In vitro transformation of apoA-I-containing lipoprotein subpopulations: role of lecithin:cholesterol acyltransferase and apoB-containing lipoproteins

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Abstract Two populations of apoA-I-containing lipoproteins are found in plasma: particles with apoA-II [Lp(AI w AII)] and particles without apoA-II [Lp(AI w/o AII)]. Both are heterogeneous in size. However, their size subpopulation distributions differ considerably between healthy subjects and patients with coronary artery diseases. The metabolic basis for such alterations was studied by determining the role of lecithin:cholesterol acyltransferase (LCAT) and apoB-containing lipoproteins (LpB) in the size subpopulation distributions of Lp(AI w AII) and Lp(AI w/o AII). ApoB-free and LCAT-free plasmas, prepared by affinity chromatography, and whole plasma were incubated at 4°C and 37°C for 24 hr. After incubation, Lp(AI w AII) and Lp(AI w/o AII) were isolated by anti-A-II and anti-A-I immunosorbents. Their size subpopulation distributions were studied by nondenaturing gradient polyacrylamide gel electrophoresis. At 4°C most Lp(AI w AII) particles were in the range of 7.0–9.2 nm Stokes diameter. Incubation of plasma at 37°C resulted in an overall enlargement of particles up to 11.2 nm and larger. These particles were enriched with cholesteryl ester and triglyceride and depleted of phospholipids and free cholesterol. Removal of LpB or LCAT from plasma prior to incubation greatly reduced their enlargement. At 4°C, Lp(AI w/o AII) contained mostly particles of 8.5 and 10.1 nm. Incubation at 37°C abolished both subpopulations with the formation of a new subpopulation of 9.2 nm. This transformation was identical in apoB-free plasma but was not seen in LCAT-free plasma. Our study shows that transformation of Lp(AI w AII) requires both LCAT and LpB. However, LpB is not necessary for the transformation of Lp(AI w/o AII) in vitro. The relevance of these in vitro studies to in vivo lipoprotein metabolism was demonstrated in a subject with hepatic triglyceride lipase deficiency.—**Cheung, M. C., and A. C. Wolf.** In vitro transformation of apoA-I-containing lipoprotein subpopulations: role of lecithin:cholesterol acyltransferase and apoB-containing lipoproteins. *J. Lipid Res.* 1989. **30:** 499–509.

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The high density lipoproteins (HDL) represent a heterogeneous population of particles differing in density, size, and lipid-protein composition. At least eight major apolipoproteins (apoA-I, A-II, A-IV, C-I, C-II, C-III, D, and E) are found in HDL with A-I and A-II together representing 85–90% of the total protein. Using affinity columns containing antibodies specific for A-I and A-II, we recently demonstrated that while nearly all HDL particles contain A-I, only 50–75% of these particles contain A-II (1). Characterization of HDL particles with and without A-II, isolated from healthy normolipidemic subjects, shows that these two populations of particles contain numerous subpopulations of various sizes. When studies were extended to subjects with coronary heart diseases (CAD) and subjects with familial combined hyperlipidemia (FCHL) who are at risk for CAD, considerable differences in the size subpopulation distribution were noted between these subjects and the healthy normolipidemic subjects (2). In order to understand the metabolic basis for such alterations, in vitro studies were performed to determine how various plasma factors affect the size distribution of HDL particles with and without A-II. This report describes the effects of two such factors: the enzyme lecithin:cholesterol acyltransferase (LCAT) and the apoB-containing lipoproteins (LpB).

METHODS

Plasma

All blood samples used in this study were obtained from subjects after a 12–14 hr overnight fast. Upon separation of plasma at 4°C by low-speed centrifugation, preservatives were promptly added to final concentrations of 0.5 g/l so-

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; Lp(AI w AII), apoA-I-containing lipoproteins with apoA-II; Lp(AI w/o AII), apoA-I-containing lipoproteins without apoA-II; LpB, apoB-containing lipoproteins; gPAGE, gradient polyacrylamide gel electrophoresis; FFA, free fatty acid; CAD, coronary heart disease; FCHL, familial combined hyperlipidemia; LTP, cholesteryl ester transfer protein.

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dium azide, 0.01 g/l chloramphenicol, and 0.005 g/l gentamycin. The plasma samples were stored at 4°C unless used for incubation.

Preparation of B-free and LCAT-free plasma

ApoB-free plasma was prepared by passing plasma through either anti-B immunosorbent or dextran sulfate-cellulose, which has been shown to selectively remove LpB (3). (The dextran sulfate-cellulose was a gift from Kanegafuchi Chemical Industry, Osaka, Japan.) Plasma free of LCAT was prepared by either quantitative immunoprecipitation with rabbit anti-LCAT immunoglobulins or by immunoadsorption using affinity-purified goat antibodies specific for LCAT. The complete removal of apoB and LCAT, and the quantitative recovery of A-I and A-II, in the respectively processed plasmas were verified by specific A-I, A-II, B, and LCAT immunoassays (4–7).

Incubation studies

In vitro incubation studies were designed to assess the effects of LpB and LCAT on Lp(AI w AII) and Lp(AI w/o AII) subpopulation distribution. Five-ml aliquots of plasma, apoB-free plasma and LCAT-free plasma were separately incubated at 37°C for 24 hr in sterilized glass vials. Similar aliquots were kept at 4°C as controls. At the end of the incubation, Lp(AI w AII) and Lp(AI w/o AII) were immediately isolated from each plasma aliquot for lipid, protein, and size subpopulation analyses.

Isolation of A-I containing lipoproteins

ApoA-I-containing lipoproteins with and without A-II were isolated from plasma by a previously established two-step immunoaffinity chromatography procedure (1, 8, 9). Briefly, to isolate particles with A-II [Lp(AI w AII)], plasma was passed through a column packed with anti-A-II immunosorbent. Nonadsorbed proteins were washed with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, and 0.05% NaN_3 (Tris buffer). The bound lipoproteins were eluted with 3 M NaSCN in 0.02 M sodium phosphate buffer, pH 7.0, and immediately filtered through a column packed with Sephadex G-25 (Pharmacia LKB Biotechnology Inc.) to remove the thiocyanate. To isolate particles without A-II [Lp(AI w/o AII)], plasma devoid of A-II (nonadsorbed proteins from the anti-A-II immunosorbent) was incubated with anti-A-I immunosorbent. The nonadsorbed proteins and bound lipoproteins were sequentially eluted from the immunosorbent and processed as described above. Recoveries of A-I and A-II from the immunosorbents were greater than 90% (1, 9). All lipoproteins were isolated at 4°C and for further studies, concentrated by MicroConFilt concentrator (Biomolecular Dynamics, Beaverton, OR) to volumes equal to or less than starting plasma volume.

Gradient polyacrylamide gel electrophoresis (gPAGE)

The size subpopulations of Lp(AI w AII) and Lp(AI w/o AII) isolated from plasma, apoB-free plasma and LCAT-free plasma before and after 37°C incubations were separated by nondenaturing gPAGE using precast 4–30% gradient gels (Pharmacia LKB Biotechnology Inc.). A high molecular weight calibration protein mixture (HMW Calibration Kit, Pharmacia LKB Biotechnology Inc.) was included in each gel run. Electrophoresis was carried out in 0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA buffer, pH 8.35, at 10°C, 125 V for 24 hr. Gels were stained overnight with 0.04% Coomassie Brillant Blue G-250 dissolved in 3.5% perchloric acid, destained in 7% acetic acid until the background was clear, and scanned by a laser densitometer (Pharmacia LKB Biotechnology Inc.). Particle size and percent distribution of the various subpopulations between 7.0 and 17.0 nm, based on protein staining, were calculated as previously described (9) using the LKB 2400 Gelscan XL™ software. To obtain a semi-quantitative assessment of changes in particle size in incubated plasma, four size intervals: 7.0–8.2 nm, 8.2–9.2 nm, 9.2–11.2 nm, and 11.2–17.0 nm were used as a basis for comparison.

Compositional studies

Phospholipid, total cholesterol, unesterified cholesterol, and triglyceride were analyzed by enzymic methods (10). The difference between total and unesterified cholesterol mass was considered as cholesteryl ester. Unless otherwise specified, all lipid determinations were performed on an ABA bichromatic analyzer (Abbott Laboratories, Irvine, TX) with proper adjustment of specimen to reagent volume to optimize measurements in the HDL particles. ApoA-I, A-II, B, and LCAT were quantitated by specific radioimmunoassays (4–7). Protein composition of isolated lipoproteins was studied by sodium dodecyl sulfate (SDS) PAGE using the method of Laemmli (11). Protein bands were visualized by staining in 0.035% Coomassie R250 dissolved in isopropanol–acetic acid–water 25:10:65 and destained in the same solvent. Total protein was determined by the method of Lowry et al. (12) using bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) as the standard.

RESULTS

Subjects

The age, sex, lipid, apolipoprotein, and LCAT profiles of the studied subjects are shown in **Table 1**. These five subjects were laboratory personnel of the Northwest Lipid Research Center or their relatives. With the exception of subject 5 who had elevated cholesterol, the remaining four subjects were normolipidemic. Two of the subjects had

TABLE 1. Lipid and protein profile of study subjects

Subject	Age	Sex	Plasma CH	Plasma TG	LDL CH	HDL CH	Plasma ApoA-I	Plasma ApoA-II	Plasma ApoB	Plasma LCAT
	yr					*mg/dl*				*μg/ml*
1	20	M	117	39	55	55	132	31.5	49	3.32
2	47	M	183	79	124	43	123	21.2	77	4.28
3	19	F	189	68	115	60	155	27.7	92	5.42
4	21	M	197	93	111	67	183	30.6	104	5.39
5	50	F	296	136	150	119	197	44.0	121	6.56

CH, cholesterol; TG, triglyceride.

HDL cholesterol levels greater than the 90th percentile values reported for their age and sex (13), and one subject had an unusually low LDL cholesterol. All subjects had normal plasma LCAT concentrations. Their A-I and A-II concentrations paralleled their HDL cholesterol levels, while their B concentrations corresponded well with their plasma cholesterol concentrations. In addition, a subject with no measurable hepatic triglyceride lipase (HTGL) activity after heparin infusion (14) was also studied.

Characteristics of plasma Lp(AI w AII) and Lp(AI w/o AII) before and after incubation

The size subpopulation distributions of Lp(AI w AII) isolated from the plasmas of five subjects are shown in **Fig. 1A**. Upon separation of the lipoproteins by gPAGE, the distribution of particles in the control plasmas in the 7.0–8.2-nm, 8.2–9.2-nm, 9.2–11.2-nm, 11.2–17-nm intervals was 19 ± 5%, 48 ± 8%, 27 ± 7%, and 6 ± 2%, respectively. Incubation of plasma considerably altered this size subpopulation distribution. There was a substantial decrease of particles in the 8.2–9.2-nm interval to 22 ± 4% with a concomitant increase in the larger 9.2–11.2-nm and 11.2–17-nm intervals to 48 ± 12% and 16 ± 6%, respectively. Thus, incubation of plasma at 37°C resulted in enlargement of Lp(AI w AII) particles to 11.2 nm or larger. **Fig. 2** (lanes 2 and 4) is a photograph of a gradient gel showing these changes.

Figs. 1B and 2 (lanes 3 and 5) depict the size subpopulations of Lp(AI w/o AII) isolated from plasma before and after incubation. For all subjects, before incubation of samples, two distinct subpopulations with apparent mean hydrated Stokes diameters of 10.1 nm for Lp(AI w/o AII)$_1$ and 8.5 nm for Lp(AI w/o AII)$_2$ were observed. The relative proportion of these two subpopulations varied among the subjects. Together they represented 74 ± 7% of all Lp(AI w/o AII) size subpopulations. Particles larger than

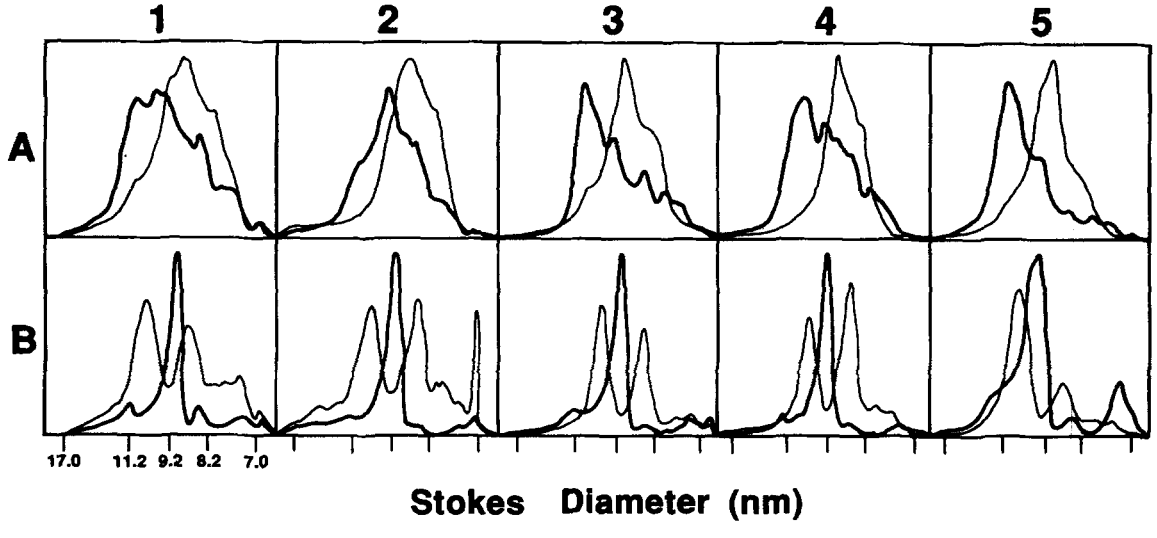

Fig. 1. Densitometric scans after gPAGE of (A) Lp(AI w AII) and (B) Lp(AI w/o AII) of subjects 1–5. The scans denote lipoproteins isolated from control (—) and 37°C-incubated (▬) plasma. These scans represent the region of the gel where particles of HDL size migrate. Indicated in all scans are the nm markers of the size intervals used as a basis for comparison: 7.0–8.2 nm, 8.2–9.2 nm, 9.2–11.2 nm, and 11.2–17.0 nm.

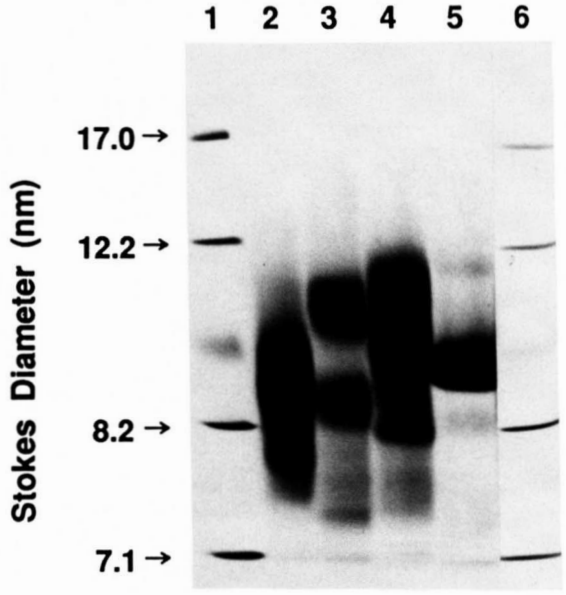

Fig. 2. Photograph of a gradient gel showing the size populations of Lp(AI w AII) and Lp(AI w/o AII) isolated from control plasma and from plasma incubated at 37°C. Lanes 1 and 6: HMW Calibration Proteins (LKB Pharmacia Biotechnology, Inc.); lanes 2 and 4: Lp(AI w AII) of 4°C and 37°C plasma, respectively; lanes 3 and 5: Lp(AI w/o AII) of 4°C and 37°C plasma, respectively.

Lp(AI w/o AII)$_1$ and smaller than Lp(AI w/o AII)$_2$ were also present in varying quantities in most subjects. After the plasmas were incubated, Lp(AI w/o AII)$_1$ and Lp(AI w/o AII)$_2$ virtually disappeared. Instead, a population of particles with mean Stokes diameter intermediate to these two subpopulations (9.2 ± 0.2 nm) was formed. This new population represented 67 ± 8% of the Lp(AI w/o AII) particles in the five incubated plasmas examined. In subjects 1–4, 37°C incubation also resulted in reduction of particles smaller than Lp(AI w/o AII)$_2$. The reverse occurred, however, in subject 5 whose 4°C-plasma contained little Lp(AI w/o AII)$_2$ and only trace quantities of particles in the 7.0 to 8.2 nm region.

The consistent transformations of Lp(AI w AII) and Lp(AI w/o AII) observed in the five subjects after 37°C plasma incubation suggest a physical aggregation or fusion of lipoproteins induced by either increased temperature and/or some process(es) involving plasma factors. To evaluate these possibilities, two preparations of HDL (d 1.063–1.21 g/ml) isolated by conventional sequential ultracentrifugation, as well as Lp(AI w AII) and Lp(AI w/o AII) isolated from plasma kept at 4°C, were incubated at 37°C and their size subpopulations were compared to the controls kept at 4°C. Incubation of HDL prepared by ultracentrifugation had no apparent effect on its particle size. Hence 37°C incubation alone did not result in the alteration of HDL particles observed above. However, incubation of Lp(AI w AII) resulted in a slight enlargement of particle size. In Lp(AI w/o AII) there was a decrease in Lp(AI w/o AII)$_1$, in particles smaller than Lp(AI w/o AII)$_2$, and the

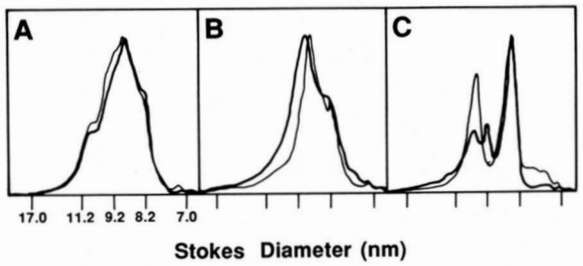

Fig. 3. Effect of 37°C incubation of (A) HDL (d 1.063–1.21 g/ml) prepared by ultracentrifugation, (B) Lp(AI w AII), and (C) Lp(AI w/o AII) of subject 4 isolated from plasma kept at 4°C. The scans represent lipoproteins before (—) and after (▬) incubation.

formation of a new subpopulation of particles intermediate in size to Lp(AI w/o AII)$_1$ and Lp(AI w/o AII)$_2$. Examples of these changes are illustrated in **Fig. 3**.

We reported earlier that Lp(AI w AII) and Lp(AI w/o AII) isolated by our immunoaffinity chromatography procedure contained considerably more active LCAT than HDL prepared by sequential ultracentrifugation (15). The lack of change at 37°C observed in HDL on one hand and the alteration of isolated Lp(AI w AII) and Lp(AI w/o AII) on the other hand suggested the involvement of LCAT. Indeed, analysis of the lipoproteins presented in Figs. 3B and 3C showed a 30% decrease in unesterified cholesterol (mass) and a 17% increase in cholesteryl ester after 37°C incubation. Since incubation of isolated Lp(AI w AII) and Lp(AI w/o AII) did not produce changes to the same extent as seen in plasma, other plasma components such as the lower density lipoproteins may also be involved.

Incubation studies on LCAT-free plasma

To study the specific role of LCAT in the alteration of Lp(AI w AII) and Lp(AI w/o AII) in vitro, LCAT was selectively removed from the plasma of two subjects (1 and 2) by specific anti-LCAT antibodies. Complete removal of LCAT was verified by radioimmunoassay. Cholesteryl ester transfer protein (LTP-1) activity in the two LCAT-free plasmas was 76% and 100% of whole plasma activity, respectively. These LCAT-free plasmas were incubated at 37°C. Results from both subjects were comparable; an example is shown in **Fig. 4**. Comparison of Figs. 4A and 4B

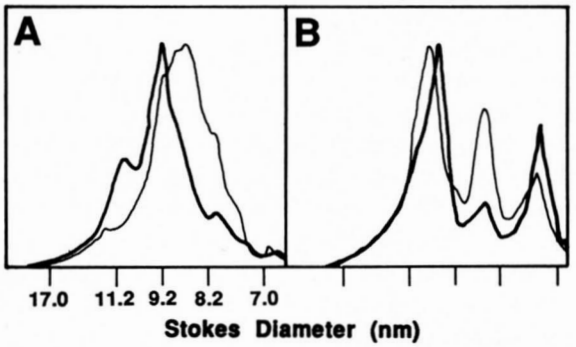

Fig. 4. Densitometric scans after gPAGE of (A) Lp(AI w AII) and (B) Lp(AI w/o AII) isolated from control (—) and incubated (▬) LCAT-free plasma of subject 1.

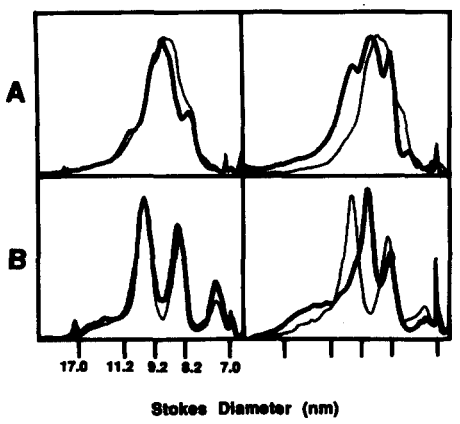

Fig. 5. Effect of addition of purified LCAT on (A) Lp(AI w AII) and (B) Lp(AI w/o AII) transformation in LCAT-free plasma. The scans represent lipoproteins isolated from 4°C (—) and 37°C (▬) incubated LCAT-free plasma in the absence of (left panel) and presence of (right panel) purified LCAT.

with Figs. 1A and 1B (subject 1), respectively, shows that Lp(AI w AII) and Lp(AI w/o AII) isolated from control LCAT-free plasma were comparable to those isolated from control whole plasma. Incubation of LCAT-free plasma at 37°C again resulted in a shift of Lp(AI w AII) particles to the 9.2–11.2-nm size interval (from 28% to 48%). However, the enlargement of particles to the 11.2–17-nm size interval was less than that observed when whole plasmas were incubated (from 6% to 12% vs. 24%) (Fig. 4A). The alterations in Lp(AI w/o AII) upon incubation of LCAT-free plasmas were also different from those seen in whole plasma. Most noticeably, the size and relative quantity of Lp(AI w/o AII)$_1$ and particles larger than Lp(AI w/o AII)$_1$, were not substantially altered (4% and 1% change, respectively) but the relative amount of Lp(AI w/o AII)$_2$ was decreased from 25% to 14% and particles smaller than Lp(AI w/o AII)$_2$ increased from 20% to 26% (Fig. 4B).

To determine whether LCAT was indeed the missing factor in LCAT-free plasma, resulting in the inability of the A-I containing lipoproteins to transform, a physiological concentration of purified LCAT was added to another affinity-isolated LCAT-free plasma sample and the mixture was incubated. A control 37°C incubation without added purified LCAT was also performed. In this subject, minimal changes in lipoprotein size were observed in incubated LCAT-free (**Fig. 5**, left panel). Upon addition of LCAT, transformations resembling those of incubated whole plasma occurred (Fig. 5, right panel). The LCAT used in this study was purified to homogeneity from human plasma by the established method of Chen and Albers (16) using a combination of dextran sulfate-Mg^{2+} precipitation and consecutive chromatography on phenyl-Sepharose, DEAE-Sepharose, Affi-Gel Blue, and hydroxylapatite columns. It exhibited a single protein band slightly below that of commercially purified bovine albumin on SDS-PAGE.

Incubation studies of apoB-free plasma

To determine the role of LpB in the conversion observed,

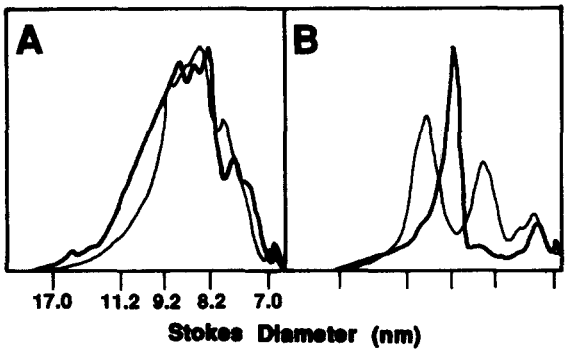

Fig. 6. Densitometric scans after gPAGE of (A) Lp(AI w AII) and (B) Lp(AI w/o AII) isolated from control (—) and incubated (▬) apoB-free plasma of subject 1.

apoB-free plasma was prepared from two subjects (1 and 3) as described in Methods. The LCAT mass and LTP-1 activity of these apoB-free plasmas were between 86 and 100% of the whole plasmas. An example of the results is shown in **Fig. 6**. Lp(AI w AII) and Lp(AI w/o AII) isolated from apoB-free plasma were comparable to those from whole plasma stored at 4°C (compare Fig. 6A and 6B with Fig. 1A and 1B for subject 1). When apoB-free plasma was incubated at 37°C, a slight enlargement of Lp(AI w AII) particles within 9.2–11.2-nm region was observed. Subpopulations within the 8.2–9.2-nm interval were unaltered (Fig. 6A). Thus the absence of LpB significantly reduced the enlargement of Lp(AI w AII). In contrast, incubation of apoB-free plasma resulted in an alteration of Lp(AI w/o AII) identical to that observed in whole plasma (Fig. 6B). Similar results were obtained with subject 3. Thus, the conversion of Lp(AI w/o AII) upon incubation of plasma at 37°C was independent of LpB.

To confirm that the absence of LpB was indeed responsible for the failure of Lp(AI w AII) to transform in apoB-free plasma, we added some d < 1.050 g/ml lipoproteins (isolated by conventional ultracentrifugation using a 60 Ti rotor) to another affinity-isolated apoB-free plasma. The mixture was then incubated as described. As shown in **Fig. 7**, little or no change in the particle size of Lp(AI w

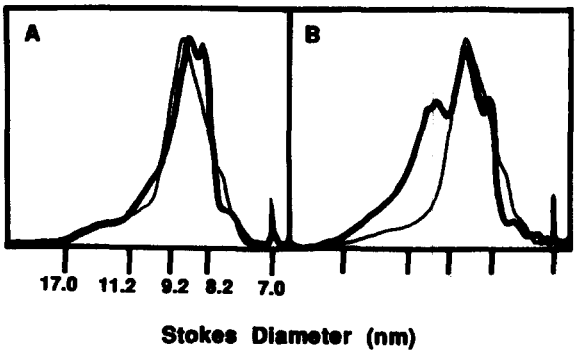

Fig. 7. Effect of addition of d < 1.050 g/ml lipoproteins on Lp(AI w AII) transformation. The scans represent lipoproteins isolated from 4°C (—) and 37°C (▬) incubated apoB-free plasma in the absence of (A) and presence of (B) d < 1.050 g/ml lipoproteins.

TABLE 2. Lipid composition of Lp(AI w AII) isolated from plasma before and after incubation

Subject[a]	4°C FC	CE	TG	PL	Lipid/ Protein	37°C FC	CE	TG	PL	Lipid/ Protein
	%					%				
1	7	31	7	54	0.70	4	43	15	38	1.02
2	7	29	12	51	0.69	7	38	18	37	1.19
3	10	28	16	47	0.79	5	32	17	46	0.77
4	13	18	7	62	0.78	10	28	16	47	0.96
5	5	28	6	61	0.87	4	33	12	51	0.96
Mean ± SD	8.4 ± 2.6	26.8 ± 4.4	9.6 ± 3.7	55.1 ± 5.9	0.77 ± 0.07	6.1 ± 2.0	34.9 ± 5.3	15.5 ± 2.2	43.6 ± 5.4	0.98 ± 0.13

FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid.
[a]The apoA-I-containing lipoprotein particles of subjects 3 to 5, but not of subjects 1 and 2, were adsorbed with either anti-B immunosorbent or dextran sulfate-cellulose prior to lipid analyses.

AII) was observed when apoB-free plasma was incubated. However, transformations comparable to those of incubated whole plasma were seen in the reconstituted apoB-containing plasma. This reconstitution experiment thus confirms that LpB plays an important role in the transformation of Lp(AI w AII).

Chemical composition of Lp(AI w AII) and Lp(AI w/o AII)

The lipid and protein compositions of Lp(AI w AII) and Lp(AI w/o AII) isolated from plasma before and after incubation are shown in **Table 2** and **Table 3**, respectively. The affinity-isolated particles of subjects 3–5, but not of subjects 1 and 2, were absorbed with either anti-B immunosorbent or dextran sulfate-cellulose prior to chemical analyses. Irrespective of LpB removal prior to analysis, in all subjects studied, incubation resulted in an increase in esterified cholesterol and a decrease in phospholipids in Lp(AI w AII) and Lp(AI w/o AII). Overall, the unesterified cholesterol contents decreased from 8% to 6% while the triglyceride contents increased from 10% to 15% (Tables 2 and 3). Thus, incubation resulted in a depletion of polar lipids with an enrichment of core lipids in both populations of apoA-I-containing particles. However, while 37°C incubation resulted in a slight increase in the lipid–protein ratio in Lp(AI w AII), the reverse was true for Lp(AI w/o AII) Tables 2 and 3).

To determine whether the observed LCAT-induced size changes of transformed Lp(AI w AII) and Lp(AI w/o AII) could be due to an accumulation of free fatty acids (FFA) and lysolecithin in the particles, the FFA and lysolecithin contents of native and transformed Lp(AI w AII) and Lp(AI w/o AII) isolated from the plasma of another subject were analyzed. Nonesterified FFA were measured by the NEFA C kit (Wako Pure Chemicals, Osaka, Japan). To quantitate the lecithin and lysolecithin contents, the lipids in the native and incubated plasma, apoA-I-free plasma, Lp(AI w AII), and Lp(AI w/o AII) of this subject were extracted with chloroform–methanol 2:1 as described by Folch et al. (17). The dried lipid was then redissolved in a known amount of chloroform and applied on a thin-layer chromatography plate containing silica gel H

TABLE 3. Lipid composition of Lp(AI w/o AII) isolated from plasma before and after incubation

Subject[a]	4°C FC	CE	TG	PL	Lipid/ Protein	37°C FC	CE	TG	PL	Lipid/ Protein
	%					%				
1	7	34	10	49	0.73	4	47	13	36	0.63
2	7	29	12	51	0.97	8	38	20	33	0.99
3	7	26	12	55	0.78	4	31	24	40	0.68
4	10	21	10	59	0.84	8	31	8	53	0.59
5	8	27	9	56	0.82	5	34	12	49	0.70
Mean ± SD	7.7 ± 1.2	27.5 ± 4.3	10.8 ± 1.3	54.0 ± 3.6	0.83 ± 0.08	5.9 ± 1.8	36.2 ± 6.1	15.4 ± 5.8	42.5 ± 7.5	0.72 ± 0.14

FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid.
[a]The apoA-I-containing lipoprotein particles of subjects 3 to 5, but not of subjects 1 and 2, were adsorbed with either anti-B immunosorbent or dextran sulfate-cellulose prior to lipid analyses.

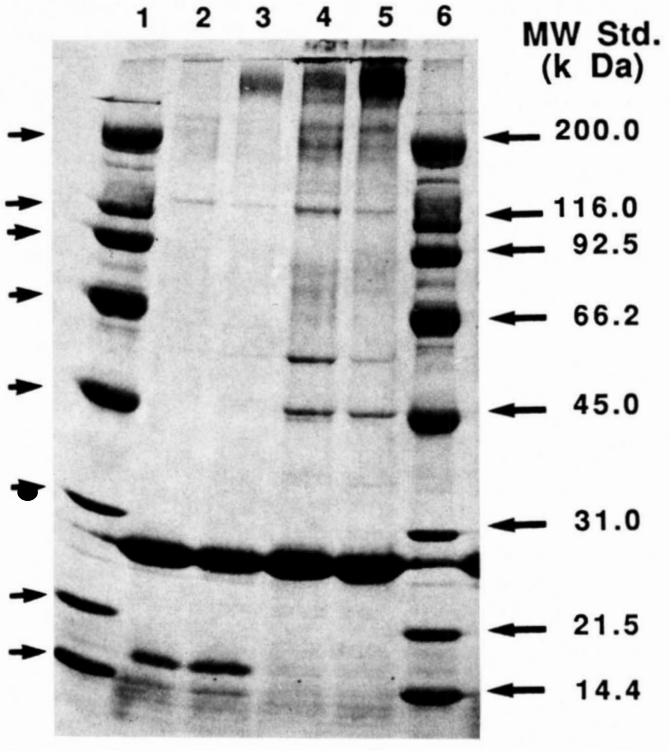

Fig. 8. SDS PAGE pattern of Lp(AI w AII) and Lp(AI w/o AII) isolated from control and incubated plasma of subject 2. Lanes 1 and 6: molecular weight standards from Bio-Rad Laboratory; lanes 2 and 3: Lp(AI w AII) isolated from control and incubated plasma, respectively; lanes 4 and 5: Lp(AI w/o AII) isolated from control and incubated plasma, respectively.

(Analabs, Foxboro Analytical, Norwalk, CT). The phospholipid subspecies were separated with a solvent system of chloroform–methanol–acetic acid–water 100:60:16:8 (v/v) and quantitated by the method of Bartlett (18). All calculations were performed based on inorganic phosphorus with no adjustment to subspecies molecular weight.

The concentration of FFA in the control (4°C) plasma of this subject was 0.238 meq/l. After 37°C incubation, the plasma FFA concentration increased to 0.401 meq/l. In both control and incubated plasma, approximately 99% of the FFA was recovered in the apoA-I-free plasma fractions. In the control plasma of this subject, lysolecithin represented 5% of the total phospholipids. Upon incubation, the percentage of lysolecithin in plasma increased to 23. This increase was located entirely in the apoA-I-free plasma fraction where lysolecithin represented 45% of the total phospholipid contents. In apoA-I-containing lipoproteins isolated from control plasma, lysolecithin was 5% of the total phospholipid contents. Corresponding lipoproteins from incubated plasma contained slightly less (3%) lysolecithin. Thus, the particle size changes observed in incubated plasma were not associated with an accumulation of FFA and lysolecithin.

The protein components of Lp(AI w AII) and Lp(AI w/o AII) isolated from plasma before and after incubation were studied by SDS-PAGE. In addition to A-I and A-II, Lp(AI w AII) also contain proteins with molecular weights comparable to the C peptides (**Fig. 8**, lanes 2 and 3). Based on densitometry, and assuming equal chromogenicity of the proteins, these small molecular weight particles amounted to 12% of the total stained A-I and A-II bands in Lp(AI w AII). Similar small molecular weight proteins were also detected in Lp(AI w/o AII), although to a lesser extent (8%) (Fig. 8, lanes 4 and 5). SDS-PAGE studies of particles isolated from the plasma of three subjects (1, 2, and 5) before and after incubation, showed no significant difference in the composition of proteins of molecular weight less than 200 kDa. In subject 2, a slightly darker protein band much larger than 200 kDa was found in particles isolated from incubated plasma (Fig. 8, lanes 3 and 5), but this was not evident in the SDS-PAGE of the 37°C particles from the other two subjects.

Effect of incubation on the distribution of apoA-I and LCAT between Lp(AI w AII) and Lp(AI w/o AII)

The distribution of apoA-I and LCAT between Lp(AI w AII) and Lp(AI w/o AII) in plasma at 4°C and 37°C was studied by quantitating the apoA-I and LCAT mass in the various plasma fractions and isolated lipoproteins. In the first five subjects, 60 ± 16% and 62 ± 12% of plasma apoA-I was found associated with apoA-II at 4°C and 37°C, respectively. Thus, incubation did not result in significant changes in the distribution of apoA-I between Lp(AI w AII) and Lp(AI w/o AII). On the contrary, in all subjects studied, incubation altered the distribution of LCAT among the various lipoproteins. At 4°C, 20 ± 2%, 64 ± 8%, and 15 ± 8% of plasma LCAT was found in Lp(AI w AII), Lp(AI w/o AII), and apoA-I-free plasma, respectively. This distribution is consistent with our earlier finding (15). At 37°C, considerably less LCAT (39 ± 8%) was found associated with Lp(AI w/o AII), while relatively more LCAT was recovered in Lp(AI w AII) (31 ± 20%) and the apoA-I-free plasma fraction (30 ± 12).

Characterization of Lp(AI w AII) and Lp(AI w/o AII) isolated from a subject with hepatic triglyceride lipase (HTGL) deficiency

The apoA-I-containing lipoproteins of a male subject with HTGL deficiency (14) were isolated by the two-step affinity chromatography procedure described in Methods. His plasma cholesterol, triglyceride, and phospholipid contents at the time of this study were 581, 4104, and 1005 mg/dl, respectively. Plasma apoA-I (148 mg/dl) and apoA-II (40 mg/dl) concentrations were normal, though HDL cholesterol measured by the polyanion-cation precipitation method was low (20 mg/dl). In view of the high triglyceride contents, the immunoaffinity-isolated Lp(AI w AII) and Lp(AI w/o AII) were further absorbed with dextran sulfate-

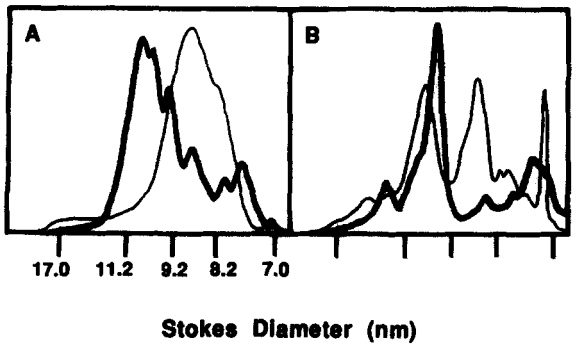

Fig. 9. Densitometric scans after gPAGE of (A) Lp(AI w AII) and (B) Lp(AI w/o AII) of a subject with HTGL deficiency (—). The scans of subject 2 (—) are shown here for comparison. Both isolations were from plasma kept at 4°C.

cellulose to remove all apoB-containing particles prior to gPAGE, protein and lipid analyses. **Fig. 9** shows their particle size distribution. Compared to the normolipidemic subjects in this study, the average sizes of the subpopulations of the Lp(AI w AII) of the HTGL-deficient subject were considerably larger, with 56% of the particles having a diameter greater than 9.2 nm. In Lp(AI w/o AII), there was little Lp(AI w/o AII)$_1$ and Lp(AI w/o AII)$_2$. Instead, a prominent population of particles between the sizes of Lp(AI w/o AII)$_1$ and Lp(AI w/o AII)$_2$, with a Stokes diameter of 9.5 nm was observed. This population of particles represented 54% of all his Lp(AI w/o AII). As illustrated in Fig. 9, the size subpopulation profiles of Lp(AI w AII) and Lp(AI w/o AII) of the HTGL-deficient subject appeared similar to the 37°C transformed Lp(AI w AII) and Lp(AI w/o AII) of the five subjects shown in Fig. 1. Lipid composition studies showed that comparable to in vitro transformed particles, both populations of particles were enriched in core lipids (56% triglycerides, 5% cholesteryl ester) and low in polar lipids (5% unesterified cholesterol and 34% phospholipid.) The lipid-protein ratios of Lp(AI w AII) and Lp(AI w/o AII) were 0.98 and 0.62, respectively.

DISCUSSION

Several laboratories have reported enlargements of HDL particles upon incubation of human plasma at 37°C in vitro (19–22). However, the exact natures of such enlargements have not been studied. Using immunoaffinity chromatography and nondenaturing gPAGE, we were able to gain further insight into the changes observed. HDL is made up of two populations of particles differing in their apoA-II contents: Lp(AI w AII) and Lp(AI w/o AII). The enrichment of lipids in the enlarged Lp(AI w AII) particles of incubated plasma, and the increase in esterified cholesterol and decrease in free cholesterol and phospholipids in both the Lp(AI w AII) and Lp(AI w/o AII) particles of incubated plasma, suggest the involvement of lower density lipoproteins and LCAT in the observed transformations. Their individual roles were assessed by incubating LCAT-free plasma and apoB-free plasma prepared by affinity chromatography. To avoid the known effects of ultracentrifugation on selected HDL subpopulations, LCAT, and LTP-1, affinity chromatography, rather than ultracentrifugation, was used to prepare apoB-free plasma (9, 15). LCAT-free plasma, instead of addition of the known chemical inhibitors, was used to delineate the role of LCAT on the transformation of HDL particles, because it has been demonstrated that the effect of LCAT inhibitors on HDL particle enlargement varies with the chemical used (19, 20). Studies performed in our laboratory using sodium iodoacetate, *p*-chloromercuriphenylsulfonate (PCMPS), and 5,5'dithiobis-(2-nitrobenzoic acid) confirmed such observations. Of these three inhibitors, PCMPS was the only LCAT inhibitor that generated results consistent with incubating LCAT-free plasma prepared by immunoaffinity chromatography. These variations could be due to either the nonspecific nature of the chemical inhibitors or the differential effects of these chemicals on the phospholipase A_2 and transacylase activities of LCAT recently described by Jauhiainen et al. (23, 24). To circumvent these, we used affinity-prepared LCAT-free plasma in this study. Selective removal of either LCAT or LpB prior to incubation significantly reduced the enlargement of Lp(AI w AII) particles, suggesting that the increased lipid contents of 37°C enlarged Lp(AI w AII) particles probably originated from LpB. Furthermore, results of our reconstituted apoB-containing and LCAT-containing plasma experiments confirmed that both cholesterol esterification and additional lipids were necessary for the enlargement of Lp(AI w AII). In the absence of LpB, the various Lp(AI w/o AII) populations were still transformed to a single population of particles comparable to that observed in whole plasma at 37°C. However, it did not occur in LCAT-free plasma, indicating that cholesterol esterification but not LpB is necessary for the transformation of Lp(AI w/o AII) in vitro. The observation that both LCAT and LpB are necessary for the transformation of Lp(AI w AII) is in agreement with earlier studies on the conversion of HDL_3 to HDL_2 (25–29). However, to our knowledge, the ability of a subpopulation of HDL particles, viz. Lp(AI w/o AII), to transform in the absence of LpB, has never been reported.

The effect of 37°C incubation on the distribution of apoA-I and LCAT between Lp(AI w AII) and Lp(AI w/o AII) was also studied. We found that the distribution of apoA-I in plasma between these two populations of particles at 4°C and 37°C was comparable. Thus, the increase in temperature had probably not created additional Lp(AI w AII) particles from Lp(AI w/o AII) particles and vice versa. The present data however, do not rule out the likely event of apolipoprotein exchange between Lp(AI w AII) and Lp(AI w/o AII). Unlike apoA-I, the distribution of LCAT in plasma was temperature-dependent. At 4°C, two-thirds

of plasma LCAT in the five subjects studied was associated with Lp(AI w/o AII). The remaining one-third distributed fairly evenly between Lp(AI w AII) and the apoA-I-free plasma fraction. This distribution was comparable to that reported earlier (15). However at 37°C, approximately equal proportions of LCAT were distributed among Lp(AI w/o AII), Lp(AI w AII), and the apoA-I-free plasma fraction. At present, we do not know the significance of this observation. It is possible that LCAT normally distributes among physiological lipoproteins in an equilibrium state and the distribution is sensitive to the physical-chemical properties of the lipoproteins. Compositional and conformational changes in the transformed Lp(AI w AII), Lp(AI w/o AII), and possibly the lower density lipoproteins, probably disrupted such an equilibrium, resulting in the redistribution of LCAT among the lipoproteins. Earlier findings that the association of LCAT with lipoproteins is labile, and can be easily perturbed by ultracentrifugation and other experimental environments (15, 30, 31) support this interpretation. Furthermore, it has been demonstrated that although nascent and discoidal HDL are preferred substrates of LCAT, this enzyme can act on a variety of lipoprotein substrates (32–35), including non-apoA-I-containing particles (36). We do not know the nature of LCAT distribution in incubated apoA-I-free plasma. However, we do know that in unincubated apoA-I-free plasma, LCAT is distributed over a broad size range, with a significant proportion in the size regions of low density and very low density lipoproteins (36).

In all our incubation studies HTGL was absent. This enzyme has been postulated to be involved in the catabolism of HDL_2, the more buoyant, larger HDL particles (37). Our observation that the Lp(AI w AII) particles from the HTGL-deficient subject were bigger than those from normolipidemic subjects supports this hypothesis. The astonishing similarities between the apoA-I-containing lipoproteins of the HTGL deficient subject and the in vitro transformed apoA-I-containing lipoproteins of the other five subjects strongly indicates the relevance of our in vitro observations to in vivo lipoprotein metabolism. We present the following putative mechanisms leading to the transformed products observed in these studies. In the absence of appropriate degradation, as in the case of HTGL deficiency and in our 37°C incubation studies, in Lp(AI w AII) continuous cholesterol esterification by LCAT and lipid transfer from LpB by LTP-1 to these HDL particles resulted in the core lipid-rich transformed Lp(AI w AII) particle observed. The conversion of lecithin to lysolecithin in the LCAT reaction, and the subsequent association of lysolecithin with the apoA-I-free plasma fraction, provides an explanation for the observed decrease in phospholipid in these particles.

For Lp(AI w/o AII), we postulate that the two major native subpopulations, Lp(AI w/o $AII)_1$ and Lp(AI w/o $AII)_2$ were independently transformed to the single 9.2-nm subpopulation observed. The transformation of Lp(AI w/o $AII)_1$ to the slightly smaller 9.2-nm subpopulation was due mainly to cholesterol esterification and the loss of surface phospholipid as lysolecithin within the particle. Transformation of Lp(AI w/o $AII)_2$, and particles smaller than Lp(AI w/o $AII)_2$, to the 9.2-nm population could conceivably occur by the mechanism of particle fusion proposed for both the small HDL in LCAT-deficient plasma and for synthetic model apoA-I-phosphatidylcholine complexes, upon addition of exogenous LCAT to either system (38, 39). Fusion of small Lp(AI w/o AII) subpopulations induced by cholesterol esterification results in their near absence after incubation. In the incubation study where purified LCAT was added to LCAT-free plasma, transformation of Lp(AI w/o $AII)_2$ was less than transformation of Lp(AI w/o $AII)_1$, and of particles smaller than Lp(AI w/o $AII)_2$ (Fig. 5B). This further suggests independent transformation of the size subpopulations of Lp(AI w/o AII).

In conclusion, the present study clearly demonstrates the occurrence of multiple mechanisms in the transformation of HDL subpopulations in vitro. Transformation of Lp(AI w AII) involves both cholesterol esterification and additional lipids from LpB. Transformation of Lp(AI w/o AII), on the other hand, requires cholesterol esterification but not additional lipids. Although we have not directly addressed the role of LTP-1 in this study, that the transfer of lipids from LpB is necessary for the enlargement of Lp(AI w AII) suggests that LTP-1 probably plays some role in this event. However, data obtained with LCAT-free and apoB-free plasma in which LTP-1 was present showed that LTP-1 can only induce limited enlargement of Lp(AI w AII) particles in the absence of LCAT. Since the transformation of Lp(AI w/o AII) particles did not require the transfer of lipids from LpB, the role of LTP-1 in the transformation of this population of HDL particles may involve modulating the lipid contents of the various Lp(AI w/o AII) subpopulations during transformation. Evidence for the existence of an HDL conversion factor has been reported recently (40). Whether this putative factor plays a role in the transformation of Lp(AI w AII) and Lp(AI w/o AII) described in this study is not known. Upon incubation of LCAT-free plasma, we often noticed a decrease in Lp(AI w/o $AII)_2$ and an increase in particles smaller than Lp(AI w/o $AII)_2$ (Fig. 4). The significance of these changes is currently being investigated. Finally, our observation that during the LCAT reaction, lipids from LpB were transferred primarily to Lp(AI w AII) supports the concept that the two major apolipoprotein-specific HDL subpopulations—Lp(AI w AII) and Lp(AI w/o AII)—are metabolically distinct.

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