

# The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion

Sari LUSA,\* Matti JAUHAINEN,†‡ Jari METSO,† Pentti SOMERHARJU\* and Christian EHNHOLM†

\*Institute of Biomedicine, Department of Medical Chemistry, University of Helsinki, Helsinki, Finland, and †National Public Health Institute, Department of Biochemistry, Mannerheimintie 166, FI-00300, Helsinki, Finland

1. Phospholipid transfer protein (PLTP) mediates conversion of high-density lipoprotein (HDL<sub>3</sub>) to large particles, with concomitant release of apolipoprotein A-I (apoA-I). To study the mechanisms involved in this conversion, reconstituted HDL (rHDL) particles containing either fluorescent pyrenylacyl cholesterol ester (PyrCE) in their core (PyrCE-rHDL) or pyrenylacyl phosphatidylcholine (PyrPC) in their surface lipid layer (PyrPC-rHDL) were prepared. Upon incubation with PLTP they behaved as native HDL<sub>3</sub>, in that their size increased considerably. 2. When PyrPC-rHDL was incubated with HDL<sub>3</sub> in the presence of PLTP, a rapid decline of the pyrene excimer/monomer fluorescence ratio (E/M) occurred, demonstrating that PLTP induced mixing of the surface lipids of PyrPC-rHDL and HDL<sub>3</sub>. As this mixing was almost complete before any significant increase in HDL particle size was observed, it represents PLTP-mediated phospholipid transfer or exchange that is not directly coupled to the formation of large HDL particles. 3. When core-labelled

PyrCE-rHDL was incubated in the presence of PLTP, a much slower, time-dependent decrease of E/M was observed, demonstrating that PLTP also promotes mixing of the core lipids. The rate and extent of mixing of core lipids correlated with the amount of PLTP added and with the increase in particle size. The enlarged particles formed could be visualized as discrete, non-aggregated particles by electron microscopy. Concomitantly with the appearance of enlarged particles, lipid-poor apoA-I molecules were released. These data, together with the fact that PLTP has been shown not to mediate transfer of cholesterol esters, strongly suggest that particle fusion rather than (net) lipid transfer or particle aggregation is responsible for the enlargement of HDL particles observed upon incubation with PLTP. 4. ApoA-I rHDL, but not apoA-II rHDL, were converted into large particles, suggesting that the presence of apoA-I is required for PLTP-mediated HDL fusion. A model for PLTP-mediated enlargement of HDL particles is presented.

## INTRODUCTION

A number of epidemiological studies have demonstrated that plasma low-density lipoprotein (LDL) levels correlate positively with coronary heart disease (CHD) predisposition while high-density lipoprotein (HDL) levels display a strong inverse correlation [1–4]. It is therefore important to understand what regulates the distribution of cholesterol between different lipoprotein classes.

Human plasma HDLs comprise a spectrum of lipoprotein particles in the density range of 1.063–1.21 g/ml. These particles have a hydrophobic core of cholesterol esters and triacylglycerols covered by polar lipids and apolipoproteins. Apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II) are the main apolipoproteins of HDL. The HDL fraction is heterogeneous in terms of size, composition and density; the two main classes being HDL<sub>2</sub> and HDL<sub>3</sub> [5–9]. An increase in the concentration of HDL<sub>2</sub> has been connected to the anti-atherogenic property of HDL [10,11]. It is, however, clear that additional information on the formation, interconversion and metabolic relationships between the different subclasses of HDL has to be obtained before the mechanism underlying the protective role of HDL in atherogenesis can be understood.

The HDL subpopulations are interconvertible and several plasma factors have been shown to participate in the interconversion [12–17]. Recent studies have demonstrated that plasma phospholipid transfer protein (PLTP) *in vitro* promotes conversion of HDL<sub>3</sub> into a population of large particles with the simultaneous release of apoA-I molecules [18,19]. The complete cDNA sequence encoding the human PLTP was reported recently [20].

To study the mechanism of the PLTP-mediated size conversion of HDL we prepared reconstituted HDL (rHDL) particles containing either pyrenylacyl phosphatidylcholine (PyrPC) as a surface lipid marker or pyrenylacyl cholesterol ester (PyrCE) as a core lipid marker. These pyrenyl derivatives display two components in their fluorescence emission spectrum: one that originates from excited monomers and the other which originates from excimers that are formed upon collision of an excited monomer with a ground-state one [21]. The ratio of excimer to monomer intensities (E/M) depends on the frequency of collisions between pyrenyl residues, and is thus proportional to the local concentration of the pyrenyl derivative. Therefore, dilution of the pyrenyl compound, whether due to lipid exchange or particle fusion, can be followed by monitoring E/M. Based on such E/M measurements, together with chromatographic and

Abbreviations used: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; CETP, cholesteryl ester transfer protein; DPPC, dipalmitoyl-phosphatidylcholine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PLTP, phospholipid transfer protein; PyrCE, pyrenylacyl cholesterol ester; PyrPC, pyrenylacyl phosphatidylcholine; rHDL, reconstituted HDL.

‡ To whom correspondence should be addressed.

electron microscopy analyses, we now provide evidence that particle fusion is the mechanism for the PLTP-mediated enlargement of HDL particles.

## EXPERIMENTAL

### Materials

1-Palmitoyl-2-oleyl phosphatidylcholine was from Avanti Polar Lipids, Alabama, U.S.A. 1-Palmitoyl-2-[1-<sup>14</sup>C]palmitoyl phosphatidylcholine ([<sup>14</sup>C]DPPC, specific radioactivity 55 mCi/mmol) and [1 $\alpha$ ,2 $\alpha$ (n)-<sup>3</sup>H]cholesterol oleate (specific radioactivity 49 Ci/mmol) were from Amersham, Bucks., U.K. PyrCE was obtained from Molecular Probes, Oregon, U.S.A. PyrPC was synthesized as described previously [22]. All other lipids were from Sigma Chemical Company, St. Louis, U.S.A. Butyl-Toyopearl 650(M) and CM-Toyopearl 650(M) were obtained from Merck, Germany. Mono Q HR 5/5, Superose 6 HR columns, heparin-Sepharose CL-6B and high-molecular-mass electrophoresis calibration standards were from Pharmacia, Uppsala, Sweden. Acrylamide was from Merck, Germany and *N,N'*-bisacrylamide from LKB, Sweden. Coomassie Brilliant Blue G-250 was obtained from SERVA, Heidelberg, Germany. Heparin (5000 units/ml) was obtained from Medica, Helsinki, Finland. All other reagents were of analytical grade.

### Isolation of lipoproteins

HDL<sub>3</sub> was isolated from fresh human plasma at a density of 1.125–1.21 g/ml by sequential ultracentrifugation using KBr for density adjustments [23], washed by refloatation at  $\rho = 1.21$  g/ml and dialysed against 10 mM phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.02% KCl. Dialysed HDL<sub>3</sub> was stored at 4 °C under nitrogen.

### Purification and assay of PLTP from human plasma

PLTP was purified and assayed essentially as described elsewhere [18,24,25]. PLTP activity is expressed as nmol of phosphatidylcholine transferred/h. PLTP preparations were free of cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase activity.

### Preparation of rHDL

Reconstitution of HDL particles containing either pyrenyl-labelled cholesterol esters (PyrCE) or phospholipids (PyrPC) was performed essentially as described by Pittman et al. [26]. A lipid mixture containing cholesterol, 1-palmitoyl-2-oleylphosphatidylcholine, cholesterol linoleate, triolein and PyrCE or PyrPC (9.1:59:20.5:9.1:2.3, by weight) was prepared by dissolving the lipids in CHCl<sub>3</sub>/methanol (2:1, v/v). The mixture was dried under nitrogen and further under vacuum for 2 h. The dry lipid was then dispersed in 20 mM Tris/1 mM EDTA, pH 7.4, and sonicated for 5 × 1 min in a 40 °C water bath under an argon atmosphere using the microtip of a Branson sonicator. Delipidated HDL protein, dissolved in 3 M urea/20 mM Tris/1 mM EDTA, pH 7.4, was added dropwise during additional cycles of sonication (5 × 1 min) to reach the final 4:1 protein/lipid weight ratio. The sample was then adjusted to a density of 1.063 g/ml with KBr solution and centrifuged in a Beckman model L3-50 ultracentrifuge at 140 000 *g* for 24 h at 15 °C. The centrifuged preparation was fractionated into three portions, the top fraction (6 ml) containing free lipids, the middle fraction (6 ml)

and the bottom fraction (8 ml) containing free apoproteins and rHDL. The density of the bottom fraction was adjusted to 1.23 g/ml and centrifuged at 130 000 *g* for 40 h at 15 °C. rHDL, recovered in the top fraction (2 ml), was concentrated to 200  $\mu$ l, applied on a Superose 6 HR gel-filtration column and eluted with 10 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4.

### Incubation of HDL<sub>3</sub> or rHDL with PLTP

Incubations were carried out in 200 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA. Samples (400  $\mu$ l) were incubated in stoppered plastic vials at 37 °C in a shaking water bath. Control samples were kept at 4 °C. After the incubation the samples were stored on ice before analysis. Further details of the individual experiments are given in the Figure legends. In this context it is important to note that the activity of PLTP used in all incubations (1.75  $\mu$ mol/h per ml) was well below that in native plasma (4.0 ± 0.5  $\mu$ mol/h per ml).

### Analysis of particle size

Particle sizes of HDL<sub>3</sub> and rHDL were analysed by native gradient gel electrophoresis as described by Blanche et al. [27]. Self-made 4–26% polyacrylamide gradient gels (8.0 cm × 8.0 cm) were used. Stained gels were scanned and analysed with the Bio Image System (Millipore Co, U.S.A.). Calibration was performed with high-molecular-mass electrophoresis calibration standards from Pharmacia. Particle size was also assessed by gel filtration on a Superose 6 HR column [18] and by negatively stained electron microscopy.

### Fluorescence measurements

Fluorescence measurements were performed using a Hitachi F-4000 fluorescence spectrophotometer equipped with a thermostatically controlled cuvette holder. Pyrenyl lipids were excited at 344 nm and the monomer and excimer emission intensities were measured at 378 nm and 475 nm respectively.

### Other methods

SDS/PAGE was performed as described by Laemmli [28]. Western blotting was performed according to Towbin et al. [29]. Protein was determined using human serum albumin as standard [30]. Cholesterol, triacylglycerols and phospholipids were determined with enzymic methods [31]. ApoA-I and apoA-II were assayed by immunoturbidometry [32].

## RESULTS

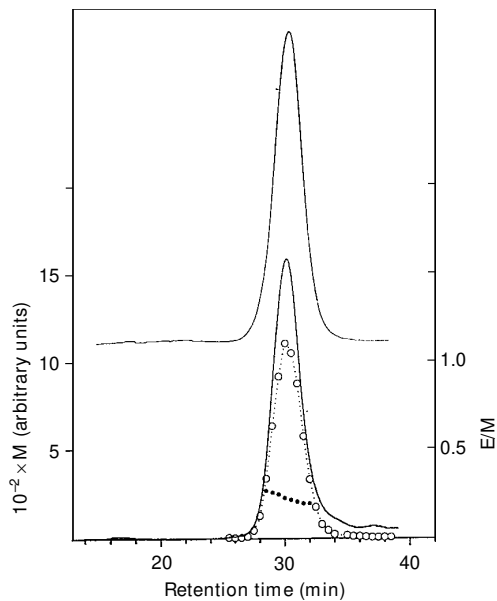
To study the mechanism of HDL size conversion, rHDL particles containing either pyrenyl-labelled phosphatidylcholine as surface lipid marker or fluorescent cholesterol ester as core lipid marker were prepared. These rHDL particles are useful tools to study the mechanism of HDL interconversion as the excimer to monomer intensity ratio (E/M) is proportional to the local probe concentration and thus determination of E/M provides information on mixing of lipids between labelled (rHDL) and unlabelled (HDL<sub>3</sub>) particles.

The lipid and apoprotein composition of rHDL particles was similar to that of HDL<sub>3</sub> (Table 1). Size-exclusion chromatography demonstrated that rHDL has a size similar to HDL<sub>3</sub> (Figure 1). In addition, the rHDL protein and PyrCE elution profiles coincided, indicating that the fluorescent probe was homogeneously incorporated into the rHDL particles. This was supported by the observation that E/M varied only slightly over the

**Table 1** Characterization of reconstituted HDL particles

Diameters of the particles were analysed by: A, Superose 6 HR gel filtration analysis; and B, 4–26% native gradient polyacrylamide gel analysis. The values in parentheses are the coefficients of variation between different preparations. Five native HDL<sub>3</sub> and three each of rHDL, apoA-I-rHDL and apoA-II-rHDL preparations were analysed.

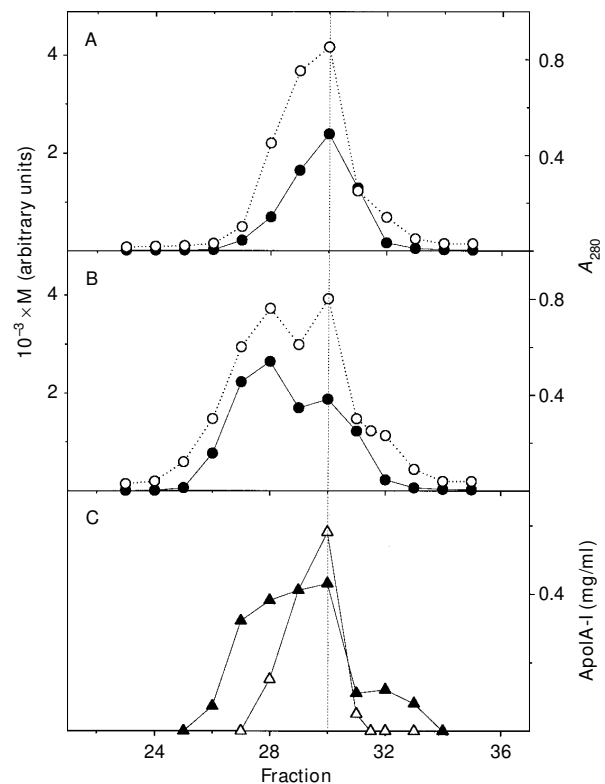
Lipoprotein	Mass (%)				Diameter (nm)		
	Protein	Phospholipid	Cholesterol	Tg	A	B	A-I/A-II (mol/mol)
Native HDL <sub>3</sub>	45 (5)	34 (7)	19 (10)	2 (12)	8.2 (1)	8.4 (2)	1.6
rHDL	39 (3)	36 (8)	17 (7)	8 (14)	7.8 (2)	7.9 (1)	1.4
ApoA-I-rHDL	48 (2)	31 (3)	18 (6)	3 (25)	7.8 (2)	7.8 (1)	
ApoA-II-rHDL	50 (4)	28 (14)	17 (5)	5 (16)	7.6 (2)	7.6 (3)	

**Figure 1** Size-exclusion chromatography of rHDL particles

rHDL (lower trace) was applied to a Superose 6HR size-exclusion chromatography column equilibrated with 10 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4, and eluted at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were analysed for monomer (○), and for excimer/monomer (E/M; ●) fluorescence emission intensities. The elution profile of native HDL<sub>3</sub> (upper trace) is shown for comparison. rHDL and HDL<sub>3</sub> absorbances have been recorded on the same relative absorbance scale ( $A_{280}$ ). M is the monomer fluorescence intensity.

rHDL peak. Electron microscopy of rHDL revealed a population of homogeneous spherical, non-aggregated, particles. We conclude that rHDL particles consist of homogeneous particles that are structurally similar to native HDL<sub>3</sub>.

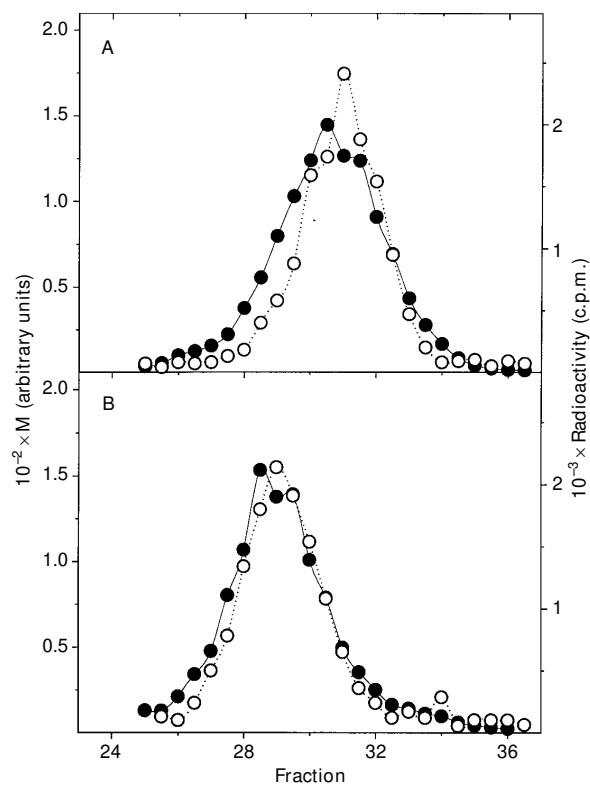
When incubated with PLTP, the average size of rHDL particles increased, accompanied by the release of apoA-I, i.e. rHDL behaves like native HDL<sub>3</sub> (Figure 2). ApoA-I particles (fractions 31–33; Figure 2, panel C) contained only traces of lipid. The release of apoA-I from rHDL particles following incubation in the presence of PLTP was further studied using ultracentrifugation. Samples of rHDL were incubated in the presence and absence of PLTP for 24 h at 37 °C. After incubation the rHDL particles were isolated by ultracentrifugation at a density of 1.21 g/ml. Of the apoA-I present in the rHDL, 3% was released from the particles following incubation in the absence of PLTP and 11% was released following incubation with PLTP.

**Figure 2** Effect of PLTP on the size of rHDL particles

PyrCE-rHDL was incubated at 37 °C in the absence (panel A) or presence of PLTP (700 nmol/h) (panel B). After a 24 h incubation, the mixture was applied on to a Superose 6 HR column equilibrated with 10 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4, and eluted at a flow rate of 0.5 ml/min. Fractions were analysed for  $A_{280}$  (○) and pyrene monomer fluorescence intensity (M; ●). Panel C illustrates the distribution of apoA-I in the eluted fractions following gel filtration of PyrCE-rHDL incubated in the absence (△) or in the presence (▲) of PLTP.

This release of apoA-I is of similar magnitude to that previously observed for native HDL [33].

To confirm that the pyrenyl lipids mimic the behaviour of the natural ones, rHDL particles containing both fluorescent cholesterol ester (PyrCE) and radioactively labelled phospholipid ( $[^{14}C]$ DPPE) were prepared and incubated with HDL<sub>3</sub> for 24 h in the presence and absence of PLTP. Size-exclusion chro-

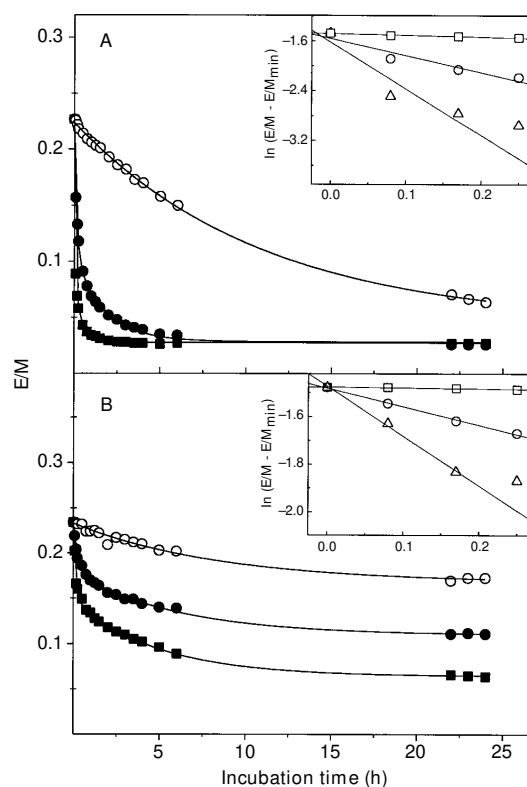


**Figure 3** Incubation of surface- and core-lipid-labelled rHDL with native HDL<sub>3</sub> in the presence of PLTP

A mixture of PyrCE/[<sup>14</sup>C]PC-rHDL and HDL<sub>3</sub> was incubated at 37 °C in the absence (panel A) or presence (panel B) of PLTP. After a 24 h incubation, the mixture was applied on a Superose 6 HR column equilibrated with 10 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4, at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were analysed for radioactivity (○) and pyrene monomer fluorescence intensity (M; ●).

matography of the incubation mixtures showed that the fluorescence and radioactivity profiles coincided and were similarly shifted after incubation with PLTP (Figure 3). Parallel results were obtained with particles co-labelled with PyrPC and [<sup>3</sup>H]cholesterol ester (results not shown). These data demonstrate that both the surface and the core lipids of rHDL are properly incorporated into the rHDL particles, and consequently PyrCE and PyrPC should correctly report on the behaviour of the natural core and surface lipid constituents respectively. It is noteworthy that detectable amounts of none of these labelled lipids could be observed in those fractions (Figure 2, panel C; fractions 32 and 33) where apoA-I, released upon incubation with PLTP, was found to elute. This indicates that very little, if any, lipid associates with the released apoA-I.

The mechanism of PLTP-induced size increase of HDL was studied by incubating rHDL particles containing either PyrPC as the surface lipid marker (PyrPC-rHDL) or PyrCE as the core lipid marker (PyrCE-rHDL) with HDL<sub>3</sub>. Since the ratio of pyrenyl lipid excimer to monomer fluorescence intensities (E/M) is proportional to the local pyrene concentration, mixing of the lipids of rHDL particles with those of HDL<sub>3</sub> results in a decrease in E/M. As shown in Figure 4 (panel A) a very rapid decrease in E/M occurred upon addition of PLTP into a mixture of PyrPC-rHDL and HDL<sub>3</sub>, indicating that extensive mixing (dilution) of the rHDL surface lipids with those of HDL<sub>3</sub> was taking place. Since this mixing was almost complete before any significant

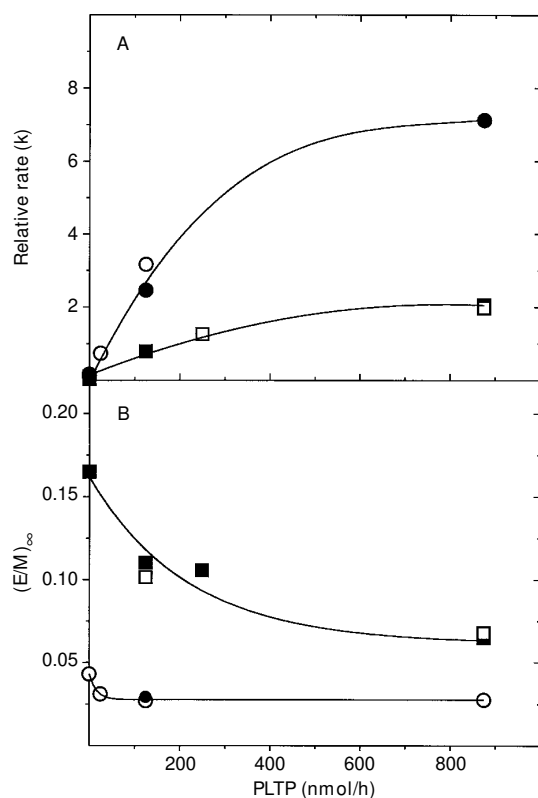


**Figure 4** Effect of PLTP on mixing of the surface and core lipids of rHDL and HDL<sub>3</sub>

Panel A: PyrPC-rHDL and a 10-fold excess of HDL<sub>3</sub> were incubated in the absence (○) or presence of 125 (●) or 875 (■) nmol/h of PLTP at 37 °C. The ratio of excimer to monomer fluorescence (E/M) is plotted as a function of incubation time. The lines are best fits to the data assuming a mono- (in the absence) or bi-exponential (in the presence of PLTP) decay respectively. To obtain true initial rates of E/M change,  $\ln(E/M - E/M_{\min})$  was plotted against time for the initial 0.2–0.25 h period (inset) and  $K$ , the relative rate constant, was obtained from the slope by using the protocol described previously ([54,55]). Panel B: As in panel A, but PyrCE-rHDL replaced PyrPC-rHDL. Again best fits to the data were obtained when mono- and bi-exponential decays were assumed in the absence and presence of PLTP respectively.

increase in particle size could be observed (see below), it is most likely due to PLTP-mediated phospholipid transfer or exchange between rHDL and HDL<sub>3</sub> particles. The rate of PyrPC mixing increased with the amount of PLTP added (Figure 5, panel A). E/M decreased also in the absence of PLTP, albeit much more slowly than in the presence of PLTP. The half-time of this PLTP-independent process, which very likely corresponds to spontaneous interparticle transfer of PyrPC, was 10.8 h, a value similar to that found previously for spontaneous transfer of natural PC between HDL-like particles [34]. Both in the presence and absence of PLTP the exchange of PyrPC appears to proceed to completion (Figure 5, panel A).

When the core-labelled PyrCE-rHDL was used, PLTP again induced a decrease in the E/M ratio, but the rate of decrease was 2–3-fold lower than that observed with PyrPC-rHDL in the presence of an equal amount of PLTP [Figure 4 (panel B) and Figure 5 (panel A)], indicating that the PLTP-induced mixing of the core lipids is a considerably slower process than that of the surface phospholipids. Furthermore, unlike in the case of surface-labelled PyrPC-rHDL, the estimated extent of core lipid mixing at equilibrium (see the legend to Figure 5, panel B) depends on the amount of PLTP added (Figure 5, panel B). The rate, and particularly the extent, of PyrCE dilution (mixing) at equilibrium



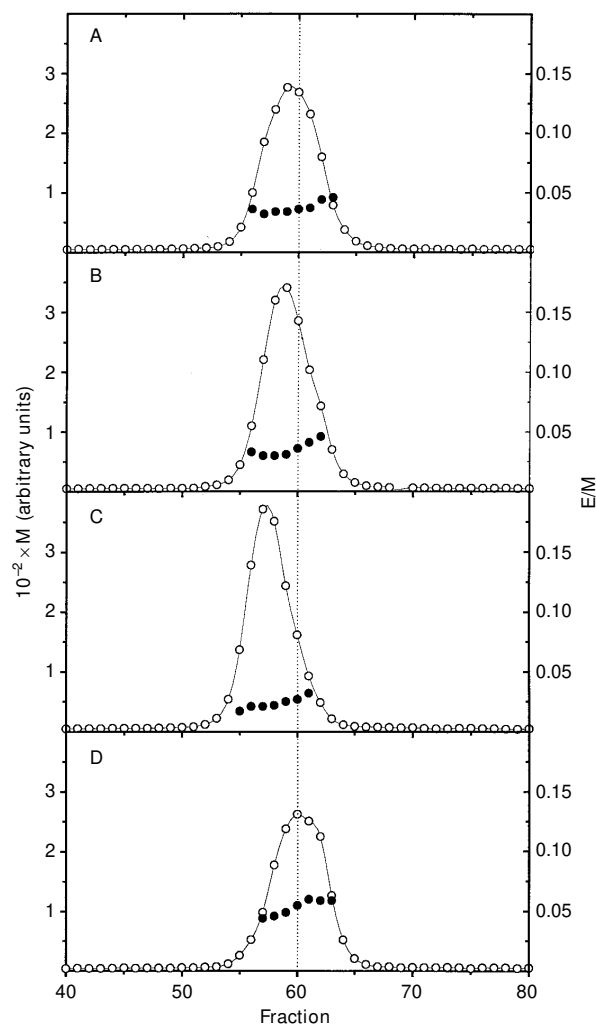
**Figure 5** Effect of PLTP concentration on the rate and extent of excimer to monomer fluorescence ratio (E/M) decrease for PyrCE-rHDL and PyrPC-rHDL

Panel A: The initial rate of E/M decrease ( $k$ ), derived from the data shown in the insets of Figure 4, is plotted versus PLTP concentration for PyrPC-rHDL (●, ○) and PyrCE-rHDL (■, □) donors. The open and closed symbols refer to data from two different experiments. Panel B: The extrapolated equilibrium value of E/M, obtained from the fits shown in Figure 4, are plotted against the amount of PLTP added. Symbols are the same as in panel A. The lines in panels A and B are just to guide the eye.

correlates closely with the appearance of enlarged particles (see below), thus indicating that PyrCE dilution and particle growth are due to a common process, most probably particle fusion.

When the PyrCE-rHDL particles were incubated in the absence of PLTP a slow decrease in the E/M ratio could be observed (Figure 4, panel B). However, unlike with PyrPC-rHDL, this decrease is unlikely to be due to spontaneous exchange of PyrCE molecules between the lipoprotein particles, because spontaneous diffusion of cholesterol esters through an aqueous medium is orders of magnitude slower than that of phospholipids; here the rates (but not the extent, see legend to Figure 4) of the spontaneous (PLTP-independent) decrease of E/M were similar for PyrCE and PyrPC ( $t_{1/2} = 8.2$  and  $10.8$  h respectively). Furthermore, the E/M ratio of PyrCE levels off after the initial period of decrease, which should not occur if the decrease was a result of spontaneous lipid transfer (compare with the data in Figure 4, panel A). We propose that the decrease is due to spontaneous fusion of a fraction of the PyrCE-rHDL particles with HDL<sub>3</sub> particles. From the extrapolated equilibrium (0.17) and the initial (0.24) values of E/M, it can be calculated that < 30% of the PyrCE-rHDL particles participates in this spontaneous fusion.

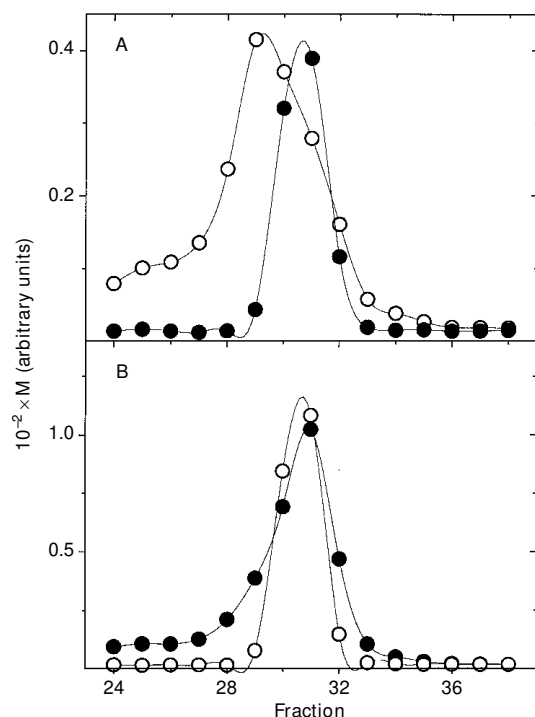
To study further the mechanism of PLTP-mediated size increase of HDL, PyrCE-rHDL was incubated with an excess of



**Figure 6** Time course of the PLTP-mediated conversion of a mixture of rHDL and HDL<sub>3</sub>

A mixture of PyrCE-rHDL and HDL<sub>3</sub> was incubated at 37 °C in the absence or presence of PLTP. The incubations were performed for 1 h (panel A), 3 h (panel B) or 24 h (panel C). After the incubations the mixtures were subjected to size-exclusion chromatography on two Superose 6HR columns connected in tandem and the fractions were analysed for monomer (○) and excimer fluorescence and the E/M ratio (●). Panel D illustrates data for a 24 h incubation performed in the absence of PLTP (a control).

HDL<sub>3</sub> in the presence of PLTP at 37 °C. Samples were taken from the incubation mixture after 1, 3 and 24 h and subjected to size-exclusion chromatography (Figure 6). As expected, a time-dependent shift of the PyrCE profile occurred in accordance with the results shown in Figure 3. In addition, the E/M ratio decreased over the whole peak with time, but more so in the leading fractions where the enlarged particles are enriched. To estimate if a quantitative correlation between the E/M ratio and the particle size exists, the particle masses in peak fractions of HDL, incubated with PLTP for 1, 3 or 24 h, were determined (results shown in Figure 6) and found to be 120, 150 and 225 kDa, respectively. Corresponding E/M values were 0.036, 0.031 and 0.021. These values indicate that there is a reasonable correlation between the enlargement of the particle size and the decrease in E/M ratio. In addition, based on the doubling of the particle mass after 24 h incubation in the presence of PLTP, it



**Figure 7** Apolipoprotein specificity of PLTP-mediated size conversion of rHDL particles

rHDL particles containing either apoA-I (panel A) or apoA-II (panel B) as sole protein component were incubated at 37 °C in the absence (●) or presence (○) of PLTP. After a 24 h incubation, the mixture was applied on a Superose 6 HR column equilibrated with 10 mM Tris/1 mM EDTA/150 mM NaCl, pH 7.4, and eluted at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were analysed for pyrene monomer fluorescence intensity (M).

can be estimated that, on average, each HDL particle has undergone one round of fusion.

The release of apoA-I from HDL<sub>3</sub> upon incubation with PLTP [18,19,33] indicated that apoprotein detachment plays an important role in the PLTP-mediated HDL conversion. To study this further, rHDL particles containing either apoA-I (A-I-rHDL) or apoA-II (A-II-rHDL) as their sole protein component and PyrCE label in their core were prepared. The size and lipid composition of these particles were similar to those of HDL<sub>3</sub> (Table 1). When A-I-rHDL was incubated in the presence of PLTP for 24 h, the original particles (mean diameter of 7.8 nm) were converted into large ones with an average diameter of 11.0 nm (Figure 7, panel A). In contrast, incubation of A-II-rHDL under identical conditions caused neither a change in particle size nor a shift in the PyrCE elution profile. Thus the presence of apoA-I molecules appears to be required for the PLTP-mediated HDL conversion.

## DISCUSSION

Three recent studies [18,19,33] have demonstrated that PLTP can promote conversion of an apparently homogeneous population of HDL<sub>3</sub> particles into a new population with an increased average size. During this conversion process, which occurs in the absence of other lipoproteins or CETP, apoA-I is released [18,19,33]. These findings were confirmed and extended to rHDL particles in the present study.

The usefulness of rHDL particles as acceptors of cholesterol from cells [35–38] or as models to study the role of apoproteins

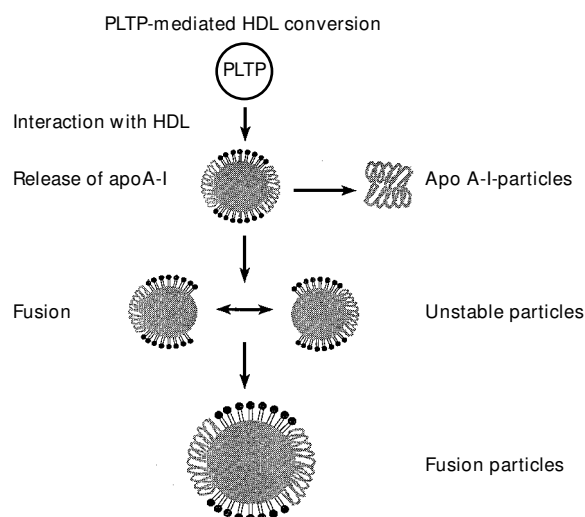
in the interconversion of HDL populations [39–41] has been demonstrated. In this study we used rHDL particles containing fluorescent pyrenyl lipid reporters, either in their core (PyrCE) or in the surface lipid layer (PyrPC). Compositional analysis and structural studies demonstrated that the rHDL particles closely resemble HDL<sub>3</sub>, as has also been reported previously [40]. The rHDL particles were also functionally similar to HDL<sub>3</sub> as their size increased considerably upon incubation with PLTP. They thus seem to be a good model to study the mechanism of PLTP-mediated HDL conversion.

The PLTP-mediated HDL conversion could result (i) from aggregation of HDL particles, (ii) from disproportionate transfer of lipid molecules between HDL subpopulations or (iii) from particle fusion. The data presented provide evidence that mere aggregation of particles can be excluded, since mixing of both the surface and core lipids of rHDL with those of HDL<sub>3</sub> was observed. Thus mixing must result from net lipid transfer or particle fusion. While unambiguous distinction between these two mechanisms cannot be made at present, several observations disfavour net lipid transfer as the mechanism. First, our results demonstrate that the transfer must also involve core lipids, i.e. cholesterol esters, since fluorescently and radioactively labelled cholesterol ester molecules were extensively incorporated into the large particles. It has, however, been demonstrated repeatedly that PLTP does not catalyse the transfer of cholesterol esters [41,42]. Thus, and since our PLTP preparation did not contain any CETP activity, the observed dilution of PyrCE and its incorporation into the enlarged particles is unlikely to result from net transfer of cholesterol esters. Further support for this is provided by the notable differences in the kinetics of PyrCE and PyrPC dilution (Figure 4). Secondly, if net transfer of lipids by PLTP from one HDL subpopulation to another were the mechanism whereby the enlarged particles are formed, then lipoprotein particles smaller than the original ones should also be formed. However, no lipid-containing particles smaller than the original ones could be detected. The small particles eluting after the original HDL peak only consisted of released apoA-I (Figure 2).

In contrast, the rapid dilution of PyrPC is obviously due to PLTP-mediated phospholipid transfer or exchange. Since this process is practically complete before any significant size change is observed in HDL (Figures 4 and 6), it is unlikely to be directly coupled to particle enlargement. However, this phospholipid transfer process may modify the particle surface in such a way that it becomes more susceptible to fusion, leading to the formation of enlarged particles.

The fact that the (apparent) equilibrium value of E/M for the PyrCE probe is dependent on the amount of PLTP added [Figure 4 (panel B) and Figure 5 (panel B)] suggests that the interaction between PLTP and HDL is not purely catalytic, but that some kind of stoichiometric interaction could be involved. However, calculations showed that even at the highest PLTP concentration used (0.17 nmol of PLTP) there is only 1 PLTP molecule per 25 HDL particles; thus it is obvious that simple, non-catalytic complex formation cannot be responsible for the observed behaviour. We presently favour the possibility that PLTP indeed acts catalytically, but each PLTP molecule can mediate only a limited number of fusion cycles and is then inactivated (possibly due to aggregation or complexation with the apoA-I molecules released from HDL particles). This would explain the observed PLTP concentration dependency [Figure 4 (panel B) and Figure 5 (panel B)].

All data presented are compatible with particle fusion being the mechanism of PLTP-induced HDL conversion. Although the molecular events behind the fusion remain to be clarified, we



**Scheme 1** Model for PLTP-induced fusion of HDL particles

PLTP interacts with the surface of HDL and induces an increase in the surface pressure either by penetrating the surface or by increasing the amount of surface lipid by net lipid transfer. ApoA-I molecule(s) are displaced from the particles because of the increased pressure. This leads to formation of particles that are unstable because of partially exposed hydrophobic cores. Two unstable particles interact through their hydrophobic core surfaces and fuse to a thermodynamically stable particle. The fusion product may then participate in additional rounds of fusion with other fusion products or original particles.

propose a tentative model (Scheme 1). According to this model, PLTP first interacts with HDL and mediates a rapid transfer of phospholipid molecules between particles, as evidenced by the data shown in Figure 4 (panel A). While this process does not seem to correlate directly with the progression of the fusion, it could predispose the particles to this process. Possibly, net transfer of phospholipid molecules to a HDL subpopulation takes place, thus leading to an increase in surface pressure, which in turn leads to the release of apoA-I molecules from the surface layer. It has been shown that an increase in surface pressure can lead to detachment of apoA-I, while apoA-II is much more resistant in this respect [43–47]. PLTP could also cause the displacement by directly interacting with apoA-I. That detachment of apoA-I is indeed playing an important role in the fusion process is supported by the finding that rHDL reconstituted with apoA-I as the only protein component was susceptible to PLTP-mediated enlargement, while rHDL reconstituted with apoA-II alone was not (Figure 7). However, independent of the actual mechanism of apoA-I release, the HDL particles should become unstable after dissociation of apoA-I because there is not enough amphiphilic surface to cover the hydrophobic lipid core (Scheme 1). As a result, the unstable particles adhere to each other and fuse to form a more stable particle. The primary fusion product may then undergo additional cycles of fusion.

The finding that rHDL particles having apoA-I as their only protein component were converted into larger particles in the presence of PLTP, whereas no size change of apoA-II-rHDL was observed, could be physiologically relevant, since human HDL is made up of two main populations of particles: both contain apoA-I, but only one of them apoA-II [7]. Clinical studies have shown that the levels of these two populations are altered in subjects at risk from coronary artery disease [48,49]. Thus the distinct reactivity of PLTP towards these two HDL subpopulations may be of physiological importance.

The PLTP-mediated fusion of HDL particles could be physio-

logically important also because of the release of lipid-poor apoA-I. Lipid-poor apoA-I has been suggested to function as a precursor of pre $\beta$ -HDL, a lipoprotein species that probably serves as the initial acceptor of cellular unesterified cholesterol in the process of reverse cholesterol transport [50,51]. Besides PLTP other proteins have been shown to release lipid-poor apoA-I *in vitro*. Thus Yokoyama and co-workers have shown that incubation of human HDL in the presence of insect haemolymph lipid transfer particle causes a preferential dissociation of apoA-I in connection with the enlargement of HDL particles [52]. Furthermore, Barter and colleagues recently reported that CETP and hepatic lipase can promote shedding of apoA-I from the surface of HDL [53]. The mechanisms of apoA-I release in these processes remain to be demonstrated. It also remains to be shown what the contribution of each of these processes is to the formation of lipid-poor apoA-I *in vivo*.

We thank Ms. Ritva Kevala, Ms. Titta Tapiola, and Ms. Tarja Grundström for expert technical assistance and Mrs. Tuula Kaleva for typing the manuscript. This research was supported by grants from the Paulo Foundation and the Jansson Foundation (M.J.) and from the Sigrid Juselius Foundation (C.E. and P.S.), Helsinki, Finland. Part of this work was presented in abstract form at the 22nd Meeting of the Federation of European Biochemical Societies, July 4–9, 1993, Stockholm, Sweden and at the 10th International Atherosclerosis Society Meeting, October 9–14, 1994, Montreal, Canada.

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