Apoprotein B of lipoprotein(a) of human plasma

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Lipoprotein(a) was purified by agarose-gel chromatography from human plasma from which lipoproteins of $S_f > 0$ had been removed either by sequential or by density-gradient ultracentrifugation. After delipidation, the apoprotein B of this lipoprotein was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It could not be distinguished from the apoprotein B of low-density lipoproteins (ρ 1.019–1.063 g/ml). A significant increase in the concentration of apoprotein B in plasma from which the $S_f > 0$ lipoproteins had been removed was observed in six subjects 4 h after a fatty meal.

ApoB is the major protein moiety of plasma lipoproteins of $S_f > 0$. (S_f is a 'flotational' coefficient, a modification of the sedimentation coefficient, with units s^{-1} , since lipoproteins float rather than sediment in the ultracentrifugal field.) These are the chylomicrons and the lipoproteins of VLDL, IDL and LDL. A precursor-product relationship has been established between VLDL ApoB and LDL ApoB (Bilheimer *et al.*, 1972; Sigurdsson *et al.*, 1975).

ApoB is also the major apoprotein of a lipoprotein that is present in plasma from which the $S_f > 0$ lipoproteins have been removed by ultracentrifugation (ρ 1.063 g/ml infranatant). This lipoprotein has been isolated from fractions of plasma obtained by ultracentrifugation at background densities ranging from 1.055 to 1.10 g/ml (Enholm *et al.*, 1970; Albers *et al.*, 1972; Jürgens & Kostner, 1975). It is characterized immunochemically by the presence of determinants of LDL due to ApoB, its major apoprotein, and by the presence of Lp(a) determinants due to its content of ApoLp(a) (Enholm *et al.*, 1970; Albers *et al.*, 1972; Jürgens & Kostner, 1975). The hydrated density, molecular weight and electrophoretic mobility of this lipoprotein are all higher than those of LDL (Enholm *et al.*, 1972; Albers *et al.*, 1972; Kostner, 1976). It is probable that this lipoprotein is identical with the sinking pre- β -lipoprotein ('SPB') (Albers *et al.*, 1972), although it has

been pointed out that neither of them is a clearly defined fraction (Kostner *et al.*, 1981).

Although ApoB of the lipoprotein that is the subject of the present study could not be distinguished immunochemically from LDL ApoB (Albers *et al.*, 1972; Enholm *et al.*, 1972), it has been shown that it is not a metabolic product of VLDL or LDL (Krempler *et al.*, 1979, 1980). Hence it was important to obtain some insight into the nature of the ApoB of this lipoprotein. This was so because it has been shown that an elevated concentration of Lp(a) in plasma is associated with the occurrence of premature ischaemic heart disease (Albers *et al.*, 1977) with a high risk of myocardial infarction (Kostner *et al.*, 1981) and that the presence of sinking pre- β -lipoprotein in plasma is associated with an increased risk of coronary heart disease (Rhodas *et al.*, 1978). It has been shown recently that two species of ApoB are associated with lipoproteins of $S_f > 0$: a low-molecular-weight species present in chylomicrons and a species with higher molecular weight present in VLDL, IDL and LDL (Kane *et al.*, 1980). Accordingly, the first purpose of this study was to ascertain which, if either, of the two species is associated with Lp(a) present in plasma from which $S_f > 0$ lipoproteins have been removed. The second objective was to follow the concentration of ApoB in the ρ 1.063 g/ml infranatant of plasma in the course of a postprandial hyperlipidaemia.

Experimental

Subjects, dietary manipulation and sample preparation

Seven healthy male volunteers participated in this study. Blood was obtained from the cubital vein

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Abbreviations used: HDL, high-density lipoproteins; VLDL, very-low-density lipoproteins; LDL, low-density-lipoproteins; IDL, intermediate-density lipoproteins; ApoB, apoprotein B; ApoA, apoprotein A; Lp(a), lipoprotein(a); ApoLp(a), apolipoprotein(a).

after an overnight fast and at hourly intervals after a standard fatty meal consisting of 250 g of cheese (66.4 g of fat). Blood was transferred to plastic vials that contained 1 mg of disodium EDTA for each 10 ml of blood. Plasma was separated by centrifugation within an hour after each venepuncture. Gentamycin (0.1 mg; Essex Chemie, Lucerne, Switzerland) was added to each ml of plasma and the samples were stored at 4°C until they were analysed or used for other purposes. The ages of the volunteers, their fasting triacylglycerol concentrations and the concentration of ApoB in plasma from which lipoproteins of $\rho < 1.063$ g/ml were removed by ultracentrifugation are given in Table 1.

Isolation of lipoproteins

Chylomicrons were a gift from Dr. N. B. Myant of this Unit. They were prepared by ultracentrifuging urine from a patient with chyluria in a Beckman Ti 60 rotor for 16 h at 5°C and 100 000 g. The thick layer of chylomicrons was separated by tube slicing, the chylomicrons resuspended in saline (0.9% NaCl) of ρ 1.006 g/ml and respun under the conditions used for their isolation.

LDL were prepared by sequential ultracentrifugation by the method of Havel *et al.* (1955) in a Beckman 40.3 rotor. They were diluted 10 times with a saline solution of ρ 1.063 g/ml and respun under the conditions used for their isolation.

The infranatant that remained in the centrifuge tubes after LDL was removed and transferred to fresh tubes, the spent ones were rinsed with an NaCl/KBr solution of ρ 1.063 g/ml (ρ 1.063 g/ml salt solution) and the washings added to the infranatant, which was respun under the conditions used for the isolation of LDL. After the spin, the upper 2 ml from each tube was discarded, the infranatant transferred to volumetric flasks and made up to volume of the original sample of plasma with the ρ 1.063 g/ml saline solution. These samples are referred to as ρ 1.063 g/ml infranatant. Lp(a) was

purified from the ρ 1.063 g/ml infranatant by the method of Enholm *et al.* (1970) as modified by Krempler *et al.* (1979). Alternatively the fraction of plasma from which Lp(a) was purified was isolated from plasma by discontinuous-density-gradient ultracentrifugation (Cheung *et al.*, 1980) in a Beckman VTi 50 rotor. At the end of the centrifugation several bands could be seen. The most prominent ones have been identified previously and shown to represent the major lipoprotein classes VLDL, LDL and the bulk of HDL (Cheung *et al.*, 1980). More recently, additional bands belonging to lipoproteins of hydrated densities greater than LDL but less than HDL have also been observed (Cheung *et al.*, 1981). In most plasma samples investigated in this study a prominent band was discernible between that of LDL and HDL. This band was more conspicuous in ultracentrifuged plasma of those subjects in whose $\rho > 1.063$ g/ml fraction of plasma the concentration of lipoproteins that react with anti-LDL serum was higher than 100 mg/l (Table 1). This band was separated from the rest of the ultracentrifuged plasma by consecutive tube slicing and the lipoproteins present were concentrated by a second ultracentrifugation: the background density of the separated material was brought to 1.10 g/ml by dialysing it against an NaCl/KBr solution of this density containing 0.1 mg of gentamycin/ml and 1 mg of NaN₃/ml. The solution was respun in a Beckman 40 rotor at 10°C and 105 000 g for 18 h. The upper 2 ml were separated by tube slicing and dialysed against the salt solution used for the purification of Lp(a) (see below). On double diffusion of this fraction against anti-Lp(a) serum, precipitin lines were formed (Fig. 1). When saline solutions were ultracentrifuged under identical conditions but in the absence of plasma and the tubes were sliced at the height corresponding to that of the relevant fraction, the density of the recovered solution was between 1.081 and 1.089 g/ml, as measured by weighing known volumes delivered from precision syringes.

The Lp(a)-rich fraction obtained by either method was purified by agarose-gel chromatography by the method of Krempler *et al.* (1979) on Bio-Gel A5m (100–200 mesh; BioRad Laboratories, Richmond, CA, U.S.A.). The size of the column was 1.8 cm $\times$ 100 cm with a flow rate of 7 ml/h. The void volume (V_0) was determined with Blue Dextran 2000 and the volume of the stationary phase (V_1) with ²²Na⁺. The values for the partition coefficient and molecular weight were obtained as described by Albers *et al.* (1972). The presence of antigens in the fractions was tested by double diffusion against anti-LDL, anti-ApoA (Boehringerwerke A.G., Marburg, Germany) and anti-Lp(a) antisera. The fractions in which ApoB and Lp(a) could be detected immunochemically were pooled and the lipo-

Table 1. The concentration of triacylglycerols in plasma and ApoB in the ρ 1.063 g/ml infranatant of seven subjects under fasting conditions

Abbreviation: ND, not determined.

Subject	Age (years)	Concn. (mg/l)	
		Triacylglycerol	ApoB
1	34	620	90
2	55	1920	230
3	28	1850	50
4	28	ND	160
5	26	750	90
6	53	1390	210
7	39	580	120

Fig. 1. Precipitin lines formed on double diffusion of Lp(a) against anti-Lp(a) antiserum

Central well, antiserum; wells 1–6, fractions 2–7 respectively of the first peak (Fig. 3) eluted from Bio-Gel column in the course of purification of Lp(a).

protein was concentrated by ultracentrifugation in a Beckman 40 rotor at 10°C for 16 h at 105 000 *g*. After the spin the upper 10 ml in each tube was removed with a Pasteur pipette and the lipoprotein present at the bottom of the tube was recovered, care being taken to redissolve any gelatinous material present in the centrifuge tube.

The immunoprecipitate of all lipoproteins present in the ρ 1.063 g/ml infranatant and that react with anti-LDL serum was prepared as follows: the ρ 1.063 g/ml infranatant was dialysed against 0.15 M-NaCl containing 1 mg of NaN_3 /ml and 1 mg of gentamycin/ml and an amount of anti-LDL serum determined by preliminary quantitative precipitin tests was added. The samples were left at 4°C for 24 h and the precipitate was removed by centrifugation in an MSE Mistral centrifuge at 2200 *g* for 15 min. The supernatant was removed and tested for antigen and antibody excess by Ouchterlony double diffusion. The sediment was resuspended in 0.15 M-NaCl containing NaN_3 and gentamycin (as used for dialysis) and spun under the same conditions. This operation was repeated twice.

Polyacrylamide-gradient-gel electrophoresis

This was performed in commercial gels (PAA 2/30; Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described (Reichl *et al.*, 1982). The samples were prestained by adding to them one-

tenth of their volume of a saturated solution of Sudan Black in propane-1,2-diol.

Agarose-gel electrophoresis

Native samples, or samples prestained with Sudan Black in the case of polyacrylamide-gradient-gel electrophoresis, were electrophoresed in 1.5% agarose (Indubiose; Pharmindustrie, Clichy, France) in 0.1 M-barbital buffer, pH 8.6. The conditions of electrophoresis were identical with those used for immunoelectrophoresis, except that the time of the run was 5 h.

Immunoelectrophoresis

The concentration of lipoproteins that contain ApoB was determined as described previously (Reichl *et al.*, 1977). The relative concentration of Lp(a) was assayed by the method of Walton *et al.* (1974). The antibody was a gift from Professor K. Walton.

Crossed immunoelectrophoresis was performed by the method of Laurell (1973). A strip of agarose in which samples were run in one dimension was embedded in agarose containing 1% anti-LDL serum and the samples were electrophoresed in a direction perpendicular to that of the first electrophoresis under the conditions used for immunoelectrophoresis in one dimension.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The method described by Kane *et al.* (1980) was used to characterize ApoB according to its size. Samples of soluble lipoproteins were prepared as described for that method. Samples of immunoprecipitates were dissolved in 0.1 M-phosphate buffer containing 10% sodium dodecyl sulphate. The amount of ApoB in the sample was estimated from the amount assayed in the solution from which it had been precipitated; the amount of total protein in the sample dissolved in the sodium dodecyl sulphate-containing buffer was determined by the method of Bensadoun & Weinstein (1976) and the sodium dodecyl sulphate concentration adjusted in such a way that for each mg of protein there was 20 mg of sodium dodecyl sulphate (Kane *et al.*, 1980).

Other methods

Triacylglycerols were determined enzymically by using a standard method (Dow Chemical Co., 1979).

Results

Isolation and purification of Lp(a)

When Lp(a) was purified by agarose-gel chromatography from the Lp(a)-enriched portion of the ρ 1.063 g/ml infranatant (Krempler *et al.*, 1980),

the first major fraction eluted with a V_e of 102 ml and the second at V_e 140 ml; purified LDL eluted at V_e 118 ml (Fig. 2). Every fraction was tested with anti-LDL, anti-Lp(a) and anti-ApoA antiserum. Material from the first peak reacted with antisera to LDL and Lp(a) but not to ApoA; the opposite was true for the material that eluted with the second major fraction.

When Lp(a) was purified by agarose-gel chromatography from the Lp(a)-enriched fraction obtained from plasma by discontinuous-density-gradient ultracentrifugation, the elution pattern from the agarose column did not differ significantly from that observed when the ρ 1.063 g/ml infranant served as starting material for this purification (Fig. 2). Using V_e values of the first major fraction, its molecular weight was found to be approx. 5×10^6 and was almost double that of LDL.

These findings were substantiated by submitting the purified Lp(a) samples to polyacrylamide-gradient gel electrophoresis, as shown in Fig. 3. It can be seen that Lp(a) becomes virtually stationary as a narrow band nearer to the site of application than isolated LDL. Lp(a) purified from the ρ 1.063 g/ml infranant and that purified from material obtained by discontinuous-density-gradient ultracentrifugation migrated equal distances from the line of application. When either of the Lp(a)-enriched fractions of plasma was electrophoresed on gradient polyacrylamide gels, minor bands of Sudanophilic material in addition to the band of Lp(a) could be seen; one of them becomes virtually stationary at a site similar to that of purified LDL (Fig. 3).

Lp(a) purified from either of the enriched fractions has an electrophoretic mobility in agarose that is distinctly higher than that of LDL (results not shown). When co-electrophoresed with LDL and

assayed by crossed immunoelectrophoresis against anti-LDL antiserum two separate rockets are formed (Fig. 4).

Characterization of the ApoB of lipoproteins of $\rho > 1.063$ g/ml

To characterize the species of ApoB associated with Lp(a) this lipoprotein was delipidated and

Fig. 3. Electrophoretogram of lipoproteins on polyacrylamide gradient gels

The lipoproteins were prestained with Sudan Black and electrophoresed for 2000 V-h. Lane 1, LDL; lane 2, Lp(a) purified from ρ 1.063 g/ml infranant; lane 3, Lp(a) purified from lipoproteins obtained by discontinuous-density-gradient ultracentrifugation; lane 4, Lp(a) as in lane 2 plus LDL; lanes 5 and 6, Lp(a) as in lane 4 plus LDL.

Fig. 2. Absorbance of eluates from Bio-Gel A5m of the lipoproteins of background density 1.081–1.087 g/ml obtained by discontinuous-density-gradient ultracentrifugation of plasma (O) and of LDL (ρ 1.019–1.063 g/ml) obtained by sequential ultracentrifugation (Δ)

The horizontal bars indicate immunoreactivity against anti-Lp(a) (□), anti-LDL (▨) and anti-ApoA (■) antisera. The void volume and the volume of the stationary phase plus the void volume are indicated by arrows.

Fig. 4. Crossed immunoelectrophoresis of a mixture of Lp(a) and LDL

The lipoproteins were electrophoresed in the first dimension on 1.5% agarose in barbital buffer, pH 8.6, at a gradient of 5 V/cm for 5 h. They were then electrophoresed under the same conditions in the second dimension for 16 h in agarose that contained 2% anti-LDL antiserum.

subjected to sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis. In all samples investigated in this study, only the larger species of ApoB, or in some cases this species and its fragments, could be seen (Fig. 5). In no sample could the smaller chylomicron-derived ApoB be detected (Fig. 5). When the ApoB of this fraction was co-electrophoresed with ApoB of isolated LDL the identity of the bands in sodium dodecyl sulphate gels could be seen (Fig. 5). It was important to ascertain whether plasma from which the $S_f > 0$ lipoproteins were removed by ultracentrifugation contained also only this larger species of ApoB. Therefore, all lipoproteins that are present in this fraction of plasma and express the ApoB antigen were immunoprecipitated with anti-LDL serum. When the washed immunoprecipitate was delipidated with sodium dodecyl sulphate and subjected to gradient electrophoresis again, only the larger species of ApoB and its fragments could be detected (Fig. 5). Only one species of ApoB could be detected, irrespective of whether the plasma sample was

obtained from fasting subjects or after a fat meal (results not shown).

Effect of a test meal on the concentration of ApoB-containing lipoproteins in plasma from which the $S_f > 0$ lipoproteins were removed

When the concentration of ApoB in the ρ 1.063 g/ml infranatant was measured in the course of a postprandial lipidaemia a significant increase could be observed. Because the absolute amount of ApoB in this fraction varies considerably from subject to subject (Table 1) we have expressed the changes induced by the test meal as percentages of the baseline values measured in fasting plasma. A maximum increase was observed 3 h after the test meal; the increase was statistically significant ($P < 0.05$). The concentration of ApoB-containing lipoproteins after the test meal was increased, with a maximum 4 h after the meal. Although there was a considerable inter-individual variation of this response, the increase at 3 h after the test meal was statistically significant at $P < 0.01$. The changes in triacylglycerol levels in the total plasma of these subjects in the course of the postprandial hyperlipidaemia were also expressed as percentage changes of the values in fasting plasma of each individual. There was no correlation between the change in ApoB concentration in the ρ 1.063 g/ml infranatant and the change in plasma triacylglycerol concentration ($r = -0.0116$, $n = 6$). When the effect of postprandial hyperlipidaemia on the concentration of Lp(a) antigen was tested in whole plasma or in plasma from which lipoproteins of $S_f > 0$ were removed no changes could be detected.

Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of LDL, Lp(a) and of the immunoprecipitate obtained by adding a titrated volume of anti-LDL antiserum to the ρ 1.063 g/ml infranatant

(1), LDL of subject 1; (2), LDL of subject 2; (3), Lp(a) purified from a lipoprotein fraction obtained by discontinuous-density-gradient ultracentrifugation; (4), Lp(a) purified from a lipoprotein fraction obtained by sequential ultracentrifugation; (5), co-electrophoresis of LDL as in gel (2) and Lp(a) as in gel (3); (6), immunoprecipitate of lipoproteins of the ρ 1.063 g/ml infranatant that react with anti-LDL antiserum. (7) Chylomicrons prepared from urine of a patient with chyluria. The ApoB nomenclature is that of Kane *et al.* (1980).

Discussion

The results of the present study show that the ApoB of the high-molecular-weight lipoprotein present in the ρ 1.063 g/ml infranatant of human plasma cannot be distinguished by sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis from LDL ApoB. However, it is possible that the two apoprotein B forms differ, for example, in their content of sialic acid.

The high-molecular-weight lipoprotein that we have studied was purified in the same way as 'LDL-a-1' (Albers *et al.*, 1972) and as Lp(a) (Krempler *et al.*, 1980). The molecular weight of our preparation (approx. 5×10^6) is similar to that of the LDL-a-1 of Albers *et al.* (1972) (5.4×10^6) and to that of the Lp(a) of Kostner (1976) (5.2×10^6); the electrophoretic mobility is higher than that of LDL and it resembles Lp(a) in containing the Lp(a) antigen. Hence, in all these respects our preparation is identical with purified Lp(a) obtained by others. It differs only from the latter in the cut-off density of the isolated fraction of plasma from which

it has been purified; the upper cut-off at ρ 1.063 g/ml instead of ρ 1.055 g/ml was chosen to minimize contamination with LDL. Lp(a) purified in the same way as the present preparation has been used by others for turnover studies of its ApoB; those studies have shown its metabolic independence on VLDL ApoB and LDL ApoB (Krempler *et al.*, 1979, 1980). The present results complement those findings in showing that the two independent metabolic pools share the same species of ApoB. The physiological meaning of this result is not apparent. It may be that Lp(a) serves as an alternative pathway for ApoB-linked export of cholesterol from cells of the intestinal mucosa or liver when conventional pathways are overloaded. In this context it is important to note that patients with familial hypercholesterolaemia secrete into their plasma an ApoB-containing lipoprotein of $\rho > 1.019$ g/ml that is not derived from VLDL (Soutar *et al.*, 1979).

ApoB-containing lipoproteins other than Lp(a) are known to be present in the ρ 1.063 g/ml infranant (Albers *et al.*, 1972). A lipoprotein the size of LDL and expressing the Lp(a) antigen has also been identified in this fraction of human plasma (Weisgraber & Mahley, 1980). It seems that the Lp(a) antigen is associated with several discrete lipoprotein species; the high-molecular-weight Lp(a) has been studied most thoroughly, probably because it is the most abundant of them. In the present study we also found lipoproteins the size of LDL in the ρ 1.063 g/ml infranant; their concentration was too low to permit isolation from the amount of plasma that was available and the characterization of their ApoB. However, when all lipoproteins of the ρ 1.063 g/ml infranant that react with antibodies to ApoB are immunoprecipitated and the ApoB of this precipitate is analysed by sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis, only ApoB of the LDL species is detected.

The concentration of lipoproteins of the ρ 1.063 g/ml infranant that react with antibodies to ApoB increases in the course of a postprandial hyperlipidaemia. In accordance with the findings of others (Albers *et al.*, 1975) we found no change in the concentration of the Lp(a) antigen in whole plasma or in the ρ 1.063 g/ml infranant. It remains to be shown whether the increase in the concentration of immunoreactive ApoB after a fatty meal is due to the high-molecular-weight Lp(a) or to other ApoB-containing lipoproteins present in the ρ 1.063 g/ml infranant.

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