High Density Lipoprotein Conversion Mediated by Human Plasma Phospholipid Transfer Protein

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Phospholipid transfer protein (PLTP) was purified from lipoprotein-free human plasma, obtained upon treatment of plasma with dextran sulfate and Ca\(^{2+}\), by employing a series of column chromatography. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified PLTP showed a single main band, corresponding to the molecular mass of 78 kDa. However, isoelectric focusing of the purified preparation gave multiple bands with PI ranging from 4.3 to 5.1, indicative of microheterogeneity. Purified PLTP was shown to possess not only phospholipid transfer activity, but also high density lipoprotein (HDL) conversion activity (Tu, A.-Y., Nishida, H. I., and Nishida, T. (1990), FASEB J. 4, A2148; Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A., and Ehnholm, C. (1993) J. Biol. Chem. 268, 4032–4036). Isolated HDL\(_2\) was enlarged to the size of HDL\(_{2b}\) upon incubation with purified PLTP for 6 h at 37 °C at the PLTP/HDL\(_2\) molar ratio of approximately 1:45. Both the HDL conversion and the phosphatidylcholine transfer activities of purified PLTP were effectively inhibited by rabbit anti-PLTP immunoglobulin G. The primary importance of PLTP in the HDL enlargement that occurs in human plasma upon incubation at 37 °C was shown by the strong inhibitory effect of the anti-PLTP immunoglobulin G. The process of PLTP-mediated HDL enlargement was accompanied by the release of apoproteins, primarily apoA-I. HDL\(_2\) enlargement mediated by PLTP was effectively inhibited by the addition of free fatty acids.

Two types of lipid transfer proteins are present in plasma. Lipid transfer protein (LTP\(^{3}\)) is involved in the transfer of neutral lipids such as cholesteryl ester and triglycerides, as well as, phospholipids (PL) among plasma lipoproteins, and is often called cholesteryl ester transfer protein or CETP (1–3). Phospholipid transfer protein (PLTP) facilitates the transfer of only PL, but not the neutral lipids (4–6). It may exert a physiological role in the transfer of phospholipids from the surface coat of lipolyzed chylomicrons and very low density lipoproteins to HDL (7, 8).

Although plasma was previously shown to contain conversion factor activities transforming HDL into larger and smaller particles (9, 10), the activities were more recently attributed to the effect of LTP (11, 12). Our study, however, revealed that the conversion factor activity responsible for the HDL enlargement was exerted by PLTP (13). The same conclusion was recently reported by Jauhiainen et al. (14). In both studies (13, 14), PLTP purified to apparent homogeneity was shown to convert HDL primarily to larger particles. Jauhiainen et al. (14) also showed the absence of inhibition of the HDL conversion by monoclonal anti-LTP antibody, thus indicating an independence of the conversion to LTP. In this communication, the direct proof for the involvement of PLTP for the HDL enlargement was provided by the inhibition of both PC transfer and HDL conversion activities of purified PLTP preparations and of human plasma by the anti-PLTP IgG prepared. Although the addition of fatty acid is known to enhance the LTP-mediated conversion of HDL primarily to smaller particles (12, 15), it showed an inhibitory effect on PLTP-mediated HDL enlargement.

EXPERIMENTAL PROCEDURES

Buffer

Experiments were performed in 39 mM phosphate buffer containing 60 mM NaCl (ionic strength 0.16, pH 7.4) unless otherwise specified. The term EDTA designates 0.025% solution unless its concentration is given.

Materials

Human VLDL (d < 1.006 g/ml), LDL (d = 1.006–1.063 g/ml), HDL\(_2\) (d = 1.063–1.25 g/ml), and HDL\(_3\) (d = 1.125–1.21 g/ml) were prepared as previously described (16, 17). Apolipoproteins were prepared from VLDL and HDL as previously described (18). Cholesterol (98+%), and crystallized lyophilized bovine serum albumin were purchased from Sigma, and 2-mercaptoethanol from Aldrich. [9,10-\(^3\)H]Dipalmitoyl phosphatidylcholine (PC) was obtained from Du Pont-New England Nuclear. Dextran sulfate (sodium salt), phenyl-Sepharose CL-4B, heparin-Sepharose CL-6B, standard proteins for the calibration of the isoelectric points and molecular weights, and Ampholine for isoelectric focusing were purchased from Pharmacia LKB Biotechnology Inc. CM-cellulose, CM-52, and DEAE-cellulose, DE-52, are products of Whatman Inc. Hydroxylapatite, reagents for polyacrylamide gel electrophoresis, goat anti-rabbit IgG alkaline phosphatase conjugate, alkaline phosphatase substrate kit, Tris base and Tween 20 were obtained from Bio-Rad. All other chemicals were of reagent grade quality and were used without further purification. Glass-distilled water was used to prepare all aqueous solutions.
Preparation of Labelled PC Vesicles and PC Cholesterol Vesicles

Single bilayer PC vesicles or PC-cholesterol vesicles were prepared according to the method of Batzi and Korn (19) as previously described (18). An ethanol solution (0.3 ml) containing 12.5 μmol of egg PC, [3H]dipalmitoyl PC ([3H]DPPC) (3.7 pCi), and 0, 2.1, 4.2, or 6.2 μmol of cholesterol was rapidly injected into 4.7 ml of phosphate buffer while stirring under N2. After incubation of the mixture for 30 min at room temperature, the ethanol used for lipid injection was removed by exhaustive dialysis at 4 °C against phosphate buffer. The amounts of cholesterol present in the preparations of PC-cholesterol vesicles corresponded to 0, 14, 25, and 33 mol% of the total lipids. These vesicles were stored at 4 °C and used within 1 week after the preparation.

Labeling of LDL

The labeling of LDL was carried out essentially according to the method of Albers et al. (6). An ethanol solution (0.3 ml) containing [3H]DPPC (3.5 μCi) was injected into 4.7 ml of freshly isolated LDL preparation in phosphate buffer. The mixtures were incubated at 37 °C for 3 h and then dialyzed exhaustively at 4 °C against the same buffer. The preparations used in experiments were stored at 4 °C and used within 2 weeks of the preparation. The methods used for the determination of lipid and protein compositions of LDL were previously described (20, 21).

Determination of PLTP Activity

The PC vesicles/HDL assay system was primarily used for the determination of PLTP activity. The assay mixtures consisted of 35 μl of 1% PC vesicles, 3.5 μg of egg PC, and with the specific activity of 32.9 μCi/μmol as PC donors, HDL2 (96 n mole of PL) as the acceptor and varying amounts of PLTP in phosphate buffer containing 60 mM NaCl with a final volume of 280 μl. The assay mixtures were incubated at 37 °C for 15 min. After incubation, the vesicles in the assay mixtures were precipitated by adding 80 μl of 0.005% dextran sulfate and 40 μl of 0.2 M MgCl2 (21). After standing for 20 min in an ice bath, the mixtures were centrifuged at 2,900 x g for 20 min to obtain the supernatant solution containing HDL2. Then an aliquot of the supernatant (200 μl) was analyzed for its radioactivity. The PLTP activity was computed by subtracting the blank values, which included the spontaneous transfer, from the total PC transfer obtained in the presence of PLTP.

Inhibition of the transfer activities of plasma PLTP and purified PLTP by rabbit anti-PLTP IgG was determined by using both the PC vesicles/HDL2 assay system and LDL/HDL assay system. For the LDL/HDL assay system, the [3H]DPPC-LDL (255 n mole of PL), and acceptors, HDL2 (255 n mole of PL), were incubated with various amounts of PLTP samples in phosphate buffer at 37 °C for 30 min with a final volume of 300 ul. After the incubation, the LDL in the assay mixture was precipitated by adding 46 μl of 0.02% dextran sulfate and 30 μl of 50 mM MgCl2 (21). After standing at 4 °C for 20 min, the mixtures were centrifuged at 2,900 x g for 20 min to precipitate radiolabeled LDL. An aliquot of the supernatant (150 μl) containing HDL2 was measured for radioactivity. The PL transfer from LDL to HDL was calculated by subtracting the transfer value in the absence of PLTP from the total amounts of [3H]DPPC transferred in the presence of PLTP.

Purification Procedures

All steps were carried out at 4 °C or in an ice bath and all solutions were precooled in a refrigerator. Chromatographic runs were monitored at 280 nm with an ISCO model UA-4 or 5 absorbance monitor (ISCO, Lincoln, NE). The centrifugations were carried out with a Du Pont/Sorvall RC-5 refrigerated centrifuge. Dialysis was carried out by using Spectra/Por regenerated cellulose membranes (Spectrum Medical Industries, Inc., Houston, TX) unless otherwise noted.

Step 1: Dextran Sulfate Treatment—This step is similar to that used for the purification of lecithin-cholesterol acyltransferase (22) and LTP (23). To 1 liter of the stirred plasma, 1.5 liter of glass-distilled water and 500 ml of dialyzed dextran sulfate (10 g) was added. This was followed by the addition of 4 M CaCl2 to the final concentration of 0.1 M. The mixture was stirred gently for 15 min and then centrifuged at 16,000 x g for 20 min in order to remove the insoluble dextran sulfate-cholesterol complex.

Step 2: Phenyl-Sepharose Chromatography—This chromatography was carried out in a similar manner as described for the purification of LTP and lecithin-cholesterol acyltransferase (17, 23). To the stirred dextran sulfate supernatant fraction, solid NaCl was added to increase the ionic strength to 0.8. This mixture was applied to a phenyl-Sepharose CL-4B column (3.5 x 21 cm), which was previously equilibrated with 0.8 M NaCl containing EDTA. The column was washed with 1,000 ml of 0.8 M NaCl solution containing 50 mM phosphate buffer containing EDTA. After elution of lecithin-cholesterol acyltransferase and LTP with 800 ml of 2.9 mM phosphate buffer (pH 7.4) containing EDTA (18, 24), the PLTP fraction was eluted with 500 ml of 25% ethanol.

Step 3: CM-Cellulose Chromatography—An aliquot of 2 M acetate buffer (pH 4.5) was added to 20% ethanol fraction to yield 50 mM buffer concentration. The fraction was applied to a CM-52 column (3.6 x 16 cm), which was equilibrated with 50 mM acetate buffer. The column was washed with 300 ml of the acetate buffer containing 50 mM NaCl, and the PLTP fraction was eluted with 700 ml of 50 mM acetate buffer containing 100 mM NaCl.

Step 4: DEAE-Cellulose Chromatography—The CM-cellulose fraction was dialyzed against 4 mM phosphate buffer (pH 7.4) containing EDTA by using Spectra hollow fiber bundles (13,292) and was applied to a DE-52 column (1.9 x 9 cm) equilibrated with the phosphate buffer. The column was washed with 150 ml of the phosphate buffer containing 50 mM NaCl and EDTA and then dialyzed against 4 mM sodium phosphate buffer containing 150 mM NaCl and EDTA.

Step 5: Heparin-Sepharose Chromatography—The DEAE-cellulose fraction was dialyzed against 4 mM phosphate buffer containing EDTA (pH 7.4) and was applied to the heparin-Sepharose CL-4B column. (1.5 x 6 cm) equilibrated with 4 mM phosphate buffer containing EDTA. The column was washed with 240 ml of 4 mM phosphate buffer containing 100 mM NaCl and with 80 ml of phosphate buffer containing 200 mM NaCl. The PLTP fraction was eluted with 80 ml of phosphate buffer containing 490 mM NaCl and EDTA and then dialyzed against 4 mM sodium phosphate buffer containing 150 mM NaCl (pH 6.8).

Step 6: Hydroxylapatite Chromatography—The dialyzed Heparin-Sepharose fraction was applied to a hydroxylapatite column (1.9 x 2.5 cm), which was previously equilibrated with 4 mM sodium phosphate buffer (pH 6.8) and with 150 mM NaCl. The column was washed with 35 ml of the equilibration buffer. PLTP was eluted with 40 ml of 25 mM sodium phosphate buffer containing 150 mM NaCl. The PLTP fraction was dialyzed against 0.4 mM phosphate buffer (pH 7.4) containing EDTA and 2 mM NaN3, and then dialyzed against 4 mM sodium phosphate buffer containing 150 mM NaCl.

Gradient Gel Electrophoresis (GGE)

GGE was carried out essentially according to the method of Blanche et al. (24) by using Pharmacia 4-30% and Bio-Rad 4-20% non-denaturing polyacrylamide gradient gel. Stained gel was scanned with an ISCO model 1312 gel scanner and analyzed by using high molecular weight electrophoresis calibration standards (Pharmacia).

Preparation of Rabbit Anti-Human PLTP IgG

Approximately 150 μg of purified human plasma PLTP in 1.0 ml of 4 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl were mixed with the same volume of Freund’s complete adjuvant. The oil suspension, 2 ml per rabbit (body weight of approximately 2.4 kg), was injected into multiple intradermal sites (23). The second injection of a similar amount of PLTP in 2 ml of oil suspension (Freund’s incomplete adjuvant: 0.15 M NaCl, 1:1, v:v) was given 2 weeks after the first injection. The blood was collected from the ear vein 1 week after the second injection. IgG fraction against human plasma PLTP was purified from the serum by using dextran sulfate/ CaCl2 treatment, ammonium sulfate precipitation, and DEAE-cellulose column chromatography. The control IgG was isolated from non-immunized rabbit sera following the same procedures (23).

The specificity of anti-PLTP IgG was determined by a solid phase enzyme-linked immunosorbent assay method using goat anti-rabbit IgG conjugated with alkaline phosphatase and Bio-Rad alkaline phosphatase substrate kit according to the procedure described by the manufacturer. The color that developed was measured at 405 nm. A standard curve was constructed for 0-18 ng of purified PLTP ad-
sorbed to microtiter plates in order to approximate the reactivities of various apoproteins, lecithin-cholesterol acyltransferase, and LTP, that were adsorbed to the plates, toward the anti-PLTP IgG. Assay methods with the use of antibody adsorbed to the plates were not feasible because of a relatively low titer of our anti-PLTP IgG preparation. The enzyme-linked immunosorbent assay indicated that the anti-PLTP IgG is highly specific for PLTP. Purified apoA-I, apoA-II, apoC-III, and apoE adsorbed to the plates showed no reactivity toward the anti-PLTP IgG. Purified apoD, lecithin-cholesterol acyltransferase, and LTP gave the reactivities equivalent to 0.5, 2.2, and 1.7%, respectively, of that of purified PLTP on protein weight basis. These proteins, however, showed no significant inhibitory effect on the binding of anti-PLTP IgG to the adsorbed purified PLTP. Anti-PLTP IgG obtained did not inhibit significantly the cholesteryl ester formation by purified lecithin-cholesterol acyltransferase (22) and cholesteryl ester transfer reaction by purified LTP (23).

Analytical Methods

The protein concentration was determined by the Lowry method (25) using crystalline bovine serum albumin as the standard. The enzymatic and fluorometric method of Gamble et al. (26) was modified for the determination of small amounts of free cholesterol and cholesterol ester. The amounts of PL were determined by the method of Bartlett (27). Triglyceride contents were measured by an enzymatic assay using an enzymatic assay kit from Sigma. SDS-PAGE on a 10% polyacrylamide gel was performed using a Mini-Protein II Dual Slab Cell (Bio-Rad) as previously described (23). The molecular weight determination was carried out by comparing with those of the standard proteins as previously described (23). Isoelectric focusing was carried out on a 5% polyacrylamide gel by using a Mini-Protein II Dual Slab Cell (Bio-Rad) as previously described (23). The pH gradient was determined by using the standard proteins with known isoelectric point (Pharmacia).

RESULTS

Purification of PLTP from Human Plasma—The purification of PLTP from 1 liter of human plasma is summarized in Table I. Dextran sulfate/CaCl₂ treatment, under the conditions given, allowed the effective precipitation of all plasma lipoproteins (22, 23). This lipoprotein removal from plasma was necessary for its application to the phenyl-Sepharose column, in order to obtain a fraction containing LTP and lecithin-cholesterol acyltransferase (17, 23). This elution also reduced the contaminant protein in the PLTP fraction. Unlike CM-cellulose chromatography, DEAE-cellulose chromatography did not lead to a substantial increase in the specific activity. This step, however, was necessary to remove contaminant proteins which, otherwise, would be present in trace amounts in the final PLTP preparations.

Heparin-Sepharose served as an excellent affinity chromatography column for PLTP (6, 26, 29). Elution of PLTP with the least amount of contaminant proteins was obtained by increasing the NaCl concentration of 4 mM phosphate buffer (pH 7.4) from 200 to 400 mM. The last purification step, hydroxyapatite column chromatography, was very effective in removing remaining contaminant proteins. The final purification of PLTP was approximately 38,700-fold over the starting plasma PC transfer activity. Since about 10% of the PC transfer activity of the starting plasma was contributed by LTP under the assay conditions used, the final purification fold could be 10% higher than the value given. The hydroxyapatite fraction exhibited a single major band by SDS-PAGE and by staining with silver stain reagent (Fig. 1, Panel A). From the mobility on the polyacrylamide gel, an apparent molecular mass of 78 kDa was computed. In spite of the apparent homogeneity exhibited by SDS-PAGE, the purified PLTP showed multiple bands upon isoelectric focusing on 5% polyacrylamide gel. The isoelectric points of the band ranged from 4.3 to 5.1 (Fig. 1, Panel B). Nearly identical patterns consisting of at least 10 bands were obtained for three different purified preparations. The presence of both 0.2% Triton X-100 and 2 mM urea in the gel was essential to resolve the multiple bands. The multiple bands of LTP due to its microheterogeneity were previously resolved in the presence of Triton X-100, but without addition of urea (23), and those of lecithin-cholesterol acyltransferase were resolved in the absence of both Triton X-100 and urea (30).

Immunoinhibition of the PC Transfer Activities of Purified PLTP and Plasma PLTP—Rabbit anti-PLTP IgG was obtained from the rabbit which had received an intradermal injection of purified PLTP. When anti-PLTP IgG was added to purified PLTP, almost complete inhibition of PC transfer activity was observed with both PC vesicle/HDL₃ and LDL/HDL₃ assay systems (Fig. 2, Panel A, curves 2 and 3). The addition of control IgG did not significantly inhibit the PC transfer activity (curve 1). When purified rabbit anti-PLTP IgG was added to plasma samples, approximately 90% of total PC transfer activity of plasma was removed at high IgG concentrations (800 µg) with the vesicles/HDL₃ assay system (Panel B, curve 3). However, when the LDL/HDL₃ assay system was used, the inhibitory effect of anti-PLTP IgG was reduced; about 40% of the transfer activity remained in the plasma (curve 2). In both assay systems, the transfer activities remaining in the plasma were due to the PC transfer activity of LTP present in the plasma (6). The differences in the inhib-

| Table I Purification of PLTP from human plasmaa |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fraction        | Volume (ml)   | Total protein (mg) | Total activity (units) | Recovery (%) | Specific activity (units/mg) |
| 1. Plasmab       | 1,000         | 73,000            | 11,720,000*           | 100           | 0.16                       |
| 2. Dextran sulfate/CaCl₂ | 3,000       | 53,000            | 8,690,000*           | 74            | 0.16                       |
| 3. Phenylo-Sepharose | 500          | 59,000            | 4,930,000*           | 85            | 0.16                       |
| 4. CM-cellulose  | 150           | 35                | 3,020,000*           | 42            | 0.22                       |
| 5. DEAE-cellulose| 150           | 35                | 2,180,000*           | 42            | 0.52                       |
| 6. Heparin-Sepharose| 80           | 2.8               | 1,210,000            | 432           | 2,700                      |
| 7. Hydroxyapatite| 40            | 0.14              | 868,000              | 7.4           | 6200                       |

* Values are representative of typical preparations.
# The amounts of protein were determined by Lowry method.
1 unit 1 nmol [3H]PC transferred/h.
2% Dextran sulfate/CaCl₂ fraction was approximately 10%.
proteins from drase (30 kDa). Isoelectric focusing of purified PLTP. Approximately 2 pg of purified PLTP standard proteins serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). Isoelectric focusing of purified PLTP (B, lane 1) and low pI standard proteins (Pharmacia) (lane 2) were carried out on a 5% polyacrylamide gel containing 0.2% Triton X-100, 2.0 M urea, and Ampholine (pH 4-6) for 5 h at 0.6 watt. After treatment with 10% trichloroacetic acid overnight, the gel was also stained with silver stain reagent. The applied sample contained 0.7 M 2-mercaptoethanol. The standard proteins from top to bottom are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). After electrophoresis, the gels were stained with silver stain reagent. The percent PC transfer, as a function of mol % cholesterol in the donor vesicles. The transfer rate was reduced to only about 20% of the transfer activity obtained in the absence of cholesterol. These results are indeed in agreement with the observations made by other laboratories using partially purified PLTP (31, 32).

HDL Conversion Activity—Purified PLTP preparations also showed HDL conversion activity. The effect of PLTP

Effect of Cholesterol on the PC Transfer Activity—To determine if our purified PLTP exhibits properties identical with those of partially purified PLTP fractions previously used by other investigators (31, 32), the PC transfer activities were determined using vesicles containing 0 (curve 1), 14 (curve 2), 25 (curve 3), and 33 mol % of cholesterol as PC donors (Fig. 3). The progressive increase in the cholesterol content resulted in a marked decrease in the PC transfer activities which were plotted as a function of PLTP concentration. The percent PC transfer, as a function of mol % cholesterol in the donor vesicles (Fig. 3, inset) was obtained in the presence of 40 ng of PLTP in comparison to the activity in the absence of cholesterol. When cholesterol content of the vesicles was increased to 33 mol %, the transfer rate was reduced to only about 20% of the transfer activity obtained in the absence of cholesterol. These results are indeed in agreement with the observations made by other laboratories using partially purified PLTP (31, 32).

FIG. 3. Effect of cholesterol in the donor vesicles on the PLTP-mediated PC transfer from vesicles to LDL. The PC vesicle preparations contained 250 nmol of egg PC, 11 nmol of [3H] DPPC, and varying amounts of cholesterol; 0 (curve 1), 14 (curve 2), 25 (curve 3), and 33 mol % (curve 4). The vesicles were incubated with human plasma LDL containing 134 nmol of PL and varying amounts of purified PLTP in phosphate buffer for 20 min at 37°C. After the incubation, LDL in the assay mixtures was precipitated by adding dextran sulfate and MgCl₂ to the final concentration of 0.01% and 6 mM, respectively. After standing for 20 min in ice, the assay mixtures were centrifuged at 2,900 X g for 20 min, and the supernatant solutions containing vesicles were carefully and completely removed. The LDL precipitate was dissolved in 100 µl of 0.1 N NaOH, and an 80-µl aliquot was analyzed for radioactivity. The PLTP-mediated PC transfer activity was calculated by subtracting the blank values, which included the nonfacilitated transfer, from the total PC transfer obtained in the presence of PLTP. In the inset, the percentage of the PC transfer activity in the presence of 40 ng of PLTP, relative to the activity in the absence of cholesterol, was plotted as a function of cholesterol mol % in the donor vesicles.
concentrations on the extent of HDL enlargement was studied by incubating isolated HDL3 with varying amounts of purified PLTP at 37 °C for 6 h and by determining the GGE patterns. Upon addition of 400 ng of purified PLTP (equivalent to PLTP/HDL3 molar ratio of approximately 1:45), HDL3 was converted to a higher molecular weight species having a Stokes' diameter of 10.7 nm (Fig. 4, pattern 3), which corresponds to the average size of HDL3 (24). Heterogeneous patterns were obtained especially in the presence of low concentrations of PLTP (33 ng, pattern 7; 56 ng, pattern 6), possibly reflecting the differences in susceptibility of various HDL3 subspecies to PLTP-mediated enlargement. This heterogeneity, however, was progressively reduced by increasing the amount of PLTP in the mixtures (100 ng, pattern 5; 200 ng, pattern 4). At the highest concentration of PLTP (400 ng, pattern 3), a relatively homogeneous pattern was obtained. The persistent appearance of a very small peak with a Stokes' diameter of 7.8 nm even at high molar ratios may indicate the presence of small amounts of subspecies resisting the enlargement.

It is noteworthy that even in the presence of the lowest amount of PLTP (PLTP/HDL3 molar ratio of approximately 1:540, pattern 7), the enlargement of HDL3 was detected by the appearance of the leading peak having the diameter of approximately 9.0 nm. The Stokes' diameter of the leading peak, representing the enlarged particles, was plotted as a function of PLTP/HDL3 molar ratio (Fig. 5). The enlarged peak approached the maximal size at the molar ratio of approximately 0.011 (1:90).

To show changes in the density and sizes of HDL3 particles that occur by treating isolated HDL3 with PLTP, four fractions (d < 1.125, 1.125 < d < 1.21, 1.21 < d < 1.25, and d > 1.25 g/ml) were obtained by sequential ultracentrifugation of HDL3 samples (1.48 mg as protein) incubated with 50,000 units (8.1 μg) of purified PLTP. The recovered HDL3 fractions were analyzed by gradient gel electrophoresis and the recovery of HDL3 proteins and lipids were determined. The distribution of control HDL3-protein, incubated without PLTP, in the four fractions, was 8, 76, 9, and 7%, respectively, while that of HDL3-protein, incubated with PLTP, in the corresponding four fractions was 30, 37, 4, and 29%, respectively. It was apparent that control HDL3 gave the recovery of the HDL3 primarily at 1.125 < d < 1.21 g/ml with a small amount of the lipoprotein recovered at d < 1.125 g/ml. Upon incubation of HDL3 with PLTP (PLTP/HDL3 molar ratio of 1:150), the recovery of the lipoprotein at 1.125 < d < 1.21 ml was greatly reduced with a major portion of the lipoprotein recovered at d < 1.125 g/ml. Furthermore, the size of lipoprotein particles in both fractions were substantially larger than the corresponding control samples (data not shown). The conversion of HDL3 into less dense, larger particles was accompanied by the release of substantial amounts of apoproteins; 29% of HDL3 protein was recovered from the d > 1.25 g/ml fraction when HDL3 was incubated with PLTP. Analysis of the protein by SDS-PAGE showed that apoA-I was a major apoprotein released during the conversion. Control HDL3 incubated in the absence of PLTP gave the recovery of approximately 7% of the protein (mostly apoA-I) from the d > 1.25 g/ml fraction. This may reflect the release of relatively small amounts of apoproteins known to occur upon recentrifugation of HDL (33).

Compositional analysis of the enlarged HDL, obtained from the

![Fig. 4. Effect of purified PLTP concentration on the isolated HDL conversion.](image)

**Fig. 4. Effect of purified PLTP concentration on the isolated HDL conversion.** Isolated HDL3 (20 μg as protein) was incubated in 4 mM phosphate buffer (pH 7.4) containing 150 mM NaCl in the presence of varying amounts of PLTP at 37 °C for 6 h. GGE of the samples was carried out on Bio-Rad 4-20% nondenaturing gradient gel using a Mini-Protein II Dual Slab Cell in 0.09 M Tris-HCl and 0.08 M borate buffer (pH 8.3) for 2000 V-h. After electrophoresis the gels were stained with Coomassie Brilliant Blue G-250 in isopropanol/acetic acid/water (25:10:65, v/v) and destained with the same solvent. Densitometer profiles 1 and 2 represent the nonincubated control HDL3 sample kept at 4 °C and the control sample incubated at 37 °C in the absence of PLTP, respectively. Profiles 3, 4, 5, 6, and 7 were obtained for the samples incubated in the presence of 400, 200, 100, 50, and 33 ng of PLTP. PLTP/HDL3 molar ratios of these samples are approximately 1:45, 1:90, 1:180, 1:360, and 1:540, respectively, based on the HDL3 molecular weight of 175 kDa and the protein content of 55%. The results shown are representatives of three similar experiments. One ng of PLTP exhibited 6.2 units of PC transfer activity.

![Fig. 5. Effect of PLTP/HDL3 molar ratio on the Stokes' diameter of the enlarged HDL3.](image)

**Fig. 5. Effect of PLTP/HDL3 molar ratio on the Stokes' diameter of the enlarged HDL3.** Stokes' diameters of the enlarged particles in the densitometer profiles (Fig. 4) were plotted against the PLTP/HDL3 molar ratio.
the HDL₃ incubated with PLTP, showed that the protein, PL, free cholesterol, cholesteryl ester, and triglyceride contents of the d < 1.125 g/ml fraction were 48, 26, 1, 22, and 3%, respectively; those of the 1.125 < d < 1.21 g/ml fraction were 55, 22, 1, 19, and 4%, respectively; and those of the d > 1.25 g/ml fraction were 98, 0.1, 0.4, and 0.3%, respectively. Corresponding values obtained for control HDL₃ recovered from the 1.125 < d < 1.21 g/ml fraction were 57, 22, 1, 17, and 4%, respectively. Noticeable decreases occurred in the protein content of the enlarged HDL in the d < 1.125 and 1.125 < d < 1.25 ml fractions, recovered from the HDL₃ incubated with PLTP, as compared to control HDL₃, reflecting the release of apoproteins in the d > 1.25 g/ml fraction. Apparently the change in the density and size of HDL₃ upon incubation with PLTP was due to the apoprotein removal from HDL, resulting in the conversion of apoprotein deficient HDL₃ particles into the lighter and larger HDL₃ and HDL₄-like particles.

**Immunoinhibition of HDL Conversion Activity**—We observed that the addition of excess anti-PLTP IgG to the incubation mixtures containing isolated HDL₃ and PLTP prevented the PLTP-mediated HDL enlargement. In order to determine the contribution of human plasma PLTP to the HDL enlargement that occurs upon incubation of plasma at 37 °C, plasma samples treated with varying amounts (0–1000 μg) of anti-PLTP IgG were incubated for 8 h at 37 °C. In the absence of anti-PLTP IgG, the plasma incubation caused the enlargement of HDL₃ as previously observed (10, 34). As compared to the HDL₃ in control plasma samples kept at 4 °C (Fig. 6, patterns 1 and 8 in both A and B), the incubation for 8 h at 37 °C resulted in the formation of enlarged HDL (patterns 2 and 7). The addition of increasing amounts of anti-PLTP IgG to plasma progressively reduced the extent of HDL enlargement (patterns 3 and 4). The enlargement was almost completely prevented at the highest concentration of anti-PLTP IgG (pattern 5). The addition of non-immunized IgG (pattern 6) did not reduce the extent of HDL enlargement. We also observed that the treatment of plasma with anti-LTP IgG (23) and with anti-lecithin-cholesterol acyltransferase IgG prepared against purified lecithin-cholesterol acyltransferase (18) did not show any appreciable inhibitory effect on the enlargement.

**Effect of Fatty Acid on HDL Conversion**—To study the effect of fatty acid on the PLTP mediated HDL₃ conversion, HDL₃ preparations, preincubated with varying amounts of palmitic acid, were incubated with PLTP at 37 °C for 6 h. The HDL₃ enlarged by PLTP in the absence of palmitic acid, gave a Stokes’ diameter of 10.3 nm (Fig. 7, pattern 3). This enlargement was progressively inhibited upon increasing the amount of palmitic acid added to HDL₃ (patterns 4–8). In the presence of 1.5 and 3 μg of palmitic acid, the GGE pattern of the incubation mixtures showed considerable heterogeneity (patterns 4 and 5), possibly reflecting the differences in the affinities of HDL₃ subspecies for palmitic acid. The extent of heterogeneity in the GGE patterns was reduced with increasing amounts of palmitic acid added to HDL₃ (patterns 6–8). The inclusion of 8 μg of palmitic acid nullified the HDL enlargement (pattern 8).

**DISCUSSION**

In the present study, human plasma PLTP was purified and some basic factors influencing the PLTP-mediated HDL conversion process were defined. The HDL conversion was observed as an enlargement of HDL₃ into HDL₄-like particles. The presence of HDL conversion factor activity in plasma was first observed by Gambert et al. (10). The incubation of plasma or isolated HDL in the presence of the d > 1.25 g/ml fraction of plasma at 37 °C for 24 h caused a decrease in small HDL and an increase in large HDL. Rye and Barter (9) also showed the presence of the conversion factor. The incubation of HDL with the partially purified conversion factor led to the formation of larger and smaller HDL particles (9). The identity of HDL conversion factor (11) remained unknown and it was later concluded that the HDL conversion was indeed caused by LTP (12).

![Image](image_url)
HDL enlargement was indicated upon comparison of the HDL patterns of control plasma (Fig. 6, patterns 1 and 8 in both A and B) and those of the plasma incubated at 37 °C for 8 h (patterns 2 and 7). Although the incubation caused a rather limited increase in the Stokes's diameter of the main peaks, the appearance of shoulders with larger Stokes’s diameter suggested the presence of HDL subspecies which were rapidly converted to HDL₄-size particles. Near complete prevention of the HDL enlargement by excess anti-PLTP IgG (pattern 5) suggested the possible involvement of PLTP in the HDL enlargement in vivo.

Based on the stoichiometry of the HDL₄ conversion (Fig. 5), PLTP apparently served as a catalyst or an enhancer of the process. The lowest PLTP/HDL₄ molar ratio used (1:540, pattern 7) roughly corresponds to the physiological molar ratio assuming the plasma PLTP concentration of 2 mg/liter. At the molar ratio of 1:360 (pattern 6), a major portion of HDL₄ was converted to HDL₃ₐ₄-size particles (Stoke’s diameter of 9.3 nm) upon incubation for 6 h at 37 °C. Although at the PLTP/HDL₄ molar ratio of 1:45 (pattern 3), HDL₄ was almost completely converted to HDL₃ₐ₄-size particles, physiological relevance of the conversion at such a high molar ratio is questionable. This conversion, however, indicated the near upper limit for the size of the particles (Fig. 5) that can be produced from HDL₄.

The PLTP-mediated HDL conversion was accompanied by the release of apoproteins, primarily apoA-I in accordance with previous observations (9, 14, 36). It is interesting to note that when isolated HDL was treated with 3 M guanidine HCl (37) or heated to 70 °C (38), the apparent HDL size increased, which seemed to be correlated with the dissociation of apoA-I. Apparently, the amount of apoA-I in HDL particles regulated the size of the particles. The preferential release of apoA-I upon treatment of HDL₄ with PLTP may be the result of the weaker affinity of apoA-I for HDL₄ (16, 39). It is well known that HDL is extremely heterogeneous with respect to apoprotein and lipid compositions (40–42). The role of PLTP in the apoprotein release needs to be investigated with refined model systems and various HDL subfractions, such as HDL containing apoA-I without apoA-II and HDL containing both apoA-I and apoA-II (42).

Although pretreatment of HDL₄ with palmitic acid was shown to inhibit the enlargement of HDL₄, plasma free fatty acids are not likely to exert inhibitory effects on the HDL enlargement in vivo. Free fatty acid concentrations in plasma are very low and yet most free fatty acids are in association with albumin, thus making unbound free fatty acid concentrations extremely low (43). The effect of palmitic acid was studied in order to compare to the effect shown by fatty acids on the LTP-mediated HDL conversion (12, 15). It appears that PLTP and LTP give opposing effects on the HDL size transformation. Unlike PLTP, LTP is primarily responsible for the formation of smaller particles (11, 12). It is possible that LTP induced HDL conversions to smaller particles are enhanced by incorporation of free fatty acid by expanding the HDL surface layer. While PLTP induced HDL enlargement is prevented by the surface layer expansion which interferes with the transient formation of surface coat deficient HDL particles needed for the enlargement.

The present study suggests a number of areas which should be addressed by future investigations. Many factors influencing HDL conversion as well as the mechanism of HDL enlargement need to be clarified. Although in the plasma, PLTP is primarily responsible for the enlargement of HDL, minor but significant roles of lecithin-cholesterol acyltransferase and LTP in HDL enlargement were indicated in our prelim-

**Fig. 7. Effect of free fatty acid on PLTP-induced HDL₄ conversion.** Isolated HDL₄ (20 μg as protein) was incubated in a similar manner as given for Fig. 4 for 6 h at 37 °C in the presence of 200 ng of purified PLTP (giving the PLTP/HDL₄ molar ratio of approximately 1:90) and varying amounts of palmitic acid with the total volume of 25 μl. GGE of the incubated HDL₄ and protein staining of the gels with Coomassie Brilliant Blue G-250 were carried out as given for Fig. 4. Densitometer profile 1 represents control HDL₄ kept at 4 °C and profile 2 shows the HDL₄ incubated in the absence of PLTP. Profiles 3, 4, 5, 6, 7, and 8 represent the HDL₄ incubated in the presence of PLTP and 0, 1.5, 3.0, 4.5, 6.0, and 8.0 μg, respectively, of palmitic acid. Data given are representatives of three similar experiments.

We showed that PLTP is responsible for the enlargement of HDL (13). Jauhiainen et al. (14) also reached the same conclusion. By using anti-PLTP IgG, we provided direct evidence that PLTP is indeed responsible for the enlargement of isolated HDL as well as the enlargement of HDL in the plasma system. PC transfer activity of purified PLTP was completely abolished upon pretreatment of the assay mixtures with anti-PLTP IgG. We recently used discoidal bilayer particles containing PC, cholesterol, and apoA-I as model lipoproteins for the PLTP-mediated enlargement (35). The enlargement was effectively inhibited when the mixtures containing the discoidal bilayer particles and purified PLTP were pretreated with anti-PLTP IgG in a similar manner as given in the legend for Fig. 6. Since the apoA-I used for preparation of discoidal bilayer particles did not cross-react with anti-PLTP IgG, the inhibition must have been due to the immuno-noprecipitation of PLTP in the mixtures. No other protein was present in the mixtures. This observation as well as the high specificity of anti-PLTP IgG used in the present study indicated the primary involvement of PLTP in the HDL enlargement and not other factors or co-factors.

The possible physiological importance of PLTP-mediated...
inary studies using model lipoprotein systems as well as plasma. The contributions of lecithin-cholesterol acyltrans-
erase and LTP to HDL enhancement and maturation are to be defined under variety of conditions. Chemical character-
ization of PLTP is yet to be completed. N terminal amino acid of our purified PLTP appears

tigation. However, freshly obtained purified PLTP showed at least ten bands with PI ranging from 4.3 to 5.1 on isoelectric focusing pattern, our preliminary study showed that upon extensive treatment of PLTP with Clostridium perfringens neuramidinase (23, 30), these bands converted into two intense bands with PI values between 7.5 and 8.0. The presence of PLTP isoforms as well as their microheterogeneity, due possibly to the differences in the number of sialic acid residues, are currently under inves-

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REFERENCES
1. Pattnaik, N. M., Monteis, A., Hughes, L. B., and Zilversmit, D. B. (1978) Biochim. Biophys. Acta 550, 429-438
2. Ihn, J., Harmony, J. A., Ellsworth, J., and Jackson, R. L. (1980) Biochem. Biophys. Res. Commun. 93, 1114-1120
3. Morton, R. E., and Zilversmit, D. B. (1982) J. Lipid Res. 23, 1056-1067
4. Bremer, M. E., Ihn, J., Brainard, J. R., and Harmony, J. A. (1978) Biochim. Biophys. Acta 529, 147-169
5. Tall, A. R., Abrez, E., and Shuman, J. (1983) J. Biol. Chem. 258, 2174-2180
6. Albers, J. J., Tolleson, J. H., Chen, C., and Steinmetz, A. (1984) Arteriosclerous 4, 49-58
7. Greet, P. H. E., and Scheek, L. M. (1984) J. Lipid Res. 25, 684-692
8. Tall, A. R. (1986) Methods Enzymol. 129, 429-483
9. Rye, K.-A., and Carter, B. J. (1986) Biochim. Biophys. Acta 879, 429-438
10. Gambert, P., Lallemand, C., Athis, A., and Padiou, F. (1982) Biochim. Biophys. Acta 713, 1-8
11. Carter, B. J., Rajaram, O. V., and Chang, L. B. F., Rye, K.-A., Gambert, P., Lagrost, L., Ehnholm, C., and Fidge, N. H. (1986) Biochim. J. 254, 179-184
12. Carter, B. J., Chang, L. B. F., Newnham, H. H., Rye, K.-A., and Rajaram, O. V. (1986) Biochim. Biophys. Acta 1046, 61-69
13. Tu, A-Y., Nishida, H. I., and Nishida, T. (1986) FASEB J. 4, A2148 (abst.)
14. Jauhiainen, M., Metso, J., Pihlman, R., Blomqvist, S., van Tol, A. and Ehnholm, C.(1993) J. Biol. Chem. 268, 4092-4096
15. Lagrost, L., and Carter, B. J. (1991) Biochim. Biophys. Acta 1082, 204-210
16. Yamazaki, S., Matsunaga, T., and Nishida, T. (1983) J. Biol. Chem. 258, 12026-12035
17. Nishida, H., Kato, H., and Nishida, T. (1990) J. Biol. Chem. 265, 4876-4883
18. Nishida, H., Nakashima, T., Yen, E. A., Araki, H., Yen, F. T., and Nishida, T. (1990) J. Biol. Chem. 265, 12026-12035
19. Baten, S., and Korn, E. D. (1973) Biochim. Biophys. Acta 298, 1015-1019
20. Meusing, R. A., and Nishida, T. (1971) Biochemistry 10, 2952-2952
21. Kim, Y. C., and Nishida, T. (1979) J. Biol. Chem. 254, 9621-9626
22. Doi, Y., and Nishida, T. (1981) Methods Enzymol. 71, 553-577
23. Kato, H., Nakashita, T., Araki, H., Nishida, H. I., and Nishida, T. (1989) J. Biol. Chem. 264, 4092-4097
24. Blanche, P., Gong, E. L., Forte, T. M., and Nichols, A. V. (1981) Biochim. Biophys. Acta 666, 408-419
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
26. Gamble, W., Vaughan, M., Kruth, H. S., and Avigan, J. (1978) J. Lipid Res. 19, 918-970
27. Bartlett, G. R. (1953) J. Biol. Chem. 204, 466-468
28. Tollefsen, J. H., Ravnk, S., and Albers, J. J. (1986) J. Lipid Res. 25, 1693-1692
29. Damen, J., Rega, J., and Scherphof, G. (1982) Biochim. Biophys. Acta 712, 444-452
30. Doi, Y., and Nishida, T. (1983) J. Biol. Chem. 258, 5840-5846
31. Damen, J., Rega, J., and Scherphof, G. (1981) Biochim. Biophys. Acta 666, 535-545
32. Sweeney, S. A., and Jonas, A. (1985) Biochim. Biophys. Acta 835, 279-290
33. Kunitake, S. T., and Kane, J. (1985) J. Biol. Chem. 260, 936-940
34. Nichols, A. V., Blanche, P. J., Shore, V. G., and Gong, E. L. (1989) Biochim. Biophys. Acta 1001, 325-337
35. Nishida, H., Tu, A-Y., and Nishida, T. (1993) FASEB J. 7, A1269 (abst)
36. Nishida, H., Blanche, P. J., Shore, V. G., and Forte, T. M. (1985) Biochim. Biophys. Acta 834, 285-300
37. Nichols, A. V., Gong, E. L., Blanche, P. J., Forte, T. M., and Anderson, D. W. (1978) Biochim. Biophys. Acta 446, 226-239
38. Tall, A. R., Deckelbaum, R. J., Small, D. M., and Shipley, G. G. (1977) Biochim. Biophys. Acta 487, 145-153
39. Ibahal, J. A., Krabs, E. K., and Phillips, M. C. (1988) Biochim. Biophys. Acta 1004, 300-308
40. Albers, J. J. (1984) in Clinical and Metabolic Aspects of High Density Lipoproteins (Miller, N. E., and Miller, G. J., ed), pp. 1-44, Elsevier, New York
41. Fielding, C. J., and Fielding, P. E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3911-3914
42. Cheung, M. C., and Albers, J. J. (1982) J. Lipid Res. 23, 747-753
43. Spector, A. A. (1986) in Biochemistry and Biology of Plasma Lipoproteins (Scanu, A., and Spector, A. A., ed), pp. 247-279, Marcel Dekker, New York
44. Tu, A-Y., Nishida, H.J., and Nishida, T. (1991) FASEB J. 5, A794
45. Nishida, H., Tu, A-Y., and Nishida, T. (1992) FASEB J. 6, A371
46. Tu, A-Y. (1991) Roles of Human Plasma Phospholipid Transfer Protein in High Density Lipoprotein Metabolism, Ph.D. thesis, University of Illinois, Urbana-Champaign