] isoform distribution among lipoproteins isolated by density gradient ultracentrifugation were studied in subjects with one-band or two-band apo[a] phenotypes as assessed by gradient gel electrophoresis before and after an oral fat load. There were no significant differences in the ultracentrifugal profile between fasting plasma and postprandial plasma that was freed of triglyceride-rich particles (TRP). One-band phenotypes exhibited a single symmetrical peak in the density gradient, whereas two-band phenotypes exhibited a multi-modal distribution. Low molecular weight apo[a] isoforms were preferentially associated with low density Lp[a] whereas high molecular weight apo[a] isoforms were found with high density Lp[a] particles. Feeding a high fat meal caused no significant increase in the total plasma level of Lp[a]. However, the isolated TRP contained the apoB-100-apo[a] complex in a quantity that represented only about 1% of its total amount in the fasting plasma. In all cases the apo[a] isoforms present in TRP were also present in the fasting plasma; however, in the two-band apo[a] phenotypes the ratio of the slow over the fast migrating band was in all cases about eightfold higher in TRP than in the fasting plasma. These observations indicate that postprandially a small percentage of apoB-100-apo[a] associates with TRP and suggest that this complex may derive from de novo synthesis rather than from a pre-existing Lp[a] plasma pool. The liver would be the source of the complex due to the presence in the latter of apoB-100. — **Pfaffinger, D., J. Schuelke, C. Kim, G. M. Fless, and A. M. Scanu.** 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.* 1991. **32:** 679–683.

Supplementary key words lipoprotein[a] • apo[a] isoforms • apo[a] distribution • postprandial Lp[a]

The density heterogeneity of Lp[a] was first reported by Harvey and Schultz (1) and later examined in detail by Fless, Rolih, and Scanu (2). The latter authors recognized that Lp[a] heterogeneity can occur both in the same individual and among individuals. At least 11 apo[a] size isoforms are identifiable in the plasma by electrophoretic means (3) although any given subject exhibits either one or two apo[a] isoforms that are coded by the apo[a] gene (4). These isoforms are related to Lp[a] buoyant density (4, 5). In this study we have examined the ultracentrifugal distribution of apo[a] isoforms in subjects with single-band and double-band apo[a] phenotypes, both in the fasting plasma and in the postprandial phase after a single fat meal. These studies were prompted by our recent observations that the apoB-100-apo[a] complex has an affinity for TG-rich particles (TRP) (6) and by the hypothesis that there might be two pools of apoB-100-apo[a], one affiliated with a cholesteryl ester-rich core, CE-Lp[a], and one with a TG-rich core, TG-Lp[a]. The results obtained in this work establish that the apo[a] phenotype in the fasting plasma is also present in postprandial TRP, although in the two-band phenotype the mass percentage of the two apo[a] isoforms in TRP differs markedly from that in fasting plasma. A preliminary report of these findings has appeared (7).

MATERIALS AND METHODS

Human subjects

Five subjects were studied: four females and one male, ages 25–50 years. All were in good health and receiving no medication. All had plasma levels of Lp[a] protein that were above the normal range established in this laboratory (0.02–7.0 mg/dl). Two subjects, 1 and 2, were hypercholesterolemic; the others had normal cholesterol levels. In terms of apo[a], three had a single-band and two had a two-band phenotype (**Table 1**). The study protocol was approved by the Institutional Review Board, and informed consent was obtained from the subjects.

Abbreviations: TRP, triglyceride-rich particles; TG, triglyceride; CE, cholesteryl ester; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoprotein

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TABLE 1. Summary of baseline values of subjects studied

Subject	Sex	Age	Surface Area	Plasma				
				Total Chol.	HDL Chol.	Triglycerides	Lp[a] Protein	Apo[a] Phenotype[a]
		yr	m^2			*mg/dl*		
1	F	50	1.86	285	45	78	28.9	1.15/1.15
2	F	41	1.61	316	79	138	46.1	0.79/1.15
3	M	36	1.67	182	48	89	23.0	0.79/1.15
4	F	40	1.85	201	53	99	14.9	1.15/1.15
5	F	25	1.75	196	39	57	16.3	1.15/1.15

[a] R_f values relative to apoB-100.

Oral fat load

In a typical protocol, each subject, after 14 h fasting, reported for study early in the morning at which time 20 ml blood was drawn from the arm vein in order to establish baseline values. Each subject was then given a fat meal (60 g/m^2) which was consumed in no longer than 20 min. The composition of the fat meal is in **Table 2**. Most of the fats were saturated (64%). Blood was drawn at 2, 3, 4, and 5 h after the beginning of the fat meal. During this time the subject was only allowed to drink water, no more than 4 oz/h.

Conditions of blood collection to obtain plasma

At each time interval the blood was collected into 10-ml tubes immersed in ice and containing, at a final concentration, 0.12% Na_2EDTA and 0.01% sodium azide in addition to gentamycin sulfate (0.8 mg/10 ml blood) and kallikrein inhibitor (400 units/10 ml blood). The blood was gently mixed and centrifuged at 3,000 rpm, 4°C for 15 min. To each 10 ml of plasma, the following was added: 10 μl benzamidine (1 M), 10 μl PMSF (0.2 M), 10 μl BHT (50 mM). These preservatives were found to be essential to prevent Lp[a] degradation.

Isolation of TRP

The plasma was centrifuged in a Beckman Model L-80 ultracentrifuge in an SW40 rotor at 29,000 rpm, 15°C for 30 min. The top fractions, containing TRP, were collected and washed twice with 0.15 M NaCl, pH 7.4, under the same ultracentrifugal conditions. Aliquots were then taken for Lp[a] protein determination; the remainder was lyophilized and stored at −80°C until use.

Single-step density ultracentrifugation

Fasting plasma or postprandial chylomicron-free plasma specimens were separated by isopycnic density gradient ultracentrifugation as previously described (8). Fractions, 0.4 ml each, were collected, dialyzed against 0.15 M NaCl, pH 7.4, overnight at 4°C and then either used immediately or stored at −80°C.

Delipidation of TRP

The delipidation was carried out by extraction with ethyl ether at −20°C, using 10 ml of solvent per mg of lyophilized material. The extraction was repeated twice under the same conditions. The end product was dried under nitrogen gas and stored at −80°C until use.

Apo[a] isoform analyses in plasma and isolated lipoproteins

The plasma was brought to d 1.21 g/ml with solid NaBr and centrifuged for 20 h in a Beckman L8-80 ultracentrifuge at 40,000 rpm (20 h, 15°C) using a 50.3 Ti rotor. In each case each top fraction containing all lipoproteins was dialyzed against saline (0.15 M NaCl, 0.02% Na_2 EDTA, pH 7.4) at 4°C and either promptly subjected to electrophoresis (see below) or stored at −80°C until use. In the case of TRP, the analyses were performed on ether-delipidated particles that were dissolved in the running buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M Na_2 EDTA, 0.2% SDS, pH 7.4) prior to analysis. Analyses were also performed on d > 1.21 g/ml lipoproteins and on 0.4-ml aliquots obtained from plasma samples separated by density gradient ultracentrifugation. The electrophoretic separation (150 V, 2 h, 15°C) was carried out on 2–16% polyacrylamide gradient gel (Pharmacia Fine Chemicals) in the presence of 0.2% SDS as previously described (7). The gel was pre-focused for 5–10 min at 70 V. After the dye entered the gel, the voltage was increased to 150 V. After the dye was no longer visible, the elec-

TABLE 2. Composition of the single fat meal

Protein	9%	
Carbohydrate	27%	
Fat (60 g/m^2)	64%	saturated fatty acids 64% monounsaturated fatty acids 29% polyunsaturated fatty acids 7%
P/S ratio	0.1	
Kcal/m^2	843	
Cholesterol (mg/1000 kcal)	227	

trophoresis was continued for an additional 15 min. For scanning purposes, the Western blots were rehydrated with water and placed into the laser densitometer (LKB Vetrascan, Ultroscan XL, Laser Densitometer, Bromma, Sweden).

Western blotting

Western blot analyses were carried out in a Hoefer Model TE 22 apparatus (Hoefer Scientific Instruments) for 18 h. Immunoblotting was conducted using a specific human apo[a] antibody raised in the rabbit as the primary antibody and a goat anti-rabbit horseradish peroxidase as the secondary antibody.

Quantification of Lp[a]

This was carried out by ELISA according to the method described by Fless, Snyder, and Scanu (9).

Other analyses

The determinations of total cholesterol, triglycerides, and HDL cholesterol were conducted in an EKTACHEM DT 60 analyzer (Eastman Kodak Co).

RESULTS

In all subjects, the significant hypertriglyceridemia that followed the ingestion of the fat meal was attended by a modest elevation of the total plasma Lp[a]. From the analysis of the fasting specimens, the postprandial specimen

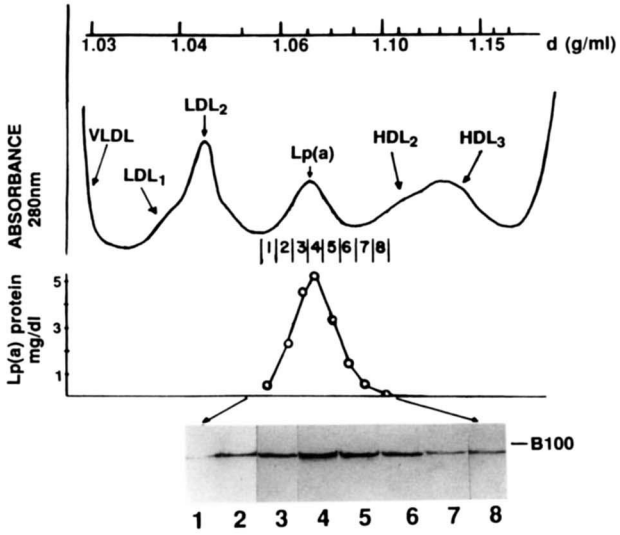

Fig. 1. Density gradient ultracentrifugal profile of fasting (single-band phenotype) indicating the major lipoprotein peaks. At the end of ultracentrifugation the fractions numbered 1–8 were collected, dialyzed, and aliquots were examined for their apoB-100-apo[a] content by ELISA. They were also analyzed by SDS-polyacrylamide density gradient (2–16%) electrophoresis, and transferred onto cellulose film; the bands were identified with a polyclonal antibody raised against human apo[a]. The position of apo[a] having apoB-100 mobility is shown.

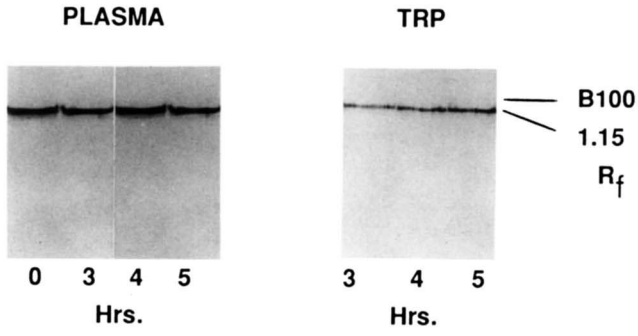

Fig. 2. Western blots of fasting plasma of single apo[a] band phenotype (left panel) and TRP isolated from the plasma of the same subject by ultracentrifugation at d < 1.006 g/ml 3, 4, and 5 h after a fat meal. The antibody was specific for apo[a].

at peak hypertriglyceridemia, and of the same specimen after removal of TRP, we estimated that the amount of apoB-100-apo[a] affiliated with TRP was 1–2% of the total plasma Lp[a]. The isolated apoB-100-apo[a] TRP was influenced by, but was not proportional to, either the mass of TRP triglycerides in the postprandial plasma or with the preprandial levels of Lp[a]. That the postprandial changes of apoB-100-apo[a] were confined to the TG-rich particles was supported by the identical single-spin profiles of fasting plasma and TRP-free postprandial plasma. The most important aspect of this study was the distribution of the apo[a] isoforms among the various ultracentrifugal lipoprotein fractions. As exemplified by subject 1 (**Fig. 1**), the TRP-free plasma showed a single symmetrical ultracentrifugal peak both by absorbance at 280 nm and apo[a] ELISA. All fractions within the peak had a single apo[a] band moving electrophoretically in a position faster than apoB-100. The same pattern was observed in all of the TRP of the plasma taken at time intervals after the fat meal (**Fig. 2**).

In the case of subject 2, exemplifying the double apo[a] band phenotype in the whole plasma, the single-spin analyses of the TRP-free plasma at 280 nm showed a heterogeneous pattern only in the Lp[a] zone between density 1.048 g/ml and 1.072 g/ml. A major and a minor band were observed by apo[a] ELISA. There was a segregation between apo[a] band pattern and buoyant density; namely the apo[a] of a low molecular weight was associated with lower hydrated density Lp[a], whereas the high molecular weight apo[a] band was in the higher density region of the gradient in the area of HDL_2 (**Fig. 3**). An intermediate band containing both apo[a] isoforms was also detected. Both apo[a] bands were found to be associated with the TRP fractions (**Fig. 4**). However, the slow:fast band ratio was 2.77 in contrast to that of fasting plasma: 0.35, i.e., about eightfold difference. The same observation was made in subject 3, also a double band phenotype.

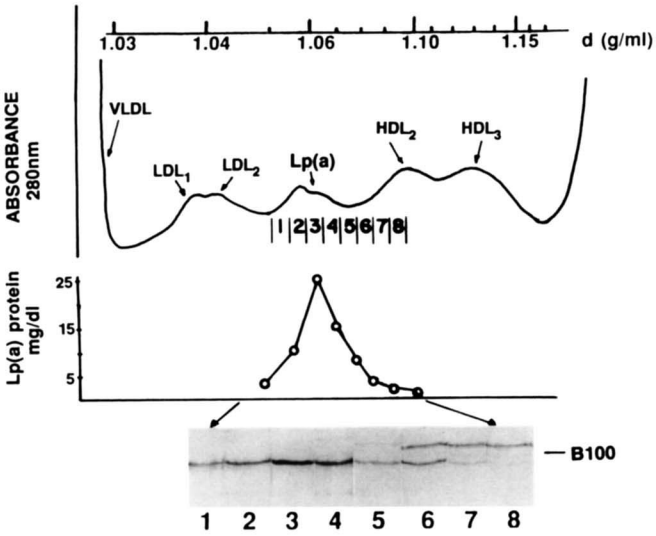

Fig. 3. Density gradient ultracentrifugation profile of fasting plasma of a double-band phenotype. For details see legend of Fig. 1.

DISCUSSION

The results of the present studies show that, in the fasting state, the plasma of subjects with one-band apo[a] phenotype exhibited a single symmetrical peak by isopycnic density gradient ultracentrifugation. The position of the peak in the gradient was between LDL and HDL in the density range between 1.050 and 1.082 g/ml. Minor amounts of apo[a] were also detected in low and high density portions of the gradient indicating that the single-step ultracentrifugal procedure used did not insure a clear separation of Lp[a] from the other lipoprotein components. In contrast, the two-band apo[a] phenotypes exhibited a heterogeneous Lp[a] density distribution, the faster of the two apo[a] isoforms being confined at the Lp[a] fractions of the lowest hydrated density, and the slow isoforms to the heavier fractions of the gradient. It was only in the middle of the gradient that we observed a component containing both apo[a] polypmorphs. As shown by us previously (2) this does not represent a discrete Lp[a] species, rather two overlapping species.

The fat load did not significantly affect the total plasma levels of Lp[a] nor its distribution in the ultracentrifugal gradient based on the comparison of the TRP-free postprandial plasma and fasting plasma. Bersot et al. (10) were the first to report on the presence of apo[a] in postprandial TRP. The subjects studied were not phenotyped with respect with apo[a]. Thus, the significance of the higher molecular weight apo[a] isoforms observed in that study is difficult to assess. In our current work the apo[a] phenotype (single or double band) in TRP was the same as that of the fasting plasma. However, in the two-band apo[a] phenotypes the TRP exhibited a slow:fast band ratio that was about eightfold higher than that of the fasting plasma. We wish also to stress that, based on the electrophoretic data and on the protocol of our Lp[a] analyses (9), the apoB-100-apo[a] complex and not apo[a] was bound to TRP, a conclusion that is also in keeping with the lack of lipophilic properties of apo[a] (6). The quantitative difference in apo[a] isoform ratio suggests that the apo[a] in TRP is probably a product of de novo synthesis and not derived by plasma transfer. This hypothesis is supported by our observation that Lp[a] particles bind poorly to TRP (6) and that in the current study there was a lack of correlation between basal plasma levels of Lp[a] and immunodetectable apo[a] in TRP. If we assume a de novo synthesis of apoB-100-apo[a] in TRP, we must also assume that the liver is the site of postprandial TG-Lp[a] in response to an increased influx of chylomicron remnants since the intestine produces neither apoB-100 nor apo[a] (4, 5). Moreover, our recent studies have shown that the postprandial peak of Lp[a] does not coincide with that of intestinal-derived chylomicrons as assessed by the retinyl palmitate curve (11). Whatever the mechanism, it is important to stress that the amount of apoB-100-apo[a] that we found in TRP was rather modest as compared to the mass of apoB-100-apo[a] in fasting plasma. In this context, we must consider the possibility that the apoB-100-apo[a] complex in TRP may follow the fate of the chylomicron remnants and thus be rapidly taken up from the circulation by the remnant receptor pathway. Whether any of the apoB-100-apo[a] affiliated to TRP escapes this route of degradation and contributes even in a minor way to the fasting plasma pool of apoB-100-apo[a] cannot be answered by the current work, although the studies by Kostner et al. (12) do not support a VLDL-like precursor for Lp[a].

From the structural standpoint, the current findings confirm and extend the previous observations by Fless et al. (2), pointing at a dependence of size of apo[a] on its affiliation to either low or high density cholesteryl ester-rich Lp[a] species. The mechanism(s) underlying this pref-

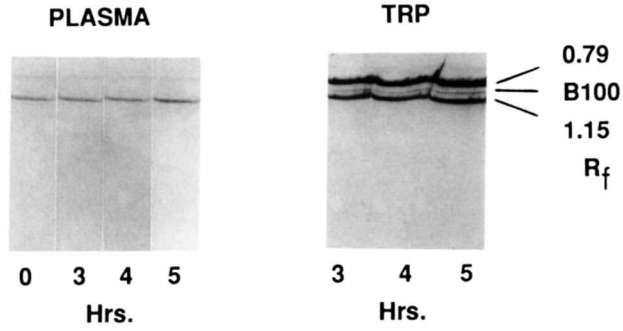

Fig. 4. Western blots of fasting plasma of double-band apo[a] phenotype (left panel) and TRP isolated from the plasma of the same subject by ultracentrifugation at d 1.006 g/ml 3, 4, and 5 h after a fat meal. The antibody was specific for apo[a].

erential affiliation is unclear. This may be simply due to an actual reduction of number of lipid-binding sites in apoB-100 induced by apo[a] or to segmental conformational changes as suggested by the studies of Zawadzki et al. (13).

From the biological standpoint our studies may have pathobiological importance in that TG-Lp[a] particles of post-prandial origin might exhibit athero-thrombotic potential. High plasma triglycerides have been associated with an increased prevalence of thrombotic events (14, 15) and apo[a] has been shown to compete for plasminogen in the fibrinolytic system (for reviews see references 5 and 16).

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