Remodelling of reconstituted high density lipoproteins by lecithin:cholesterol acyltransferase

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Abstract Discoidal reconstituted high density lipoproteins (rHDL) with a diameter of 7.9 nm, a molar ratio of egg phosphatidylcholine (PC): unesterified cholesterol (UC): cholesteryl esters (CE): apolipoprotein (apo) A-I of 33:7:0:1 and containing two molecules of apoA-I per particle were incubated with lecithin:cholesterol acyltransferase (LCAT) in the presence of low density lipoproteins as a source of additional UC and PC for the LCAT reaction. After 24 h of incubation, the rHDL had a diameter of 8.8 nm, a molar ratio of PC:UC:CE:apoA-I of 16:3:23:1 and contained three rather than two molecules of apoA-I per particle. The fact that there was no change in the concentration of rHDL-associated apoA-I indicated that the increase from two to three molecules of apoA-I per particle was achieved at the expense of a one-third reduction in the number of rHDL particles in a process that must have involved particle fusion. When the incubations were repeated in the presence of exogenous, lipid-free apoA-I, the resulting rHDL were identical in size and composition to those generated in its absence. Under these conditions, however, the increase from two to three molecules of apoA-I per rHDL particle coincided with a 50% increase in the concentration of rHDL-associated apoA-I. Thus, when lipid-free apoA-I is available, the LCAT-mediated increase in number of apoA-I molecules per rHDL particle is achieved by a direct incorporation of lipid-free apolipoprotein without any need for particle fusion and therefore without a reduction in the number of rHDL particles.–**Liang, H-Q., K-A. Rye, and P. J. Barter.** Remodelling of reconstituted high density lipoproteins by lecithin:cholesterol acyltransferase. *J. Lipid Res.* 1996. **37:** 1962–1970.

Supplementary key words apoA-I • reconstituted HDL • HDL fusion • HDL remodelling

There is now overwhelming evidence that high density lipoproteins (HDL) are anti-atherogenic (1, 2). There is also clear evidence that the HDL fraction is heterogeneous, consisting of several subpopulations of particles that differ in size, density, electrophoretic mobility, and in composition of both lipids and apolipoproteins. This heterogeneity has important implications in that specific subpopulations differ both in physiological function (3) and in their abilities to protect against the development of atherosclerosis (4, 5).

Much of the heterogeneity of HDL is the consequence of remodelling of the lipoproteins by plasma factors (6). For example, when HDL interact with triglyceride-rich lipoproteins and the cholesteryl ester transfer protein (CETP), there is a reduction in HDL cholesteryl ester content, a decrease in HDL size, and a dissociation of apolipoprotein (apo) A-I from the HDL (7). Conversely, when lecithin:cholesterol acyltransferase (LCAT) increases the cholesteryl ester content of HDL, there is an associated increase in HDL size (8–10) and an increase in the number of molecules of apoA-I per particle (9).

Two mechanisms have been proposed for the LCAT-mediated increase in the number of apoA-I molecules per particle. According to one view, as LCAT increases the cholesteryl ester content of HDL, it also promotes particle fusion (9) to form large HDL in which the number of molecules of both cholesteryl esters and apoA-I are increased. An alternate view holds that an LCAT-mediated increase in HDL cholesteryl esters is accompanied by the direct incorporation of lipid-free apoA-I into the HDL (11). The experiments described in this report seek to differentiate between these two proposed mechanisms. In studies using homogeneous preparations of reconstituted HDL (rHDL) it has been found that both the HDL fusion model and the direct incorporation model operate, with evidence suggesting that the direct incorporation of apoA-I into HDL takes precedence over particle fusion.

Abbreviations: apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; PC, phosphatidylcholine; rHDL, reconstituted high density lipoproteins; TBS, Tris-buffered saline; UC, unesterified cholesterol.

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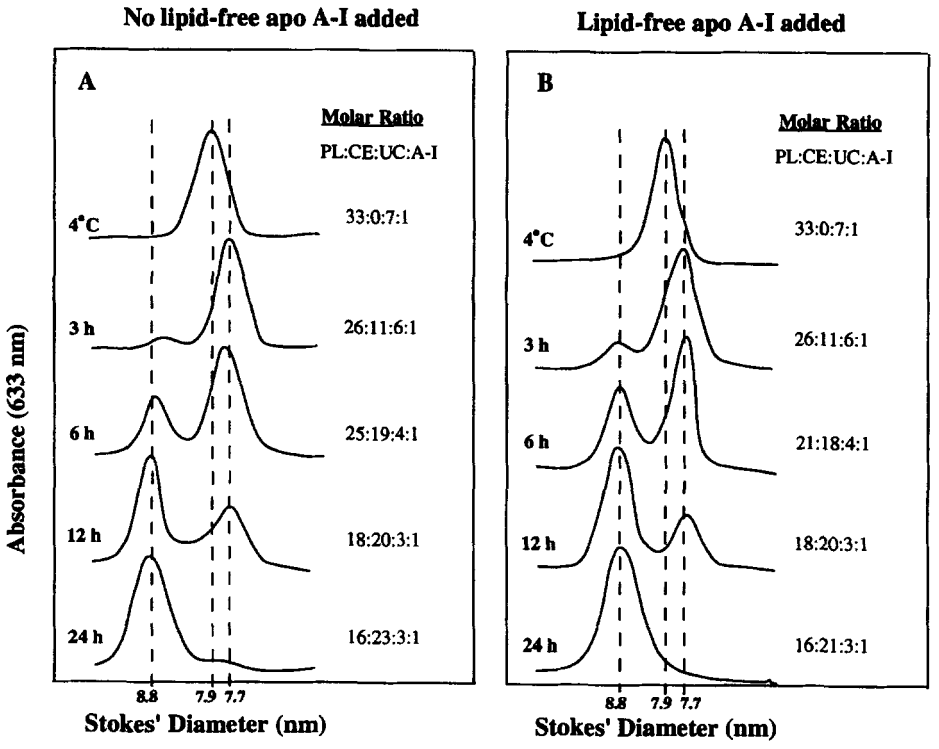

Fig. 1. Changes in size and composition of rHDL during incubation with LCAT in the absence and in the presence of added lipid-free apoA-I. Incubations were conducted at 37°C for 0 to 24 h. Incubation mixtures contained discoidal rHDL (apoA-I final concentration 0.1 mg/ml), LDL (apoB final concentration 0.4 mg/ml), LCAT (2.3 ml), bovine serum albumin (18 mg/ml final concentration), and β-mercaptoethanol (final concentration 14 mM). In the incubations shown in panel B, the mixtures were supplemented by the addition of lipid-free apoA-I at a final concentration of 0.05 mg/ml, equivalent to 50% of the apoA-I in the discoidal rHDL. The final incubation volume was 2.65 ml. After incubation, the fraction of d 1.07–1.25 g/ml was isolated by ultracentrifugation and assayed for composition. The d < 1.25 g/ml fraction was also recovered and subjected to gradient gel electrophoresis as described in Methods. The profiles showing the particle size distribution of rHDL were obtained by laser densitometric scans of stained gels.

METHODS

Isolation of lipoproteins and apoA-I

Human plasma for the isolation of lipoproteins, apoA-I, and LCAT was donated by the Transfusion Service at the Royal Adelaide Hospital. Lipoprotein fractions were separated ultracentrifugally at 4°C (12) in the density intervals, 1.019–1.055 g/ml for LDL and 1.07–1.25 g/ml for HDL. In each case samples were subjected to two successive spins at the higher density to minimize contamination by plasma proteins. The LDL and HDL were extensively dialyzed against Tris-buffered saline (TBS), pH 7.4, containing 0.01 M Tris, 0.15 M NaCl, 0.006% (w/v) NaN_3, and 0.005% (w/v) EDTA-Na_2. ApoA-I was purified to homogeneity as described previously (13).

Preparation of reconstituted HDL

Discoidal rHDL were prepared from egg phosphatidylcholine (PC), unesterified cholesterol (UC), and apoA-I (molar ratio 80:10:1) by the cholate dialysis method (14, 15). Before use, the discoidal rHDL were incubated at 37°C for 24 h with LDL at a protein ratio of 4:1 (apoB: apoA-I) and then subjected to size exclusion chromatography on a prepacked Superose 6 HR 10/30 column (Pharmacia LKB Biotechnology). This approach provided a homogeneous population of discoidal rHDL of diameter 7.9 nm as determined by non-denaturing polyacrylamide gradient gel electrophoresis (see below). The molar ratio of PC:UC:apoA-I was 33:7:1.

Isolation of LCAT

LCAT was isolated from human plasma as previously described (16, 17). No apoA-I was detected in the purified LCAT. Activity of LCAT was determined as described by Piran and Morin (18) using discoidal rHDL labeled with ^3H in the cholesterol moiety as a substrate. Activity was expressed as nmol cholesterol esterified per hour per ml of the LCAT solution. The LCAT samples

TABLE 1. Characterization of the rHDL formed during incubation with LCAT and LDL in the presence and absence of lipid-free apoA-I

Incubation Conditions	Molecules of Constituents/Particle				Electrophoretic Mobility[b]	Morphology[c]	Relative Number of Particles[d]
	PL	UC	CE	ApoA-I[a]			
4°C (no lipid-free apoA-I)	68	14	1	2	0.43	discoidal	100
4°C (+ lipid-free apoA-I)[e]	66	15	1	2	*f*	*f*	99
37°C (no lipid-free apoA-I)	52	9	67	3	0.52	spherical	62
37°C (+ lipid-free apoA-I)[e]	53	8	63	3	0.52	spherical	96

All incubations were for 24 h and contained rHDL (final apoA-I concentration 0.1 mg/ml), LDL (final apoB concentration 0.4 mg/ml), LCAT (2.3 ml), bovine serum albumin (final concentration 18 mg/ml), and β-mercaptoethanol (final concentration 14 mM). The final incubation volume was 2.65 ml. After incubation, rHDL were recovered by ultracentrifugation in the density interval 1.07–1.25 g/ml. The results represent the means of three experiments.

[a]The numbers of apoA-I molecules per rHDL particle were determined by cross-linking. The numbers of molecules of other constituents were calculated relative to apoA-I.

[b]Electrophoretic mobility was calculated as described in the Methods section.

[c]Determined by electron microscopy.

[d]The relative numbers of rHDL particles were calculated from the relative concentrations of rHDL-associated apoA-I as shown in Table 2 and the number of apoA-I molecules per rHDL particle.

[e]The amount of added lipid-free apoA-I was equivalent to 50% of the apoA-I in the discoidal rHDL.

[f]Not determined.

used in this study were stored in aliquots at -70°C until used. The LCAT activity (91 nmol cholesterol esterified per hour per ml) was checked routinely at the time of each experiment; activity remained stable for the duration of the study.

Experimental conditions

Details of individual incubations are provided in the legends to the tables and figures. Incubation mixtures were placed in sealed plastic tubes and either kept at 4°C or incubated at 37°C in a shaking water bath. Incubations were terminated by placing the tubes on ice. After incubation, samples were subjected to ultracentrifugation to recover the fraction of d < 1.25 g/ml (total lipoproteins) or the fraction of 1.07 g/ml < d < 1.25 g/ml (rHDL). When isolating the d 1.07–1.25 g/ml fraction, samples were subjected to two successive spins at the lower density to ensure complete removal of LDL. An absence of contaminating LDL was confirmed by the findings that: *i)* apoB was unmeasurable in an assay that is quantitative at concentrations down to 6 μg per ml; *ii)* there were no beta-migrating particles detectable when the samples were subjected to agarose gel electrophoresis; and *iii)* there were no detectable particles of LDL size when the samples were subjected to gradient gel electrophoresis on 3–35% non-denaturing gels as described below. The d 1.07–1.25 g/ml fraction was assayed for lipids and apoA-I to determine rHDL composition. It was also subjected to cross-linking to determine the number of molecules of apoA-I per particle, to agarose gel electrophoresis, and to electron microscopy. The fraction of d < 1.25 g/ml was assayed for apoA-I to determine the concentration of lipid-associated apoA-I (as distinct from lipid-free apoA-I which has a density > 1.25 g/ml). It was established in preliminary experiments that the fraction of d < 1.07 g/ml did not contain apoA-I under any of the experimental conditions used. Thus, the concentration of apoA-I in the fraction of d < 1.25 g/ml equates with that associated with rHDL. The fraction of d < 1.25 g/ml was also subjected to non-denaturing gradient gel electrophoresis to monitor rHDL size.

Non-denaturing polyacrylamide gradient gel electrophoresis

The particle size distribution of rHDL was determined by electrophoresis on 3–35% non-denaturing polyacrylamide gels (Gradipore, Australia) and laser

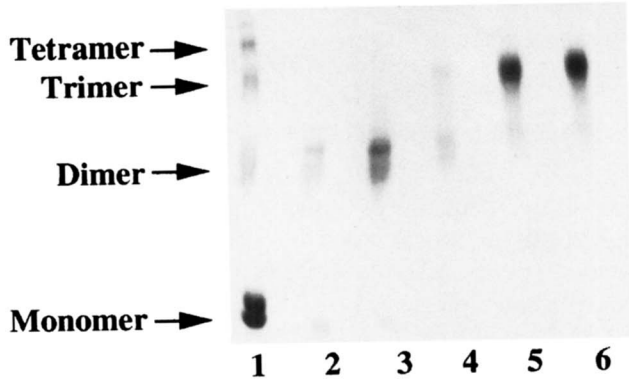

Fig. 2. Cross-linking of rHDL. Aliquots of rHDL were maintained at 4°C or incubated at 37°C under various conditions. At the end of the incubations, the rHDL were isolated by ultracentrifugation as described in the legend to Table 1 and cross-linked as described in the Methods section. The cross-linked samples were then subjected to electrophoresis on a 3–35% SDS gradient gel. Track 1 shows cross-linked, lipid-free apoA-I. Tracks 2 and 3 represent, respectively, rHDL which were either maintained at 4°C or incubated for 24 h at 37°C with no additions. The rHDL in tracks 4 and 5 were mixed with LDL and LCAT, as in Table 1, and incubated at 37°C for 3 h and 24 h, respectively. Track 6 shows rHDL after incubation at 37°C for 24 h with LDL, LCAT and exogenous lipid-free apoA-I as in Table 1.

densitometric scanning as described (19). Particle diameters were determined by reference to a calibration kit containing thyroglobulin (Stokes' radius 8.50 nm), ferritin (6.1 nm), lactate dehydrogenase (4.08 nm), and bovine serum albumin (3.55 nm) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Agarose gel electrophoresis

Lipoprotein and apolipoprotein samples were applied to 0.6% agarose gels in a Bio-Rad Mini Sub gel electrophoresis system (Hercules, CA) as described (20). The electrophoresis was run at 100 volts for 1 h at room temperature. The gels were fixed in an ethanol–water–acetic acid 60:30:10 (v/v/v) solution and stained with Coomassie Blue G-250. Electrophoretic mobilities were calculated by dividing the electrophoretic velocity (migration distance (μm)/time (s)) by the electrophoretic potential (voltage (v)/length of gel (cm)) (21).

$$\text{Mobility} = \frac{\text{Migration distance (μm)} / \text{Time (s)}}{\text{Voltage (v)} / \text{Length of gel (cm)}}$$

Cross-linking of apoA-I

The number of apoA-I molecules associated with rHDL was determined by cross-linking with bis (sulfosuccinimidyl) suberate (BS) (Pierce Chemical Co., Rockford, IL) as described (19, 22).

Electron microscopy

Electron microscopy was performed as described previously (23) with minor modification. Samples were diluted to a protein concentration of 0.1 mg/ml with a solution containing 125 mM ammonium acetate, 2.6 mM ammonium hydrogen carbonate, and 0.26 mM EDTA-Na_2, pH7.4. The samples were dialyzed against 2 × 1 L of the same buffer and then negatively stained with 2% (w/v) sodium phosphotungstate (pH 7.4) by placing equal volumes of sample and stain on a Butvar-coated 300-mesh copper grid (Probing & Structure Pty. Ltd., Australia). The sample and stain were drawn off with filter paper after a few minutes, leaving a thin film that was dried at room temperature prior to examination under the electron microscope (Phillips CM200 Transmission Electron Microscope).

Chemical analyses

All chemical assays were performed on a Cobas Fara Centrifugal Analyser (Roche Diagnostics, Zurich, Switzerland). Concentrations of total cholesterol, free cholesterol, and phospholipid were measured using enzymatic kits from Boehringer Mannheim (Germany). The concentration of esterified cholesterol was calculated as the difference between the concentrations of total (esterified plus free) cholesterol and unesterified cholesterol. Concentrations of apoA-I and apoB were measured immunoturbidmetrically using antisera to human apoA-I and apoB raised in sheep (Boehringer Mannheim, Germany). The assays were standardized using appropriate dilutions of apo calibration serum (Boehringer Mannheim, Germany). These assays have the capacity to quantitate apoA-I and apoB down to concentrations of 10 μg/ml and 6 μg/ml, respectively.

RESULTS

Effects of LCAT on the size and composition of rHDL (Fig. 1, Table 1)

Prior to being incubated with LCAT, the discoidal rHDL had an apparent of diameter of 7.9 nm and a molar ratio of PC: UC: apoA-I of 33:7:1 (**Fig. 1A**). They contained two molecules of apoA-I per particle as determined by cross-linking (**Table 1**) and appeared as

TABLE 2. Effect of LCAT on rHDL-associated apoA-I

Experiment	Recovery of ApoA-I in d < 1.25 g/ml Fraction (Percentage of control)[a]		
	4°C, + LCAT + apoA-I[b]	37°C, + LCAT - apoA-I[b]	37°C, + LCAT + apoA-I[b]
1	100	98	143
2	98	98	148
3	103	102	150
4	102	103	138
5	98	99	141
6	94	105	144
Mean ± SD	99.2 ± 3.3	100.8 ± 2.9	144.0 ± 4.4

Discoidal rHDL (final apoA-I concentration 0.1 mg/ml), LDL (final apoB concentration 0.4 mg/ml), LCAT (2.1 ml), bovine serum albumin (final concentration 18 mg/ml), and β-mercaptoethanol (final concentration 14 mM) were incubated at 37°C for 24 h. The final incubation volume was 2.5 ml. After incubation, rHDL-associated apoA-I was recovered as the ultracentrifugal fraction of d < 1.25 g/ml (see Methods).

[a]The amount of apoA-I associated with rHDL is expressed as a percentage of that in a control sample kept at 4°C with no additions.

[b]Lipid-free apoA-I was added (final concentration 0.05 mg/ml) in an amount equivalent to 50% of the apoA-I in the discoidal rHDL.

stacked discs in electron micrographs (result not shown). When incubated for 3 h at 37°C with LCAT plus LDL (as a source of additional UC and PC for the LCAT reaction), the generation of cholesteryl esters (CE) coincided with the rHDL being converted into smaller particles with a diameter of 7.7 nm and a molar ratio of PL: CE: UC: apoA-I of 26:11:6:1 (Fig. 1A). Most of these particles still contained two molecules of apoA-I per particle (**Fig. 2**) but now contained a substantial amount of CE and were probably spherical. Beyond 3 h, the 7.7 nm particles were converted progressively into particles of diameter 8.8 nm. After 24 h, virtually all of the rHDL had a diameter of 8.8 nm (Fig. 1A) and a molar ratio of PL: CE: UC: apoA-I of 16:23:3:1 (Fig. 1A). There was also an increase from two to three apoA-I molecules per particle as determined by cross-linking (Fig. 2). These particles appeared as spheres in electron micrographs (result not shown). The surface charge of the particles was assessed by agarose gel electrophoresis. The non-incubated, discoidal rHDL had an electrophoretic mobility (prebeta) identical to that of lipid-free apoA-I (result not shown). The spherical rHDL formed during 24 h of incubation at 37°C with LCAT and LDL had an increased electrophoretic mobility compared to that of the discoidal rHDL (Table 1). The spherical rHDL migrated to an alpha position comparable to that of native HDL.

When the incubation mixtures of rHDL, LDL, and LCAT were supplemented by the addition of an amount of lipid-free apoA-I equivalent to 50% of that present in the rHDL, virtually identical results were obtained. The changes in particle size and composition were the same as those in the incubations conducted without the added lipid-free apoA-I (Fig. 1B). As in the absence of lipid-free apoA-I, 24 h of incubation converted the prebeta-migrating, discoidal rHDL into alpha-migrating, spherical particles in which the number of apoA-I molecules per particle was increased from two to three.

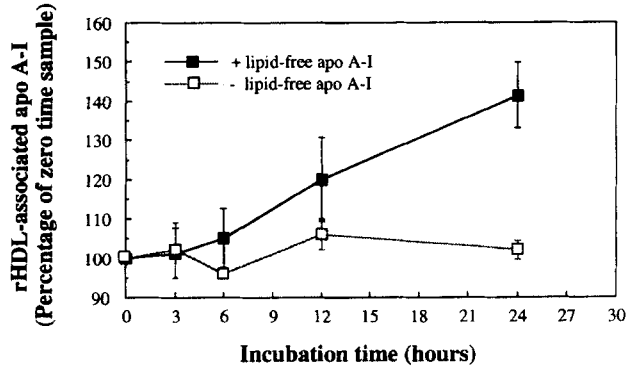

Fig. 3. Time course of the incorporation of lipid-free apoA-I into rHDL during incubation with LCAT. Incubation conditions were as described in the legend to Fig. 1. After incubation, rHDL-associated apoA-I was recovered as the ultracentrifugal fraction of d < 1.25 g/ml (see Methods). Concentrations of rHDL-associated apoA-I are expressed as percentages of those in samples kept at 4°C with no additions. The data points and bars represent the respective means and standard deviations of four experiments.

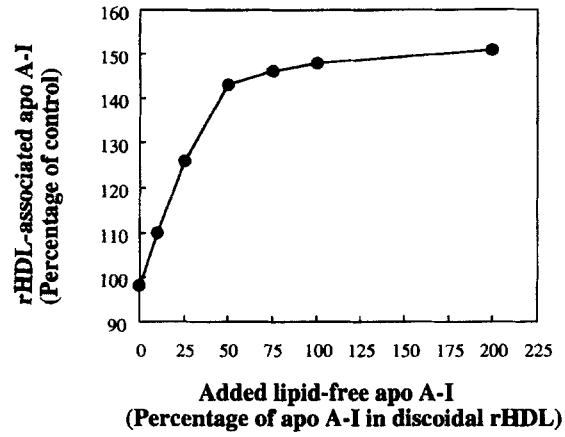

Fig. 4. Concentration dependence of the incorporation of lipid-free apoA-I into rHDL during incubation with LCAT. Incubations were conducted at 37°C for 24 h. Incubation mixtures contained discoidal rHDL (final apoA-I concentration 0.1 mg/ml), LDL (final apoB concentration 0.4 mg/ml), LCAT (0.85 ml), bovine serum albumin (final concentration 18 mg/ml), and β-mercaptoethanol (final concentration 14 mM). Exogenous, lipid-free apoA-I was added to the mixtures at concentrations ranging from 0 to 0.2 mg/ml, equivalent to 0 to 200% of that in the discoidal rHDL. The final incubation volume was 1.0 ml. After incubation, rHDL-associated apoA-I was recovered as the ultracentrifugal fraction of d < 1.25 g/ml (see Methods). Concentrations of rHDL-associated apoA-I are expressed as percentages of those in samples kept at 4°C with no additions. Each point represents the mean of two experiments.

When these incubations were conducted in the presence of LDL but in the absence of LCAT there was no change in the size or composition of the original rHDL, whether or not lipid-free apoA-I was added to the incubation mixture (result not shown).

As stated above, when discoidal rHDL were incubated with LCAT and LDL, the number of apoA-I molecules per particle increased from two to three, regardless of whether the incubation was supplemented with lipid-free apoA-I (Table 1). In the absence of added lipid-free apoA-I, this increase in the number of apoA-I molecules occurred without any alteration in the concentration of rHDL-associated apoA-I (**Table 2**). This indicated that the number of rHDL particles must have been reduced by one third (Table 1). In the presence of lipid-free apoA-I, on the other hand, the increase from two to three in the number of apoA-I molecules per particle was accompanied by a 44% increase in the concentration of rHDL-associated apoA-I (Table 2), indicating that under these conditions the number of rHDL particles was essentially unchanged (Table 1).

Incorporation of lipid-free apoA-I into rHDL (Table 2)

While the addition of lipid-free apoA-I had no observable effect on the composition, size, and electrophoretic

mobility of the rHDL formed during incubation of discoidal rHDL with LCAT and LDL (Table 1, Fig. 1), there was, nevertheless, an obvious incorporation of the lipid-free apoA-I into the HDL fraction (Table 2). When incubations were conducted in the absence of added lipid-free apoA-I, the concentration of rHDL-associated apoA-I did not change (Table 2). However, when exogenous, lipid-free apoA-I was included in the incubation mixture in an amount equivalent to 50% of that in the discoidal rHDL, there was a 44% increase in the concentration of rHDL-associated apoA-I (Table 2). This apparent quantitative incorporation of lipid-free apoA-I into the rHDL was supported by the observation in samples incubated at 37°C in the presence of LCAT that >90% of the apoA-I in the total incubation mixtures was recovered in the ultracentrifugal fraction of d < 1.25 g/ml, with no measurable apoA-I being found in the fraction of d > 1.25 g/ml, whether or not the samples had been supplemented with exogenous, lipid-free apoA-I (result not shown). In fact, measurable amounts of apoA-I were recovered in the d < 1.25 g/ml fraction only in those samples that were supplemented with exogenous apoA-I and either kept at 4°C or incubated at 37°C in the absence of LCAT. Under these circumstances >90% of the added lipid-free apoA-I was recovered in the d > 1.25 g/ml fraction (results not shown).

Time course of the incorporation of lipid-free apoA-I into rHDL (Fig. 3)

The time course of incorporation of exogenous, lipid-free apoA-I into rHDL during incubation with LCAT and LDL (**Fig. 3**) roughly paralleled the formation of the 8.8 nm particles (Fig. 1B). After 12 h of incubation, when about one-third of the rHDL had been converted into particles with a diameter of 8.8 nm (Fig. 1B), the concentration of rHDL-associated apoA-I had increased by about 20%, while after 24 h, when all of the rHDL had been converted to 8.8 nm particles, the rHDL-associated apoA-I concentration was increased by more than 40% (Fig. 3).

Concentration-dependence of the incorporation of lipid-free apoA-I into rHDL (Fig. 4)

To determine whether the concentration of lipid-free apoA-I is a limiting factor in its incorporation into rHDL, mixtures of discoidal rHDL, LCAT, and LDL were supplemented with increasing amounts of apoA-I. At concentrations up to about 50% of that in the discoidal rHDL, there was a quantitative incorporation of the lipid-free apoA-I into rHDL (**Fig. 4**). At this point, virtually all of the rHDL had been converted from particles containing two molecules of apoA-I into particles containing three molecules of apoA-I (Fig. 2). Increasing the concentration of the exogenous apoA-I to levels above 50% of that initially in the rHDL did not further increase its incorporation (Fig. 4). Under these conditions the surplus was recovered in the lipoprotein-free fraction of d > 1.25 g/ml. Thus, the amount of lipid-free apoA-I is a limiting factor in its incorporation into rHDL only when present at concentrations less than 50% of that initially in the rHDL.

Effect of LCAT activity on the incorporation of lipid-free apoA-I into rHDL (Fig. 5)

Not only was the incorporation of exogenous, lipid-free apoA-I into rHDL during incubation with LDL dependent on the presence of LCAT, but the magnitude of the incorporation was a function of the amount of LCAT added (**Fig. 5**). In incubations that did not contain added lipid-free apoA-I, however, the concentration of rHDL-associated apoA-I was uninfluenced by the presence of LCAT.

DISCUSSION

The effects of LCAT on rHDL in the present study are qualitatively similar to those reported previously for native HDL (11). In each case LCAT increased both the cholesteryl ester content and the size of HDL and also promoted the incorporation of lipid-free apoA-I into HDL. By taking advantage of the homogeneity of the

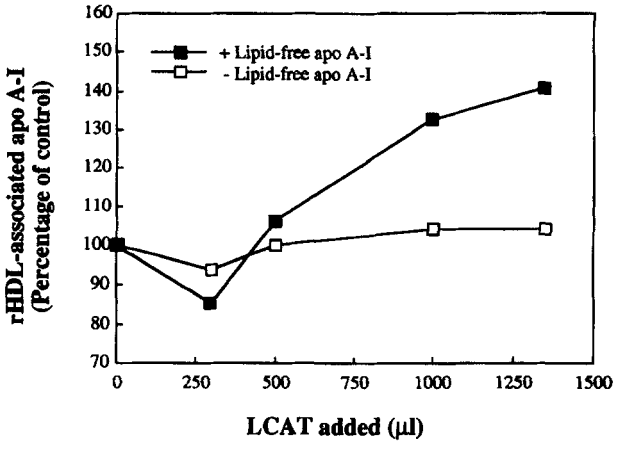

Fig. 5. Effects of LCAT activity on the incorporation of lipid-free apoA-I into rHDL. Incubations were conducted at 37°C for 24 h. The incubation mixtures contained discoidal rHDL (final apoA-I concentration 0.1 mg/ml), LDL (final apoB concentration 0.4 mg/ml), bovine serum albumin (final concentration 18 mg/ml), and β-mercaptoethanol (final concentration 14 mM). Half of the mixtures contained no added lipid-free apoA-I, while the others were supplemented by the addition of lipid-free apoA-I at a final concentration of 0.05 mg/ml, equivalent to 50% of that in the discoidal rHDL. LCAT was added in amounts ranging from 0 to 1.35 ml. The final incubation volume was 1.57 ml. After incubation, rHDL-associated apoA-I was recovered as the ultracentrifugal fraction of d < 1.25 g/ml (see Methods). Concentrations of rHDL-associated apoA-I are expressed as percentages of that in a sample kept at 4°C with no additions. The points are means obtained from duplicate incubations in a single experiment.

rHDL, we were also able to demonstrate in the present studies that the number of molecules of apoA-I per particle increased during the incubation with LCAT (Table 1).

In confirmation of previous studies reported by Nichols et al. (9), discoidal rHDL containing two molecules of apoA-I per particle are initially converted by LCAT into small spherical rHDL that still have two apoA-I molecules per particle. These small spherical rHDL are subsequently converted by LCAT into larger spherical rHDL with three molecules of apoA-I per particle. In the absence of a source of additional apoA-I, an increase from two to three in the number of apoA-I molecules per particle must be accompanied by a reduction in the number of HDL particles. Indeed, it has been postulated that LCAT promotes a fusion of small, spherical, two apoA-I-containing HDL particles into half the number of larger HDL particles, each containing four molecules of apoA-I (9). It has been further suggested that these larger HDL are unstable and spontaneously shed a molecule of apoA-I to form more stable particles, each of which now contains three molecules of apoA-I (9). The end result of this process is a halving of the number of HDL particles and the appearance of one quarter of the apoA-I in a lipoprotein-free form. While such a result has been reported (9), it has not been confirmed in the present studies.

When rHDL were incubated with LCAT in the absence of added lipid-free apoA-I in the present studies, an increase in number of apoA-I molecules per particle from two to three was confirmed. However, in contrast to the previous report (9), the number of particles was reduced by only one third rather than one by half and there was no appearance of lipid-free apoA-I. One interpretation of the present result is that any apoA-I that might have dissociated from unstable fusion products was subsequently incorporated into other rHDL which were increasing in size, thus providing an alternate mechanism for increasing the number of apoA-I molecules per particle from two to three. If such a process of direct incorporation of apoA-I was energetically more favorable than particle fusion, it follows that lipid-free apoA-I would not accumulate and the number of HDL particles would be reduced, as illustrated in **Fig. 6C** and as observed (Table 1), by one third rather than by the one half predicted by fusion alone (Fig. 6A).

The validity of this proposed process of direct incorporation of lipid-free apoA-I into HDL was tested in the present study by adding exogenous lipid-free apoA-I to the incubation mixtures. If direct incorporation of apoA-I into small rHDL particles were to take precedence over particle fusion during interaction with LCAT, the addition of exogenous lipid-free apoA-I would abolish particle fusion. Under these circumstances, the increase in rHDL apoA-I content would occur without a decrease in the number of HDL particles (Fig. 6B). This is precisely what was found experimentally (Table 1). Thus, the results of the present study are consistent with the existence of two mechanisms by which the apoA-I content of HDL particles can be increased during interactions with LCAT, with evidence suggesting that a direct incorporation of lipid-free apoA-I takes precedence over particle fusion.

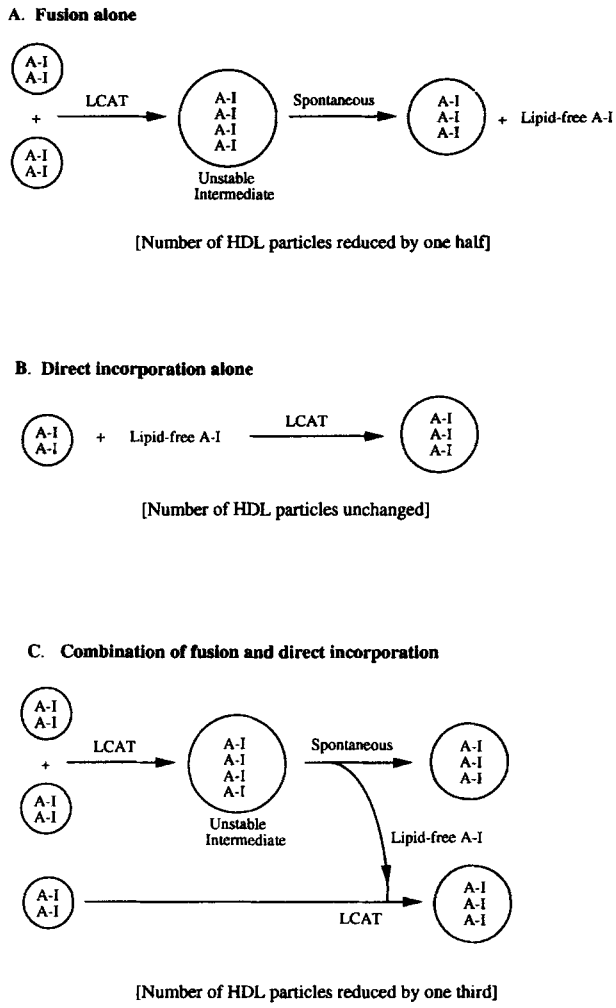

Fig. 6. Hypothetical models of the mechanisms by which LCAT increases the number of apoA-I molecules per HDL particle from two to three. A: Fusion Model. LCAT mediates the fusion of small HDL particles containing two molecules of apoA-I to form half the number of larger but unstable particles that contain four molecules of apoA-I. These larger particles spontaneously shed a molecule of apoA-I to form more stable HDL with three molecules of apoA-I per particle; a by-product of this process is the release of 25% of the apoA-I into a lipid-free pool. B: Direct Incorporation Model. LCAT mediates a direct incorporation of lipid-free apoA-I into HDL, increasing the number of molecules of apoA-I per particle from two to three without a reduction in the number of HDL particles. C: Combination of the Fusion and Direct Incorporation Models. This combination increases the number of apoA-I molecules per HDL particle from two to three and decreases the number of HDL particles by one third.

There are several potential sources of lipid-free apoA-I in plasma. In addition to that which may be released from unstable fusion products as outlined above, lipid-free apoA-I may be secreted directly from the liver and intestine (24) or released from HDL by the action of lipases (25, 26). Lipid-free apoA-I also dissociates from native HDL after a CETP-mediated reduction in their cholesteryl ester content and particle size (7). The fact that this dissociated, lipid-free apoA-I is reincorporated into the depleted HDL during their subsequent interaction with LCAT has led to the postulation of a cycle in which alternating CETP-mediated decreases and LCAT-mediated increases in the cholesteryl ester content and the size of HDL particles are accompanied by the cycling of apoA-I between HDL and a lipid-free pool (11).

These studies contribute substantially to our understanding the remodelling of HDL by LCAT. They also have several clinical implications. The existence of a process of direct incorporation of lipid-free apoA-I into HDL provides a means for retaining in the HDL fraction apoA-I that might otherwise be lost from the circulation by excretion in urine (27, 28). It also provides a means for maintaining HDL particle numbers and thus for transporting larger amounts of cholesteryl esters that might otherwise be transferred to potentially atherogenic apoB-containing lipoproteins.

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