Triglyceride-enrichment of high density lipoproteins enhances their remodelling by phospholipid transfer protein

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Abstract Phospholipid transfer protein (PLTP) converts high density lipoproteins (HDL) to larger and smaller particles during incubation in vitro. The present study shows that these conversions are enhanced in triglyceride (TG)-enriched HDL. When pig HDL were enriched with TG by incubation with cholesteryl ester transfer protein (CETP) and very low density lipoproteins, the TG/apoA-I molar ratio increased from 0.7/1 to 11.4/1 and the diameter increased from 9.0 to 9.5 nm. When unmodified pig HDL were incubated with PLTP, the diameter increased from 9.0 to 9.5 nm. Incubation of the TG-enriched pig HDL with PLTP generated larger (13.0 nm) and smaller (7.5 nm) particles. The composition of the large particles was comparable to the non-incubated, TG-enriched HDL. The small particles contained only phospholipids and apolipoprotein (apo) A-I. Studies were also carried out with spherical, reconstituted HDL (rHDL) containing only cholesteryl esters in their core. When the rHDL were enriched with TG by incubation with Intralipid and CETP, the diameter increased from 9.0 to 9.2 nm and the TG/apoA-I molar ratio became 5.7/1. When unmodified rHDL were incubated with PLTP, the diameter of 15% of the particles increased to 11.0 nm, 18% decreased to 7.6 nm, and 67% were unchanged. When the TG-enriched rHDL were incubated with PLTP, the diameter of 62% of the particles increased to 11.4 nm, 26% decreased to 7.6 nm, and 12% did not change. Preincubation with Intralipid alone or CETP alone did not enrich the rHDL with TG or enhance the size changes. These results show that the TG content of HDL has a major influence on PLTP-mediated size changes. —**Rye, K-A., M. Jauhiainen, P. J. Barter, and C. Ehnholm.** Triglyceride-enrichment of high density lipoproteins enhances their remodelling by phospholipid transfer protein. *J. Lipid Res.* 1998. **39:** 613–622.

Supplementary key words high density lipoprotein size • cholesteryl ester transfer protein

Phospholipid transfer protein (PLTP) is a hydrophobic, heat-labile 476 amino acid protein that is present in the plasma of several species, including pigs and humans (1, 2). PLTP transfers phospholipids between high density lipoproteins (HDL) and very low density lipoproteins (VLDL) (3). It also transfers phospholipids between particles within the HDL fraction in a process that results in conversion of the HDL into larger and smaller particles (1).

In 1996 Lusa et al. (4) reported that the large particles that are formed during incubations of PLTP and human HDL_3 are fusion products. These investigators also suggested that the fusion is initiated by the binding of PLTP to the surface of HDL and that, subsequent to the binding of PLTP, apolipoprotein (apo) A-I complexed with a small amount of phospholipid dissociates from the HDL surface (4). The phospholipid–apoA-I complexes that dissociate from the surface of HDL during incubation with PLTP are similar in composition to the HDL in human plasma which have pre-β-migration on agarose gels (5). As it has been established that pre-β-migrating HDL can be the initial acceptors of cellular cholesterol in plasma (6), this finding suggests that the interaction of PLTP with the surface of HDL may have a significant influence on cholesterol transport in the plasma compartment. This possibility is supported by the recent finding that the concentration of pre-β_1-

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; rHDL, reconstituted HDL; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; UC, unesterified cholesterol; CE, cholesterol ester(s); apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; TBS, Tris-buffered saline; TG, triglyceride(s); SDS, sodium dodecyl sulfate.

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migrating HDL increases when samples of whole plasma are incubated with exogenous PLTP (7).

At present little is known about factors that regulate the interaction of PLTP with the surface of HDL particles. Cheung, Wolfbauer, and Albers (8) have reported that the concentrations of triglyceride (TG) and phospholipid in HDL correlate positively with the rate of PLTP-mediated phospholipid transfers in plasma. However, it is not known whether the phospholipid and TG concentrations of HDL also influence PLTP-mediated changes in HDL size. The aim of the present study was to determine whether the amount of TG in HDL influences PLTP-mediated changes in HDL particle size.

EXPERIMENTAL PROCEDURES

Isolation of pig HDL and human VLDL

Human HDL (1.063 < d < 1.21 g/ml), human HDL_3 (1.12 < d < 1.21 g/ml) and pig HDL (1.087 < d < 1.21 g/ml) were isolated by sequential ultracentrifugation at 5°C. Density adjustments were made by addition of solid KBr to the plasma samples. The ultracentrifugation was carried out at 50,000 rpm in a Beckman Ti 50.2 rotor for 24 h at the lower densities and for 40 h at the higher densities. All the HDL fractions were re-isolated at their upper density (1.21 g/ml) by a further 40 h of ultracentrifugation at 50,000 rpm. The isolated HDL were dialyzed against 2 × 5 liters Tris-buffered saline (10 mm Tris-HCl, 150 mm NaCl, pH 7.4) containing 1 mm $EDTA\text{-}Na_2$ and 0.01% (w/v) NaN_3, (TBS), and stored at 4°C. Human VLDL were isolated from plasma by ultracentrifugation at 50,000 rpm for 24 h at 1.006 g/ml in a Beckman Ti 50.2 rotor which was maintained at 5°C. The VLDL preparations were dialyzed against TBS before use.

Preparation of spherical reconstituted HDL

The plasma samples for this part of the study were donated by the Transfusion Service, Royal Adelaide Hospital. Apolipoprotein (apo) A-I was purified from human plasma as described (9, 10). Discoidal reconstituted HDL (rHDL) containing 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), unesterified cholesterol (UC), and purified apoA-I were prepared by the cholate dialysis method (11). The discoidal rHDL were converted into spherical rHDL by incubation with low density lipoproteins (LDL) and purified lecithin:cholesterol acyltransferase (LCAT). The spherical rHDL were isolated from the LDL and LCAT by ultracentrifugation as described elsewhere (9) and dialyzed against 3 × 1 liter TBS before use.

Purification of PLTP from human plasma

The PLTP used in the experiments with pig HDL was purified and assayed as described (1, 12). The transfer activity of these preparations was 7.0 μmol of phosphatidylcholine transferred/ml PLTP per h.

The PLTP used in the rHDL experiments was isolated from human plasma (donated by the Transfusion Service, Royal Adelaide Hospital) as follows. The proteins that precipitated when whole plasma was adjusted from 35 to 45% saturation with ammonium sulfate were adjusted to a density of 1.25 g/ml and ultracentrifuged at 55,000 rpm for 26 h in a Ti 55.2 rotor. This procedure was carried out at 4°C. The d > 1.25 g/ml fraction was applied at a flow rate of 10 ml/min to an XK 50/30 column containing a Macroprep Hydrophobic Interaction gel (Bio-Rad). PLTP was eluted from the column with Milli Ro water, dialyzed against 10 mm Tris, pH 7.4, and applied at a flow rate of 10 ml/min to a pre-equilibrated XK 26/40 column packed with DEAE Sepharose Fast Flow (Pharmacia). PLTP was eluted from the DEAE column with a 0–250 mm NaCl gradient. The fractions containing PLTP were pooled, dialyzed against 10 mm Tris/50 mm NaCl/1 mm $EDTA\text{-}Na_2$, pH 7.4, and applied at 10 ml/min to a pre-equilibrated HR 10/30 column containing Heparin Sepharose Fast Flow (Pharmacia). PLTP was eluted from this column with 10 mm Tris/500 mm NaCl/1 mm $EDTA\text{-}Na_2$, pH 7.4. The active fractions from the heparin-Sepharose column were pooled, dialyzed against 20 mm Tris, pH 7.4, and applied at 1 ml/min to a pre-equilibrated HR 5/5 Mono Q column (Pharmacia). PLTP was eluted from the Mono Q column with a 0–1 m NaCl gradient. All the chromatographic procedures were carried out at room temperature on a Pharmacia FPLC system. The PLTP preparations used in the rHDL experiments were assayed using [^{14}C-DPPC]-labeled egg phosphatidylcholine vesicles and native HDL as described (12). These preparations transferred 2.3 μmol phosphatidylcholine/ml PLTP per h.

All the PLTP preparations were free of cholesteryl ester transfer protein (CETP) and LCAT activity and appeared as a single band after electrophoresis on an SDS gel and silver staining.

Purification of CETP from human plasma

The CETP used in the experiments with pig HDL was isolated from human plasma following partially the procedure for PLTP. Briefly, the non-bound fractions from the first heparin-Sepharose purification step of PLTP were combined and applied to a Mono Q column equilibrated with 25 mm Tris-HCl buffer (pH 7.4) containing 1 mm $EDTA\text{-}Na_2$. CETP was eluted with a linear NaCl gradient (0–0.5 m) at a flow rate of 1 ml/min. CETP activity eluted at a NaCl concentration of 165

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mm. The CETP preparations, which were assayed as described by Groener, Pelton, and Kostner (13), were devoid of PLTP and LCAT activities. CETP prepared in this manner had 35–47 units of activity/ml, where 1 unit is the transfer activity of 1 ml of a preparation of pooled, human lipoprotein-deficient plasma.

The CETP used for the rHDL experiments was purified exactly as described elsewhere (9). The assay for CETP activity involved the transfer of [^{3}H]cholesteryl esters ([^{3}H]CE) from [^{3}H]CE-HDL_3 to LDL (14, 15). The assay was linear as long as less than 30% of the total radioactivity counts were transferred from the HDL_3 to the LDL. The CETP used in this study had 29 units of activity/ml, where 1 unit is the transfer activity of 1 ml of a preparation of pooled, human lipoprotein-deficient plasma.

Enrichment of pig HDL and rHDL with TG

Ultracentrifugally isolated pig HDL were incubated at 37°C for 24 h with isolated human VLDL and purified CETP. Details of these incubations are presented in the legend to Table 1. When the incubations were complete, the mixtures were adjusted to a density of 1.063 g/ml and ultracentrifuged (100,000 rpm, 541,000 g) for 18 h at 5°C using a TLA 100.2 rotor in a Beckman TL-100 Tabletop ultracentrifuge. The VLDL in the d $<$ 1.063 g/ml fraction were removed by aspiration using a Pasteur pipette. The d $>$ 1.063 g/ml fraction was then adjusted to 1.21 g/ml and ultracentrifuged (100,000 rpm, 541,000 *g*) for 18 h at 5°C in a TLA 100.2 rotor. The d $<$ 1.21 g/ml fraction, which contained the TG-enriched HDL, was recovered, dialyzed against 2 × 5 liters of TBS and stored at 4°C. Recovery of HDL protein after these procedures was 70–75%.

The rHDL were enriched with TG by incubation with the phospholipid/TG emulsion, Intralipid (KabiVitrum AB, Stockholm, Sweden) and purified CETP. The incubations were carried out at 37°C for 15 min in a shaking water bath. Details of these incubations are described in the legend to Table 3. When the incubations were complete the mixtures were adjusted to 1.063 g/ml and ultracentrifuged for 16 h at 100,000 rpm in a Beckman TLA 100.4 rotor. The Intralipid was removed as the supernatant by tube slicing. The d $>$ 1.063 g/ml fraction was then adjusted to 1.21 g/ml and the TG-enriched rHDL were isolated as the supernatant after 16 h of ultracentrifugation at 100,000 rpm in a Beckman TLA 100.4 rotor. These procedures were carried out at 4°C in a Beckman TL-100 Tabletop ultracentrifuge. The TG-enriched rHDL were dialyzed against 3 × 1 liter TBS before use.

Incubation of pig HDL with PLTP

The incubations with PLTP were carried out as described elsewhere (1, 2). Briefly, pig HDL (600 μg protein) were incubated for 24 h at 37°C in the presence or absence of PLTP. The final volume of the incubation mixture was 0.5 ml. The final activity of PLTP in the incubation mixture was 800–1000 nmol phospholipid transferred/h. When the incubations were complete the HDL were subjected to gel permeation chromatography at a flow rate of 0.5 ml/min using two Superose 6HR columns connected in tandem. The columns were calibrated with protein markers for gel permeation chromatography (Gel Filtration Standards, Bio-Rad, CA). Pig HDL particle size was also assessed by non-denaturing polyacrylamide gradient gel electrophoresis using self-made 4–26% polyacrylamide gels (8.0 × 8.0 cm). The samples were electrophoresed for 15 min at 15 V, then for 20 min at 70 V, and finally for 20 h at 150 V. Stokes' diameters were determined by reference to high molecular weight standards (Pharmacia). When the electrophoresis was complete the gels were stained with Coomassie G-250 and scanned with a Bio-image System (Millipore Co).

Incubation of TG-enriched rHDL with PLTP

TG-enriched rHDL were incubated in the presence or absence of PLTP for 24 h in a shaking water bath maintained at 37°C. Details of the incubations are described in the legend to Fig. 4. When the incubations were complete, the rHDL were isolated as the d $<$ 1.25 g/ml fraction after 16 h of ultracentrifugation at 100,000 rpm in a TLA 100.1 rotor. This procedure was carried out at 4°C in a Beckman TL-100 Tabletop ultracentrifuge. The rHDL were then subjected to electrophoresis on PAA 3/40 non-denaturing polyacrylamide gradient gels (Gradipore, Sydney, Australia), stained with Coomassie Blue, and scanned with a laser densitometer (Pharmacia). These procedures are described elsewhere (16). The magnitude of the rHDL size changes that occurred during incubation with PLTP was determined by integration of peak areas of the laser densitometric scans.

Other methods

Protein concentrations were determined by the method of Lowry et al. (17), using human serum albumin as standard. Human apoA-I and pig apoA-I were assayed by immunoturbidometry (18). Cholesterol, triglycerides, and phospholipids were determined using enzymatic methods (19). LCAT activity was measured as described (20, 21).

RESULTS

TG-enrichment of pig HDL (Fig. 1, Table 1)

Pig HDL, which contain a minimal amount of TG (22), were enriched with TG by incubation for 24 h at

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TABLE 1. Composition of pig HDL before and after TG-enrichment

Incubation Conditions	Additions	Protein	CE	UC	PL	TG	TG/CE	TG/A-I
			mass %				*mass %*	*mol/mol*
4°C, 24 h	−VLDL, −CETP	46	18	4	31	1	0.06/1	0.7/1
37°C, 24 h	+VLDL, +CETP	42	10	4	29	15	1.50/1	11.4/1

Native pig HDL (1.5 mg protein) were either maintained at 4°C in the absence of VLDL and CETP or incubated at 37°C for 24 h in the presence of human VLDL (1 mg protein, 3.6 mg TG) and CETP (final activity 14 units/ml). The final volume of the incubation mixture was 1.0 ml. When the incubations were complete, the pig HDL were isolated by ultracentrifugation as described under Experimental Procedures. Composition is expressed as mass %. The values represent the mean of three separate experiments. CE, cholesteryl ester; UC, unesterified cholesterol; PL, phospholipid; TG, triglyceride; A-I, apolipoprotein A-I.

37°C with human VLDL and purified CETP. When the incubation was complete the TG-enriched pig HDL were re-isolated by ultracentrifugation as described under Experimental Procedures. The composition of the original and the TG-enriched pig HDL are shown in **Table 1**. The non-incubated pig HDL contained 1% TG by mass (molar ratio TG/apoA-I = 0.7/1), while the HDL that were TG-enriched by incubation with VLDL and CETP contained 15% TG by mass (molar ratio TG/apoA-I = 11.4/1). The TG-enrichment was accompanied by a concomitant decrease in the CE content of the particles. The TG/CE ratio of the non-incubated HDL was 0.06/1. After 24 h of incubation with CETP and VLDL this value increased 25-fold, to 1.5/1. As judged by non-denaturing polyacrylamide gradient gel electrophoresis, TG-enrichment increased the diameter of the pig HDL from 9.0 to 9.5 nm (**Fig. 1**). The recovery of apoA-I in the TG-enriched pig HDL was >98% (result not shown).

Incubation of TG-enriched pig HDL with PLTP (Fig. 1)

Unmodified pig HDL (1% TG by mass) and TG-enriched pig HDL (15% TG by mass) were incubated for 24 h with PLTP. Changes in particle size were assessed by subjecting the incubated HDL to non-denaturing polyacrylamide gradient gel electrophoresis (Fig. 1). When the unmodified HDL were incubated with PLTP their diameter increased slightly, from 9.0 to 9.5 nm. By contrast, when the TG-enriched HDL (diameter = 9.5 nm) were incubated with PLTP, populations of particles that were larger (diameter = 13.0 nm) and smaller (diameter = 7.5 nm) than the original, TG-enriched HDL were generated.

Time course of the PLTP-mediated conversion of TG-enriched pig HDL to large and small particles (Fig. 2)

In this experiment TG-enriched pig HDL containing 15% TG were incubated with PLTP for 0–24 h. Changes in particle size were monitored by non-denaturing polyacrylamide gradient gel electrophoresis (**Fig. 2**). The diameter of the TG-enriched HDL that was maintained at 4°C was 9.5 nm. Incubation for 6 h at 37°C in the presence of PLTP did not affect the size of the TG-enriched HDL. By 12 h, the diameter of the HDL had

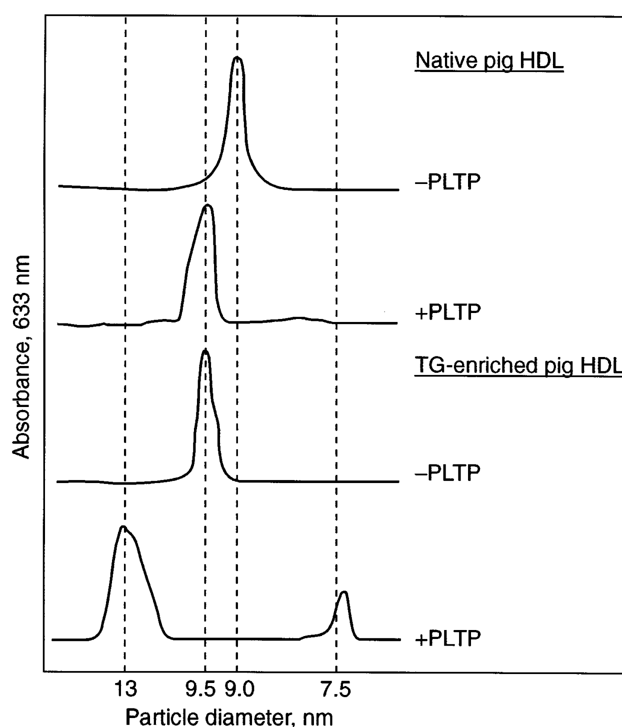

Fig. 1. PLTP-mediated changes in the size of native and TG-enriched pig HDL. Pig HDL were either maintained at 4°C or enriched with TG by incubation at 37°C for 24 h with human VLDL and purified CETP. Details of the incubations are described in the legend to Table 1. When the incubations were complete the native HDL and TG-enriched HDL were isolated by ultracentrifugation, then incubated at 37°C for an additional 24 h with PLTP as described under Experimental Procedures. When the PLTP incubations were complete the HDL were subjected to nondenaturing polyacrylamide gradient gel electrophoresis. The profiles represent laser densitometric scans of Coomassie G-250-stained gels. Particle diameters were calculated by reference to known high molecular weight standards.

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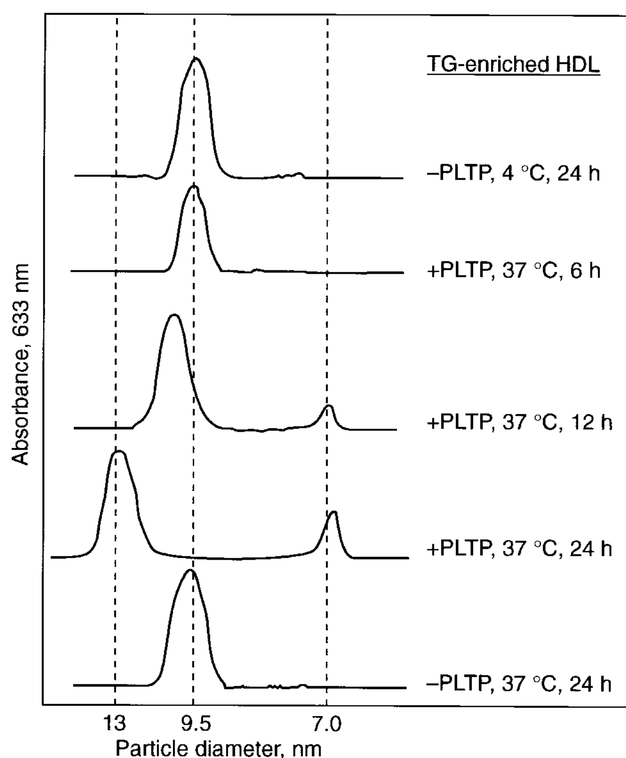

Fig. 2. Time course of the PLTP-mediated conversion of TG-enriched HDL to large and small particles. TG-enriched pig HDL containing 15% TG by mass were prepared as described in the legend to Table 1. The TG-enriched HDL (0.65 mg protein) were then incubated at 37°C for 0–24 h with PLTP. The final volume of the incubation mixture was 0.5 ml and 0.1 ml of a PLTP preparation that transferred 7 μmol phospholipid/ml PLTP per h was used. When the incubations with PLTP were complete, the HDL were subjected to nondenaturing polyacrylamide gradient gel electrophoresis. Laser densitometric scans of the stained gradient gels are shown.

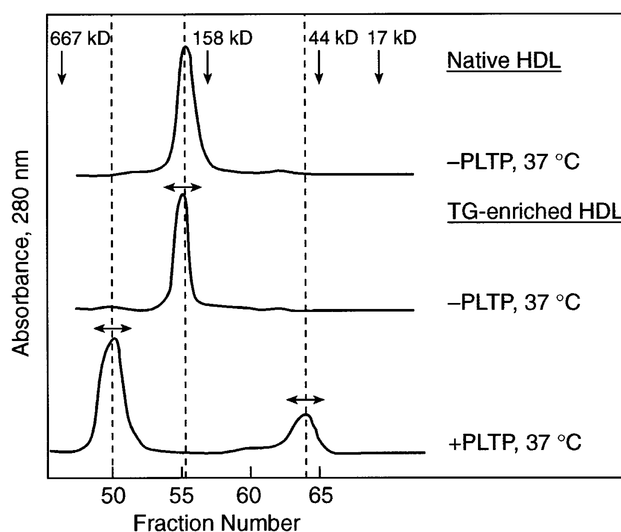

Fig. 3. Isolation of the large and small conversion products formed by incubating TG-enriched pig HDL with PLTP. Pig HDL were either maintained at 4°C or enriched with TG by incubation at 37°C for 24 h with human VLDL and purified CETP. Details of the incubations are described in the legend to Table 1. When the incubations were complete the HDL were isolated by ultracentrifugation, then incubated at 37°C for an additional 24 h with PLTP as described under Experimental Procedures. When the PLTP incubations were complete the HDL were subjected to gel permeation chromatography using two Superose 6HR columns connected in tandem. Elution profiles from the Superose columns are shown.

increased from 9.5 to 11.5 nm. A minor population of small particles (diameter = 7.0 nm) was also apparent at this time. After 24 h of incubation with PLTP, the TG-enriched HDL were converted quantitatively to large and small particles with respective diameters of 13.0 and 7.0 nm. After 24 h of incubation in the absence of PLTP the diameter of the TG-enriched HDL was unchanged at 9.5 nm.

Isolation and composition of the conversion products formed during incubation of TG-rich pig HDL with PLTP (Fig. 3, Table 2)

The large and small conversion products were resolved by subjecting the incubation mixtures directly to size-exclusion chromatography using two Superose 6HR columns connected in tandem. Elution profiles from the size-exclusion chromatographic procedure are shown in **Fig. 3**. The upper profile represents unmodified pig HDL incubated at 37°C for 24 h in the absence of PLTP. The HDL eluted in fractions 54–57. The center profile represents TG-enriched pig HDL (15% TG) after 24 h of incubation at 37°C in the absence of PLTP. This HDL eluted a little earlier (fractions 54–56) than the unmodified HDL. This is consistent with TG enrichment mediating a slight increase in HDL particle size. The lower profile represents TG-enriched pig HDL after 24 h of incubation at 37°C in the presence of PLTP. In this case the HDL eluted as two peaks in fractions 48–52 (large particles) and 62–66 (small particles). By comparison with standards of known molecular weight, the apparent molecular weight of the unmodified HDL was approximately 175 kDa. TG-enrichment of the HDL increased the apparent molecular weight to about 200 kDa. In the case of the TG-rich HDL which had been incubated for 24 h with PLTP, the apparent molecular weight of large conversion products was about 300 kDa, compared to approximately 50 kDa for the small conversion products. The composition of the large and small conversion products that were formed during the incubation of TG-rich pig HDL with PLTP was determined by pooling the fractions from the size exclusion chromatography column

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TABLE 2. Composition of the large and small conversion products formed during incubation of TG-enriched pig HDL with PLTP

Sample	Pool	Protein	CE	UC	PL	TG
				mass %		
Original TG-rich HDL	fractions 54–56	47	11	3	24	15
Large conversion products	fractions 48–52	45	13	3	23	16
Small conversion products	fractions 62–66	73	–	–	27	–

Pig HDL were enriched with TG by incubation at 37°C for 24 h with purified CETP and human VLDL as described in the legend to Table 1. The TG-enriched HDL were isolated by ultracentrifugation, then incubated at 37°C for 24 h with purified PLTP as described under Experimental Procedures. The large and small conversion products were resolved by gel permeation chromatography. The fractions obtained from the gel permeation chromatography column were pooled as shown in Fig. 3 and the composition of the pools was determined as described under Experimental Procedures. CE, cholesteryl ester; UC, unesterified cholesterol; PL, phospholipid; TG, triglyceride.

as indicated in Fig. 3. The composition of the pooled samples is shown in **Table 2**. The large conversion products (fractions 48–52) were comparable in composition to the TG-enriched HDL that had not been incubated with PLTP (fractions 54–56). The small particles (fractions 62–66), by contrast, contained only apoA-I and phospholipids and were deficient in CE, UC, and TG.

Incubation of TG-enriched rHDL with PLTP (Fig. 4, Table 3)

The effects of TG enrichment on the interaction of HDL with PLTP were also investigated in studies with spherical rHDL. Unmodified spherical rHDL containing only CE in their core were enriched with TG by preincubation at 37°C for 15 min with CETP and the phospholipid/TG emulsion Intralipid. To ensure that the rHDL were not modified by CETP or Intralipid, control pre-incubations consisting of rHDL and either CETP alone or Intralipid alone were carried out at 37°C for 15 min. When the preincubations were complete the rHDL were isolated by ultracentrifugation in the $1.063 < d < 1.21$ g/ml density range. The results in **Table 3** show that the composition of the rHDL was not affected significantly by incubation with either Intralipid alone or CETP alone. As has been reported in earlier studies from this laboratory (9), a trace amount of TG was evident in the rHDL that had been incubated with Intralipid but not CETP. This most likely represents minor contamination of the rHDL with Intralipid rather than spontaneous transfer of TG from Intralipid to the rHDL. After 15 min of incubation with Intralipid and CETP, the molar ratio of CE/apoA-I in the rHDL decreased from 21.2/1 to 17.1/1 and the molar ratio of TG/apoA-I increased from 0.7/1 to 5.7/1. This indicates that the rHDL gained TG and lost CE and is in agreement with what we have reported earlier (9). The non-denaturing gradient gel electrophoretic profiles in **Fig. 4** show that rHDL size was not affected by preincubation with either Intralipid alone or CETP alone. When the rHDL were incubated with both CETP and Intralipid, the diameter increased from 9.0 to 9.2 nm. This increase is not surprising given that these conditions enriched the rHDL with TG and the molecular volume of TG is approximately 1.4 times greater than that of CE (23). As judged by non-denaturing gradient

TABLE 3. Enrichment of rHDL with TG by incubation with Intralipid and CETP

rHDL	Additions	Incubation Conditions	Stoichiometry PL/UC/CE/TG/A-1
			mol/mol
1	+Intralipid, −CETP	4°C, 15 min	25.5/2.9/21.2/0.7/1.0
2	+Intralipid, −CETP	37°C, 15 min	26.1/1.3/21.3/0.7/1.0
3	−Intralipid, +CETP	37°C, 15 min	25.6/3.5/22.5/0.0/1.0
4	+Intralipid, +CETP	37°C, 15 min	28.9/5.1/17.1/5.7/1.0

Spherical rHDL (final CE concentration = 0.1 mm) were mixed with Intralipid alone (final TG concentration = 4.0 mm) or Intralipid and CETP (activity in incubation = 2.6 units/ml) and either maintained at 4°C or incubated at 37°C for 15 min. A control incubation containing rHDL and CETP, but no Intralipid, was also incubated at 37°C for 15 min. The final volume of each incubation mixture was 5 ml. When the incubations were complete the rHDL were reisolated in the $1.063 < d < 1.21$ g/ml density range, with two spins at both densities, then dialyzed against TBS. The stoichiometry of the reisolated rHDL was determined as described under Experimental Procedures using the mean of triplicate determinations which varied by less than 10%. PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride; A-I, apoA-I.

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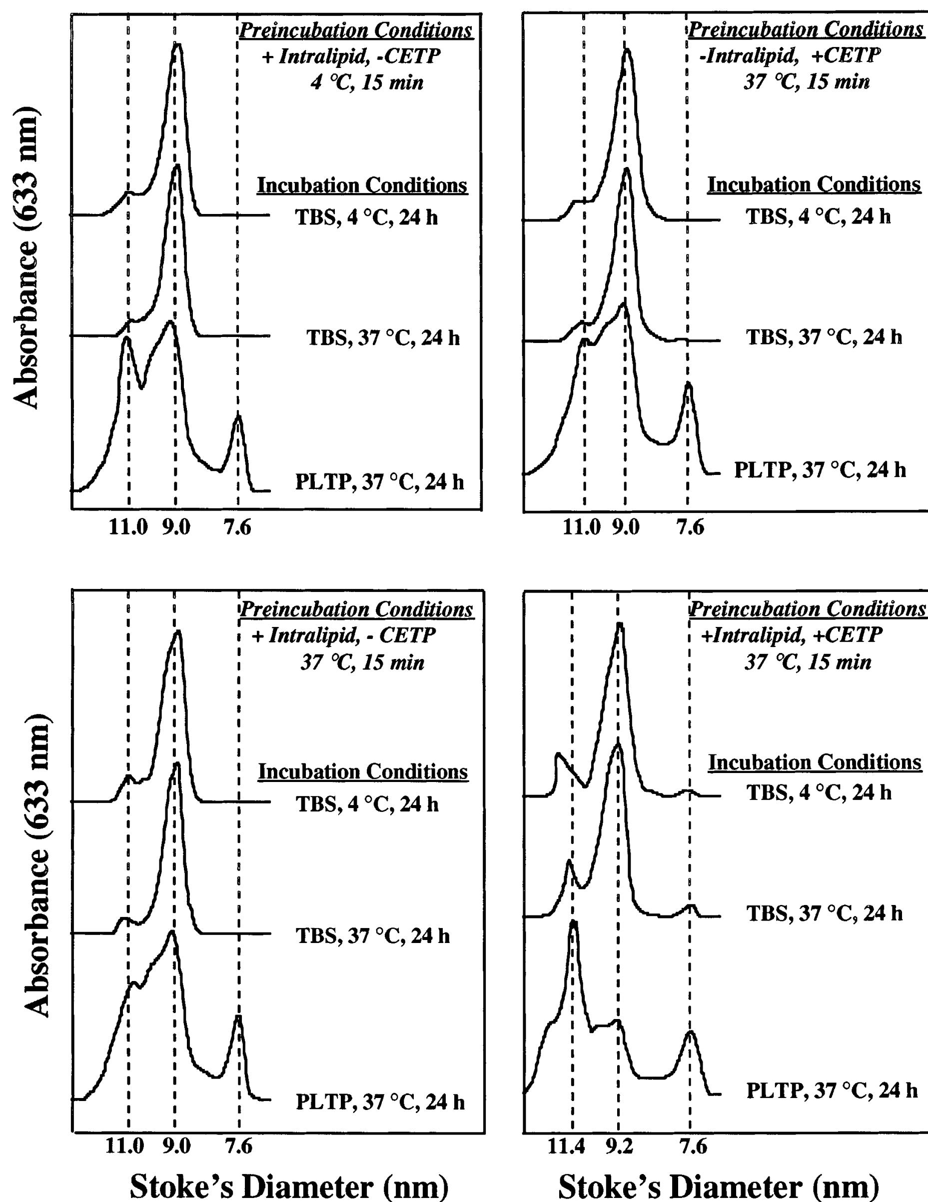

Fig. 4. PLTP-mediated changes in the size of TG-enriched rHDL. Spherical rHDL were mixed with Intralipid alone, CETP alone, or Intralipid and CETP and either maintained at 4°C or incubated at 37°C for 15 min. The rHDL were then isolated by ultracentrifugation as described in the legend to Table 3. The reisolated rHDL (final apoA-I concentration = 0.12 mg/ml) were then either maintained at 4°C for 24 h or incubated at 37°C for 24 h in the presence or absence of PLTP (0.15 ml of a preparation that transferred 2.25 μmol phospholipid/ml PLTP per h). The final volume of the incubation mixtures was 0.2 ml. When the incubations were complete the mixtures were adjusted to a density of 1.25 g/ml and the rHDL were reisolated by ultracentrifugation. The rHDL were then subjected to non-denaturing polyacrylamide gradient gel electrophoresis on PAA 3/40 non-denaturating gradient gels. Laser densitometric scans of the Coomassie-stained gradient gels are shown.

gel electrophoresis and immunoblotting, apoA-I did not dissociate from the rHDL during the 15-min incubation with Intralipid and CETP (result not shown).

The rHDL that had been preincubated with CETP and/or Intralipid were reisolated by ultracentrifugation and added to TBS or PLTP. These mixtures were either maintained at 4°C or incubated at 37°C for 24 h. When these incubations were complete the rHDL were reisolated by ultracentrifugation and subjected to non-denaturing gradient polyacrylamide gel electrophore-

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sis (Fig. 4). The rHDL that had been preincubated at 4°C in the presence of Intralipid, but without CETP, were 9.0 nm in diameter. Their size did not change when they were incubated at 37°C for 24 h with TBS. When they were incubated for 24 h in the presence of PLTP, the diameter of 15% of the particles increased to 11.0 nm while 18% decreased to 7.6 nm. The size of the remaining 67% of the rHDL did not change. Similar results were obtained for rHDL that were preincubated at 37°C with CETP alone or Intralipid alone. When the rHDL that had been pre-incubated at 37°C for 15 min with Intralipid and CETP were incubated with PLTP, the conversion to larger and smaller particles was enhanced relative to what was observed for the non-TG-enriched rHDL. The diameter of 62% of the TG-enriched rHDL increased to 11.4 nm, while 26% decreased to 7.6 nm. Only 12% of the TG-enriched particles did not change in size.

DISCUSSION

It is now well established that PLTP not only facilitates transfers of phospholipids between lipoprotein fractions, but also changes HDL particle size (1, 3). Conversion of HDL into larger and smaller particles has been demonstrated with PLTP isolated from the plasma of humans (1) and pigs (2), as well as with mouse and human recombinant PLTP (24). The results of the present study show that the PLTP-mediated conversion of HDL to larger and smaller particles is enhanced in HDL that are enriched with TG. The effect of TG enrichment was observed in experiments with both pig HDL and spherical, reconstituted HDL prepared with a synthetic phospholipid (POPC) and human apoA-I.

In the present study the small conversion products that were generated by incubating TG-enriched pig HDL with PLTP were deficient in both CE and TG. The absence of CE and TG in these conversion products is consistent with a discoidal structure (25). As judged by the work of other investigators, such particles are likely to be excellent substrates for LCAT (25, 26). If similar particles were generated in plasma by the PLTP-mediated remodelling of HDL, it is likely that they would be converted rapidly into mature, spherical HDL by activity of LCAT. It therefore follows that the generation of discoidal HDL during the interaction of PLTP with HDL may increase the rate of LCAT-mediated cholesterol esterification in plasma.

As judged by non-denaturing polyacrylamide gradient gel electrophoresis (Fig. 1), the small conversion products that were formed by incubating TG-enriched pig HDL with PLTP were 7.5 nm in diameter. Assuming that apoA-I is the sole apolipoprotein constituent in these small conversion products, their composition is consistent with a phospholipid/apolipoprotein molar ratio of approximately 14/1 (Table 2). In a recent report Zhao, Sparks, and Marcel (27) described the preparation of reconstituted, discoidal rHDL that were comparable in size and composition to these small conversion products. Zhao et al. (27) also used chemical crosslinking to establish that their reconstituted HDL contained two molecules of apoA-I/particle and exhibited an electrophoretic mobility intermediate between α and pre-β when subjected to agarose gel electrophoresis. The discoidal rHDL described by Zhao et al. (27) also mediated the efflux of unesterified cholesterol from cultured skin fibroblasts. The small conversion products that are generated during incubations of PLTP with whole plasma have also been shown to promote efflux of cholesterol from cells (7). When this result is considered together with the current data, which is consistent with the small conversion products being discoidal, it follows that PLTP may be involved in regulating the initial steps of the reverse cholesterol transport pathway.

Reverse cholesterol transport is the process whereby excess cholesterol is removed from peripheral tissues and transported to the liver for excretion or recycling (28). The primary acceptor of cellular cholesterol in plasma has been identified as a population of small, lipid-poor HDL that contain a single molecule of apoA-I and exhibit pre-β-mobility on agarose gel electrophoresis (pre-β_1 HDL) (6). Recent studies have demonstrated that the efflux of radiolabeled cholesterol from cultured skin fibroblasts into whole plasma increases by 40% when the plasma has been preincubated with exogenous PLTP (7). This finding is consistent with PLTP mediating the conversion of a proportion of the HDL in plasma into pre-β_1 HDL.

At present there is no evidence to suggest that pre-β_1 HDL are generated when isolated HDL are incubated with PLTP in vitro (1, 2). The small conversion products in the present, and in earlier studies, are large and lipid-rich relative to the pre-β_1 HDL in human plasma. There is, however, an explanation for the absence of pre-β_1 HDL in these incubations. It has been suggested that apoA-I complexed with a small amount of lipid is released from the surface of HDL during incubations with PLTP, and that these lipid–protein complexes may be comparable to the pre-β_1 HDL in plasma (4). However, if the complexes were to acquire additional phospholipids from the HDL, either by spontaneous transfer or by transfers facilitated by PLTP, they may become unstable and rearrange into larger particles with two molecules of apoA-I/particle. Such particles would be comparable to the small conversion products that are

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described in the present and earlier reports (1, 2). The notion that a single molecule of lipid-free apoA-I can acquire only a very small amount of phospholipid before becoming unstable and undergoing a structural rearrangement is consistent with what has been reported by Zhao et al. (27) who found that a single molecule of apoA-I associates with a maximum of five molecules of phospholipid.

The present findings raise the question as to whether the PLTP-mediated conversion of TG-enriched HDL into larger and smaller particles is enhanced relative to non-TG-enriched HDL in vivo. Evidence that this may be the case comes from a recent study showing that the remodelling of HDL in plasma is much greater in hypertriglyceridemic subjects than in normal subjects (29). In that study, the plasma of the hypertriglyceridemic subjects contained more small HDL than the plasma of the normolipidemic controls (29). The authors concluded that the increased concentration of small HDL was due to an increase in CETP-mediated transfers of CE and TG between HDL and other lipoprotein classes (29). However, given that the HDL of the hypertriglyceridemic subjects were enriched with TG, it is possible that the observed increase in the concentration of small HDL may have been due to the combined activities of PLTP and CETP.

One of the most interesting questions to emerge from the present study relates to the mechanism underlying the enhanced conversion of TG-enriched HDL into larger and smaller particles during incubation with PLTP. Enhanced conversion was observed for both TG-enriched pig HDL as well as for TG-enriched rHDL that contained human apoA-I. One explanation for the enhanced conversion is that both the pig HDL and the rHDL are destabilized by incorporation of TG. To this end, Sparks et al. (30) reported recently that the apoA-I molecules in TG-containing rHDL are less stable than the apoA-I in CE-containing rHDL. If this destabilization was evident throughout the entire HDL particle, it is likely that TG-containing HDL would undergo structural rearrangements to smaller, presumably more stable, particles more rapidly than their CE-containing counterparts.

It is also possible that the enhanced conversion of the TG-enriched HDL was caused by TG mediating structural changes at the HDL surface that facilitated HDL-PLTP interactions. Changes of this type could either increase the rate at which PLTP interacts with the HDL surface or increase the number of HDL binding sites recognized by PLTP. At present we do not know whether PLTP binds to the phospholipids or the apolipoproteins at the surface of HDL in vivo. There is, however, some evidence to suggest that PLTP binds to both apoA-I and apoA-II in vitro (31). Thus, the enhanced changes in HDL particle size observed in the present study may have been caused by TG altering the conformation of apoA-I at the surface of the pig HDL and rHDL in such a way that the number of PLTP binding sites increased.

There are two ways in which TG enrichment of HDL may change the conformation of apoA-I. First, substitution of TG for CE may cause unesterified cholesterol to partition from the surface into the core of the particles (32, 33). This, in turn, may alter the packing order of the phospholipids at the HDL surface (34, 35), and change the surface area that is accessible to apoA-I. If apoA-I were to then occupy all the available surface of the HDL, it follows that a change in apoA-I conformation may occur when the HDL are enriched with TG. Second, partitioning of cholesterol from the surface of HDL into the core may enhance the HDL lipid–water interfacial hydration (36) and alter the conformation of apoA-I.

In conclusion, this study shows that PLTP-mediated changes in HDL particle size are modulated by a novel, cooperative interaction between CETP, TG-rich HDL, and PLTP. The results raise, for the first time, the possibility that the size distribution of HDL in plasma may be determined by factors that regulate the concentration of TG-rich lipoproteins as well as the plasma activities of PLTP and CETP.

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