Comparative study of phospholipid transfer activities mediated by cholesteryl ester transfer protein and phospholipid transfer protein

Laurent Lagrost,[1] Anne Athias, Philippe Gambert, and Christian Lallemant

Laboratoire de Biochimie des Lipoprotéines, INSERM CJF 93-10, Faculté de Médecine, Dijon, France

Abstract In the present study, a sequential procedure was set up to separate simultaneously cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PTP), and lecithin:cholesterol acyltransferase (LCAT) from human plasma. Subsequently, phospholipid transfer activities of purified lipid transfer proteins, deprived of LCAT activity, were compared and potential interactions between the two proteins were studied. Phospholipid transfer (PT) activity was determined by using three independent assays that measured the transfer of radiolabeled phosphatidylcholine ([^{14}C]PC) either from phospholipid liposomes to high density lipoproteins-3 ($PT_{liposome \rightarrow HDL_3}$), from high density lipoproteins-3 to phospholipid liposomes ($PT_{HDL_3 \rightarrow liposome}$), or from HDL_3 to low density lipoproteins ($PT_{HDL_3 \rightarrow LDL}$). Comparative study of CETP and PTP pointed out several differences in the ability of the two proteins to transfer phospholipids. *i)* Whereas both CETP and PTP were able to mediate phospholipid transfers from [^{14}C]PC-HDL_3 to LDL, only PTP facilitated phospholipid transfers from [^{14}C]PC-liposomes to HDL_3. *ii)* As PTP did not promote the transfer of phospholipids from [^{14}C]PC-HDL_3 to liposomes, it was concluded that it functions as a phospholipid transfer protein rather than a phospholipid exchange protein. This latter point was confirmed by the ability of purified PTP to induce the net mass transfer of phospholipids from PC-liposomes to HDL_3. *iii)* While PTP presented no intrinsic cholesteryl ester transfer activity, it was able to significantly increase CETP-mediated cholesteryl ester transfers from HDL_3 to LDL. *iv)* CETP did not influence the $PT_{liposome \rightarrow HDL_3}$ activity induced by PTP. *v)* Oleic acid was able to significantly increase the cholesteryl ester transfer activity of CETP, but not the $PT_{liposome \rightarrow HDL_3}$ activity of PTP. *vi)* $PT_{HDL_3 \rightarrow LDL}$ activity of purified CETP was explained, for a large part, by the copurification of nonesterified fatty acids. Taken together, data of the present report suggest that phospholipid transfer activity of CETP and PTP could occur through distinct processes. Since, in human plasma, PTP is not only responsible for the major part of phospholipid net mass transfer but is also able in vitro to modulate the CETP-mediated transfer of cholesteryl esters between various plasma lipoprotein fractions, it could play a determinant role in lipoprotein remodeling in vivo.—**Lagrost, L., A. Athias, P. Gambert, and C. Lallemant.** Comparative study of phospholipid transfer activities mediated by cholesteryl ester transfer protein and phospholipid transfer protein. *J. Lipid Res.* 1994. **35:** 825–835.

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In human plasma, lipids can exchange between different lipoprotein classes (1). Whereas spontaneous and temperature-dependent transfers of phospholipids were described (2), they would be too slow to be of physiological importance (3). In the case of nonpolar lipids (cholesteryl esters and triglycerides), spontaneous transfers have not been reported. It results that equilibration of lipid species between lipoprotein classes, as observed in human plasma (1), depends mainly on the presence of specific factors, and recent data suggested that several proteins would account for plasma lipid transfer activities. Indeed, interspecies comparisons demonstrated that some species, among them rat and mouse, lack the cholesteryl ester and triglyceride transfer activities but not the facilitated phospholipid transfer activity (4, 5). In addition, different locations of cholesteryl ester transfer and phospholipid transfer activities have been reported in human and rabbit plasmas (6). In fact, during the past few years, two distinct lipid transfer proteins (LTP) have been identified. The cholesteryl ester transfer protein (CETP or LTP-I), a 74,000 Da glycoprotein, is responsible for the totality of plasma cholesteryl ester and triglyceride transfer activity and for only a part of the plasma phospholipid transfer activity (7). In vivo, CETP activity results in the net transfer of cholesteryl esters from HDL to lipoproteins of lower densities, VLDL and LDL, with a reciprocal net transfer of triglycerides to HDL (8). The remainder of

Abbreviations: LTP, lipid transfer protein; CETP, cholesteryl ester transfer protein; PTP, phospholipid transfer protein; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; $PT_{liposome \rightarrow HDL_3}$, transfer of phosphatidylcholine from phospholipid liposomes to HDL_3; $PT_{HDL_3 \rightarrow liposome}$, transfer of phosphatidylcholine from HDL_3 to phospholipid liposomes; $PT_{HDL_3 \rightarrow LDL}$, transfer of phosphatidylcholine from HDL_3 to LDL; PC, phosphatidylcholine; UC, unesterified cholesterol; CE, cholesteryl ester; CET, cholesteryl ester transfer; NEFA, nonesterified fatty acid; TBS, Tris-buffered saline.

[1]To whom correspondence should be addressed at: Laboratoire de Biochimie Médicale, Hôpital du Bocage, 21034 Dijon Cedex, France.

plasma phospholipid transfer activity is catalyzed by the phospholipid transfer protein (PTP or LTP-II) which does not catalyze the transfer of neutral lipids (9). PTP is a temperature-sensitive protein with reported apparent molecular weights ranging between 41,000 and 75,000 (9-11). PTP could play an important role in the catabolism of triglyceride-rich lipoproteins by transferring surface phospholipids of chylomicrons to the plasma high density lipoprotein fraction (12, 13). Recent studies also revealed that both CETP (14, 15) and PTP (11) can induce the size redistribution, or conversion, of HDL particles in the absence of other lipoprotein classes.

Although both PTP and CETP are able to mediate phospholipid transfers, the phospholipid transfer activity of the two lipid transfer proteins could be somewhat different and previous studies have suggested that phospholipid transfers would be dependent on the type of experimental system used, i.e., transfer of phospholipids either between artificial phospholipid vesicles and lipoprotein particles or between two different plasma lipoprotein fractions (9). Previous studies have also shown that CETP and PTP activities may be interdependent (10).

The aim of the present study was to characterize further the phospholipid transfer activity of CETP and PTP. To this end, we set up a single, sequential procedure in order to separate simultaneously CETP, PTP, and lecithin:cholesterol acyltransferase (LCAT) from human plasma. Subsequently, activities of purified lipid transfer proteins, used alone or in combination, were compared. In addition, as nonesterified fatty acids have been shown to modulate CETP-mediated cholesteryl ester transfers (16-18), the effect of oleic acid on phospholipid transfer activities of CETP and PTP was investigated.

MATERIALS AND METHODS

Materials

[1α,2α(n)-^{3}H]cholesterol and 1,2-di[1-^{14}C]palmitoyl-*sn*-glycerophosphocholine were purchased from Amersham (Les Ulis, France). Egg L-α-phosphatidylcholine, fatty acid-free bovine serum albumin, oleic acid, and heparin were from Sigma (St. Quentin-Fallavier, France). Phenyl-Sepharose matrix and Mono-Q HR 5/5 column were from Pharmacia (Uppsala, Sweden). Heparin-Ultrogel and Cibacron Blue-Trisacryl were from Sepracor (Villeneuve-la-Garenne, France).

Lipoprotein preparation

Lipoprotein fractions were isolated from total human plasma by sequential ultracentrifugation at a speed of 100,000 rpm (350,000 *g*) in a TLA-100.2 rotor in a Beckman TL-100 ultracentrifuge (Beckman, Palo Alto, CA). Densities were adjusted by the addition of solid KBr. LDL and HDL_3 were isolated as the plasma fractions $1.019 < d < 1.063$ g/ml and $1.13 < d < 1.21$ g/ml, respectively.

Isolation of CETP, PTP, and LCAT

CETP, PTP, and LCAT were purified from citrated human plasma. All purification steps were performed by using a FPLC system (Pharmacia) according to the sequential procedure presented in **Fig. 1.** This procedure combined some of the chromatographic steps that were

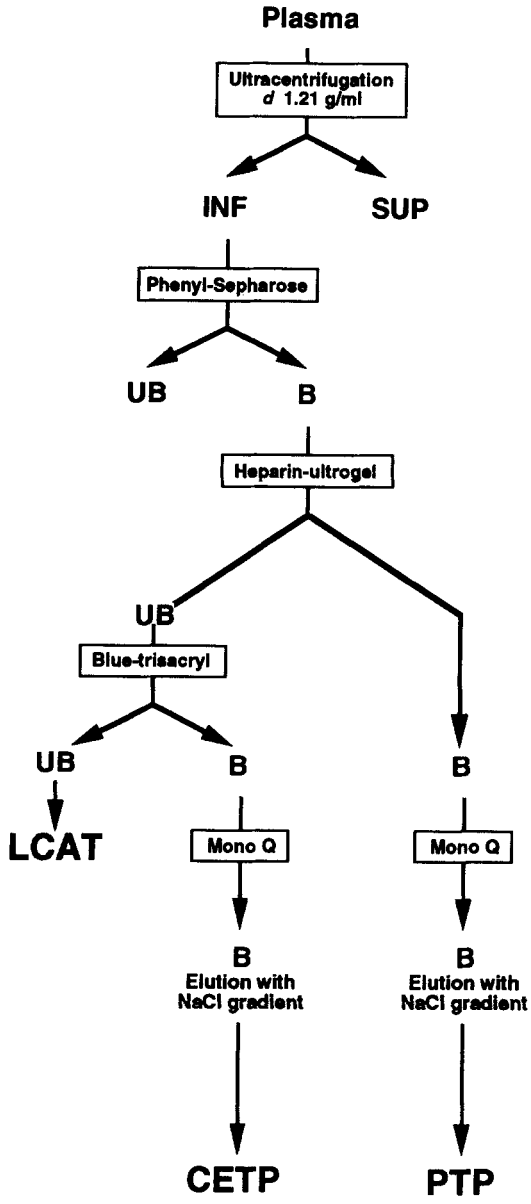

Fig. 1. Sequential procedure for the isolation of cholesteryl ester transfer, phospholipid transfer, and cholesterol esterification activities from human plasma. All chromatographic steps were carried out by using an FPLC system (see Materials and Methods section). INF, $d > 1.21$ g/ml infranatant; SUP, $d < 1.21$ g/ml supernatant; B, bound fraction; UB, unbound fraction; LCAT, lecithin:cholesterol acyltransferase-containing fraction; CETP, cholesteryl ester transfer protein-containing fraction; PTP, phospholipid transfer protein-containing fraction.

previously described to purify CETP (19, 20), PTP (9, 10, 20, 21), and LCAT (22). With the method detailed below, the ultracentrifugally isolated $d > 1.21$ g/ml plasma proteins were fractionated successively by hydrophobic interaction chromatography on a phenyl-Sepharose CL-4B column, affinity chromatographies on Heparin-Ultrogel A4R and Blue-Trisacryl columns, and anion-exchange chromatography on a Mono-Q HR 5/5 column. Chromatographic separations were performed at 4°C, with the exception of anion-exchange chromatography which was conducted at room temperature.

Briefly, 300 ml of plasma was adjusted to density 1.21 g/ml with solid KBr and subjected to ultracentrifugation for 48 h at 55,000 rpm (223,000 *g*) in a 70Ti rotor in an L7 ultracentrifuge (Beckman). The $d > 1.21$ g/ml infranatant (approximately 12 g of proteins) was recovered by tube slicing in a volume of approximately 250 ml and was applied on a 2.6 × 40 cm phenyl-Sepharose CL-4B column. Unbound proteins were washed off with a 10 mmol/l Tris, 150 mmol/l NaCl, 0.2 g/l NaN_3, 1 mmol/l EDTA, pH 7.4 buffer (TBS buffer). Once the absorbance of the effluent decreased to 0.3, the eluent was changed to water and bound proteins (approximately 200 mg), containing CETP, PTP, and LCAT activities (Fig. 1), were recovered in a 150 ml volume. The phenyl-Sepharose-bound protein fraction was supplemented with 15 ml of a 110 mmol/l Tris, pH 7.4 buffer and small amounts of precipitated proteins were removed by low speed centrifugation. Protein solution was then divided into two equal aliquotes which were applied successively to 1.6 × 20 cm Heparin-Ultrogel A4R and Blue Trisacryl columns mounted in series. Columns were washed with TBS buffer until absorbance came back to basal level. Heparin-Ultrogel and Blue-Trisacryl columns, containing PTP and CETP activities, respectively, were then separated. Heparin-bound proteins (60 ml, 76 mg of protein) and Blue-bound proteins (60 ml, 52 mg of protein) were eluted with TBS buffer containing 0.5 and 1 mol/l NaCl, respectively. Protein fractions were dialyzed overnight against TBS buffer and were further purified on a MonoQ HR 5/5 column. About 20 mg of either heparin-bound or Blue-bound proteins were applied to the MonoQ column per chromatographic cycle and lipid transfer activities were eluted according to a discontinuous gradient ranging from 0 to 500 mmol/l of NaCl. Active fractions, which eluted approximately between 100 and 200 mmol/l of NaCl, were pooled, aliquoted, and stored at −80°C.

CETP assay

CETP activity of a sample was measured as its capacity to promote the transfer of radiolabeled cholesteryl esters from HDL_3 to LDL, as previously described (18). HDL_3 containing radiolabeled cholesteryl esters ([^{3}H]CE-HDL_3) were biosynthetically labeled by a 24-h, 37°C incubation of 10 nmol of [1α,2α(n)-^{3}H]cholesterol with the $d > 1.13$ g/ml fraction obtained from 20 ml of human plasma (18). As judged by thin-layer chromatography, more than 95% of radioactivity was recovered in the cholesteryl ester moiety. An aliquot of each sample, with a volume ranging from 10 to 40 μl, was mixed with 2.5 nmol of [^{3}H]CE-HDL_3 and 10 nmol of LDL in a final volume of 50 μl. In 'TBS blank' controls, samples were replaced by TBS buffer. Mixtures were then incubated at 37°C. At the end of the incubation, the $d < 1.068$ and $d > 1.068$ g/ml fractions, containing LDL and HDL_3, respectively, were recovered in 1-ml volumes after ultracentrifugation of incubated mixtures for 7 h at 50,000 rpm (269,000 *g*) in a 50.4 Ti rotor on a L7 ultracentrifuge (Beckman). The ultracentrifugation was performed at 5°C and the relative proportions of radiolabeled cholesteryl esters recovered in the $d < 1.068$ and $d > 1.068$ g/ml fractions did not vary when samples were kept for several days at this temperature. Ultracentrifugally isolated lipoprotein fractions were then transferred into 6-ml counting vials. Two ml of scintillation fluid (OptiScint Hisafe 3, Pharmacia) was added to each vial, and the radioactivity was assayed for 5 min in a Wallac 1410 liquid scintillation counter (Pharmacia). CETP activity was calculated as the rate of total radioactivity transferred from the $d > 1.068$ to the $d < 1.068$ g/ml fraction after deduction of 'TBS blank' controls in which the relative proportion of radioactivity transferred from radiolabeled HDL_3 to LDL was constantly lower than 4%.

Phospholipid transfer assays

Phospholipid transfer (PT) activity was determined by using three independent assays that measured the transfer of radiolabeled phosphatidylcholine either from phospholipid liposomes to HDL_3 ($PT_{liposome \rightarrow HDL_3}$) (21), from HDL_3 to LDL ($PT_{HDL_3 \rightarrow LDL}$) (10), or from HDL_3 to phospholipid liposomes ($PT_{HDL_3 \rightarrow liposome}$).

$PT_{liposome \rightarrow HDL_3}$ activity was measured with radiolabeled phosphatidylcholine-containing liposomes ([^{14}C]PC-liposomes) which were prepared according to the general procedure described by Damen, Regts, and Scherphof (21). Briefly, 10 μmol of egg phosphatidylcholine containing 10 nmol of [^{14}C]phosphatidylcholine (sp act, 100 mCi/mmol) was dried under a stream of nitrogen and recovered in 1 ml of a 10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, buffer containing 0.2 g/l NaN_3 and 1 mmol/l EDTA. [^{14}C]PC-liposomes were finally obtained by dispersing phospholipids with a sonifier. Transfer of radiolabeled phospholipids was measured by incubating at 37°C an aliquot of 100 μl of each sample with [^{14}C]PC-liposomes (125 nmol of phosphatidylcholine) and HDL_3 (250 μg of protein) in a final volume of 400 μl. In '4°C blank' controls, mixtures were maintained at 4°C. Phospholipid liposomes were subsequently precipitated by addition of 300 μl of a 500 mmol/l NaCl, 215 mmol/l $MnCl_2$,

445 U/ml heparin. 'Total Count' controls received 300 μl of TBS buffer instead of the $MnCl_2$/heparin precipitant reagent. Successively, tubes were vortexed, left for 10 min at room temperature and, finally, the precipitate was removed by a 10 min centrifugation at 10,000 rpm in an Eppendorf ultracentrifuge. $PT_{liposome \rightarrow HDL_3}$ activity was calculated as the rate of total radiolabeled phospholipids ('Total Count' control) which were transferred from liposomes to HDL_3 after deduction of '4°C blank' control values. Less than 5% of total radioactivity was recovered in the supernatant after precipitation of [^{14}C]PC-liposomes in '4°C blank' control samples.

$PT_{HDL_3 \rightarrow LDL}$ was measured according to the general procedure described by Tollefson, Ravnik, and Albers (10). Radiolabeled [^{14}C]phosphatidylcholine-containing HDL_3 ([^{14}C]PC-HDL_3) were prepared directly by a 6-h, 37°C incubation of 25 nmol of [^{14}C]phosphatidylcholine, dissolved in 50 μl of ethanol, with the HDL_3 fraction isolated from 40 ml of normolipidemic human plasma. Aliquots of 100 μl of samples were incubated at 37°C with [^{14}C]PC-HDL_3 (10 μmol of phospholipids) and unlabeled LDL (50 μmol of phospholipids) in a final volume of 600 μl. In 'TBS blank' controls, samples were replaced by TBS buffer. At the end of the incubation, the $d < 1.068$ and $d > 1.068$ g/ml fractions, containing LDL and HDL_3, respectively, were recovered in 1 ml volumes after ultracentrifugation of incubated mixtures for 7 h at 50,000 rpm (269,000 *g*) in a 50.4 Ti rotor on a L7 ultracentrifuge (Beckman). Ultracentrifugally isolated lipoprotein fractions were then transferred into 6-ml counting vials. Two ml of scintillation fluid (OptiScint Hisafe 3, Pharmacia) was added to each vial, and the radioactivity was assayed for 5 min in a Wallac 1410 liquid scintillation counter (Pharmacia). $PT_{HDL_3 \rightarrow LDL}$ activity was calculated as the rate of total radioactivity transferred from the $d > 1.068$ to the $d < 1.068$ g/ml fraction after deduction of 'TBS blank' control values which never exceeded 5% of total counts.

$PT_{HDL_3 \rightarrow liposome}$ activity was measured by incubating [^{14}C]PC-HDL_3 (250 μg of protein) and phosphatidylcholine liposomes (125 nmol of phosphatidylcholine) for 20 min at 37°C in a final volume of 400 μl. [^{14}C]PC-HDL_3 and PC-liposomes were prepared as described above for the determination of $PT_{liposome \rightarrow HDL_3}$ and $PT_{HDL_3 \rightarrow LDL}$ activities, except that PC-liposomes did not contain [^{14}C]phosphatidylcholine. After incubation, phospholipid liposomes were precipitated with $MnCl_2$/heparin as described above. $PT_{HDL_3 \rightarrow liposome}$ activity was calculated as the rate of total radiolabeled phospholipids that were transferred from HDL_3 to liposomes as compared with '4°C blank' control values. In '4°C blank' controls, more than 95% of total radioactivity was recovered in the supernatant after precipitation of PC-liposomes.

LCAT assay

LCAT activity was measured according to the general procedure of Piran and Morin (23) with minor modifications. This method has been shown to correlate well with thin-layer chromatography and to constitute a rapid and accurate single-step procedure to measure cholesterol esterification activity (23). In the present study, fresh ultracentrifugally isolated HDL_3 containing radiolabeled unesterified cholesterol ([^{3}H]UC-HDL_3) were used as the substrate for LCAT. This labeled substrate was prepared by incubating 300 μl of HDL_3 (1.4 μmol of total cholesterol) for 30 min at 37°C with about 20 pmol of [1α,2α(n)-^{3}H]cholesterol (sp act, 46 Ci/mmol). A 10-μl aliquot of [^{3}H]UC-HDL_3 (about 45 nmol of total cholesterol) was subsequently mixed with 50 μl of protein samples in TBS buffer containing 4 mg/ml fatty acid-free albumin, 50 mmol/l β-mercaptoethanol in a final volume of 500 μl. In: 'TBS blank' controls, the 50-μl aliquot of studied samples was replaced by 50 μl of TBS buffer. After incubation of the mixtures under nitrogen for 1 h at 37°C, the cholesterol esterification reaction was stopped by addition of 2 ml of 1% digitonin in 95% ethanol. 'Total Count' controls received 2 ml of 95% ethanol which did not contain digitonin. The tubes were mixed for 15 sec on a vortex stirrer. Volumes of 100 μl of a 5 g/l unesterified cholesterol solution were added to all tubes which were vortexed again. Finally, tubes were centrifuged for 10 min at 1500 *g* and 500 μl of resulting supernatants containing

TABLE 1. Purification of CET, $PT_{HDL3 \rightarrow LDL}$, $PT_{liposome \rightarrow HDL3}$, and LCAT activities from human plasmas

Fraction	Specific Activity[a]			
	CET	$PT_{HDL3 \rightarrow LDL}$	$PT_{liposome \rightarrow HDL3}$	LCAT
	%/h/μg protein			
d > 1.21 g/ml Fraction	0.6 ± 0.1[b]	0.4 ± 0.1	2.1 ± 0.4	3.7 ± 0.4
Bound phenyl-Sepharose	330.8 ± 19.5	50.2 ± 1.8	245.4 ± 22.7	138.4 ± 2.1
Bound heparin	17.7 ± 1.1	597.5 ± 36.6	854.9 ± 99.8	5.4. ± 2.2
Unbound heparin/bound Blue Trisacryl	1849.6 ± 253.9	513.4 ± 59.1	27.4 ± 15.5	5.0 ± 5.4
Unbound heparin/unbound Blue	56.7 ± 11.9	12.7 ± 5.0	22.1 ± 13.2	1457.1 ± 364.2

[a] Specific activities of lipid transfer and cholesterol esterification were determined in the pool of fractions showing activity. Activities are expressed in percent per hour per μg of protein.
[b] Values are means ± SD of three different isolations.

esterified cholesterol was mixed with 2 ml of scintillation fluid (OptiScint Hisafe 3, Pharmacia) in 6-ml counting vials. Radioactivity was assayed for 5 min in a Wallac 1410 liquid scintillation counter (Pharmacia). LCAT activity was calculated as the rate of total radiolabeled cholesterol ('Total Count' control) that was esterified after deduction of 'TBS blank' control values.

Protein and lipid analyses

All chemical assays were performed on a Cobas-Bio Centrifugal Analyser (Roche Diagnostics, Zürick, Switzerland). Total cholesterol and phospholipid concentrations were determined by enzymatic methods using Boehringer (Mannheim, FRG) reagents. Protein concentrations were measured with the bicinchoninic reagent (Pierce, Rockford, IL) according to Smith et al. (24). Nonesterified fatty acid concentrations were determined by using an enzymatic kit (Wako Pure Chemicals Industries, Osaka, Japan).

RESULTS

Isolation and characterization of CETP, PTP, and LCAT

The experimental procedure set up to simultaneously purify CETP, PTP, and LCAT was as described in Fig. 1. At each purification step, cholesteryl ester transfer (CET), phospholipid transfer (PT), and cholesterol esterification (LCAT) activities were measured in isolated fractions as described in the Materials and Methods section. In the case of phospholipid transfers, activity was measured by using two different phospholipid transfer assays, in which transfers of radiolabeled phosphatidylcholine (PC) were measured either from [^{14}C]PC-liposomes to HDL_3 ($PT_{liposome \rightarrow HDL_3}$ activity) or from [^{14}C]PC-HDL_3 to LDL ($PT_{HDL_3 \rightarrow LDL}$ activity).

As shown in **Table 1,** phenyl-Sepharose chromatography allowed us to obtain a first substantial purification of cholesterol esterification and lipid transfer activities. As compared with the $d > 1.21$ g/ml plasma fraction, binding to the hydrophobic matrix of phenyl-Sepharose yielded 530-, 114-, 120-, and 40-fold purifications of CET, $PT_{liposome \rightarrow HDL_3}$, $PT_{HDL_3 \rightarrow LDL}$, and LCAT activities, respectively.

Application of the phenyl-Sepharose-bound fraction to coupled Heparin-Ultrogel and Blue-Trisacryl columns constituted a determinant step in the separation of activities. Indeed, whereas the heparin-bound fraction contained high levels of both phospholipid transfer activities, $PT_{liposome \rightarrow HDL_3}$ AND $PT_{HDL_3 \rightarrow LDL}$ activities, it contained only residual levels of CET and LCAT activities indicating that the two latter activities were exclusively catalyzed by plasma proteins that did not interact with heparin (Table 1). While virtually all of the $PT_{liposome \rightarrow HDL_3}$ activity bound to the heparin matrix, $PT_{HDL_3 \rightarrow LDL}$ activity was recovered in both heparin-bound and Blue-bound fractions (Table 1). These data indicated that $PT_{liposome \rightarrow HDL_3}$ activity would be catalyzed by a specific protein, different from CETP and LCAT. By contrast, as observed with affinity columns, $PT_{HDL_3 \rightarrow LDL}$ activity would be catalyzed by at least two proteins, one binding heparin and another one binding Blue-Trisacryl. As compared with the $d > 1.21$ g/ml plasma fraction, heparin-binding yielded approximately 400- and 1420-fold purifications of $PT_{liposome \rightarrow HDL_3}$ and $PT_{HDL_3 \rightarrow LDL}$ activities, respectively (Table 1). On the other hand, Blue-binding yielded approximately 2980- and 1220-fold purification of CET and $PT_{HDL_3 \rightarrow LDL}$ activities, respectively. The fraction that did not bind to the coupled heparin/Blue column system contained LCAT activity but no lipid transfer activities (Table 1). This protein fraction corresponded to a 400-fold purification of LCAT as compared with the $d > 1.21$ g/ml plasma fraction.

Heparin-bound and Blue-bound protein fractions were further purified by anion exchange chromatography on a monoQ column as described in Materials and Methods. With the low salt concentrations of the eluent used at the start, all of the injected proteins bound to the monoQ matrix. As shown in **Fig. 2,** proteins were progressively eluted according to a discontinuous salt gradient ranging from 0 to 500 mmol/l of NaCl. Similar elution profiles of lipid transfer activities were obtained with heparin-bound and Blue-bound protein fractions and the bulk of activity eluted approximately in the 100–150 mmol/l NaCl range in both cases (Fig. 2). After monoQ chromatography of heparin-bound proteins, pooled fractions 15–30 represented a 2450-fold increase in specific $PT_{HDL_3 \rightarrow LDL}$ activity and a 1060-fold increase in specific $PT_{liposome \rightarrow HDL_3}$ activity as compared with the $d > 1.21$ g/ml plasma fraction. Pooled fractions 15–30 recovered after monoQ chromatography of Blue-bound proteins represented a 5140-fold increase in specific CET activity and a 1750-fold increase in specific $PT_{HDL_3 \rightarrow LDL}$ activity as compared with the $d > 1.21$ g/ml plasma fraction.

In summary, the purification procedure allowed two lipid transfer protein preparations to be obtained, both of which were deprived of LCAT activity. The first one, containing PT activity but no CET activity, had the main characteristics of PTP (1). The second one, containing both PT and CET activities, had the main characteristics of CETP (1).

Phospholipid transfer activities of PTP and CETP were further compared by determining $PT_{liposome \rightarrow HDL_3}$, $PT_{HDL_3 \rightarrow liposome}$, and $PT_{HDL_3 \rightarrow LDL}$ activities in the presence of increasing amounts of each protein fraction. As shown in **Fig. 3,** both PTP and CETP induced the transfer of radiolabeled phospholipids from HDL_3 to LDL in a concentration-dependent manner. By contrast, only PTP mediated a significant concentration-dependent

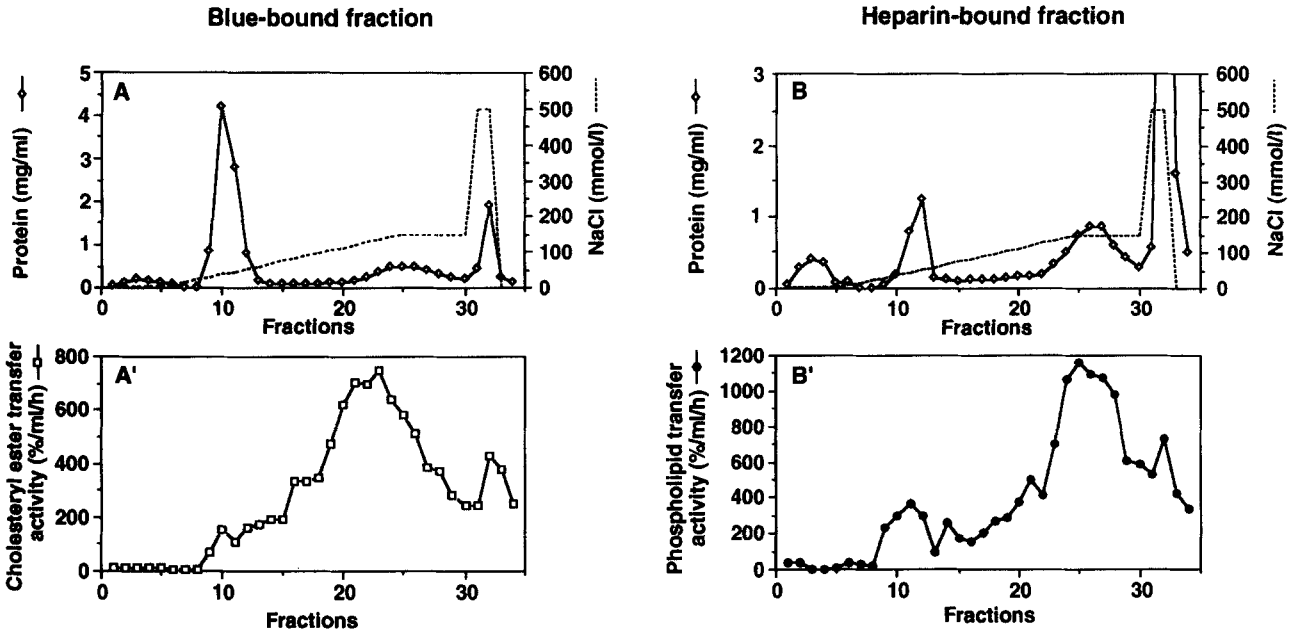

Fig. 2. Chromatography of Blue-bound (A and A') and heparin-bound (B and B') protein fraction on 5/5 HR MonoQ column. Proteins bound to MonoQ were eluted according to a discontinuous NaCl gradient ranging from 0 to 500 mmol/l (broken lines). Cholesteryl ester transfer activity (open squares) was monitored by incubating 10 μl of each fraction with 2.5 nmol of [^{3}H]CE-HDL$_3$ and 10 nmol of LDL for 90 min at 37°C in a final volume of 50 μl. Phospholipid transfer activity (closed circles) was monitored by incubating 100 μl of each fraction with 13 nmol of [^{14}C]PC-HDL$_3$ and 65 nmol of LDL for 3 h at 37°C in a final volume of 600 μl.

transfer of radiolabeled phospholipids from liposomes to HDL$_3$ (Fig. 3, upper panel). Less than 3% of the total radiolabeled phospholipid pool was transferred from liposomes to HDL$_3$ in the presence of CETP, even with the highest protein concentration studied (Fig. 3, middle panel). Neither CETP nor PTP promoted the transfer of PC from HDL$_3$ to liposomes (Fig. 3, lower panel). As observed by incubating HDL$_3$ (protein concentration, 0.63 g/l), PC-liposomes (phospholipid concentration, 313 μmol/l), and purified PTP (93 μg/ml) for 2 h at 37°C, PTP induced a 20% increase in the phospholipid mass content of HDL$_3$ particles (results not shown).

Interactions between PTP- and CETP-mediated lipid transfer activities

Potential interactions between CETP and PTP were investigated by determining the combined effects of CETP and PTP on CET, $PT_{HDL_3 \rightarrow LDL}$, and $PT_{liposome \rightarrow HDL_3}$ activities. For that purpose, substrates containing labeled and unlabeled lipid markers were incubated at 37°C for up to 3 h in the presence of either CETP, PTP, or both CETP and PTP.

As shown in **Fig. 4A,** whereas PTP increased cholesteryl ester transfer activity of CETP (Fig. 4A), the $PT_{liposome \rightarrow HDL_3}$ activity of PTP was not dependent on CETP as similar phospholipid transfer values were obtained whether CETP was present or not (Fig. 4B). Consistent data were obtained from experiments that involved a large range of CETP concentrations, varying from 0 up to 50 μg/ml of protein (results not shown). As mentioned above, CETP and PTP were able to facilitate the transfer of phospholipids between HDL$_3$ and LDL lipoprotein fractions and, as shown in Fig. 4C, both proteins induced a time-dependent redistribution of [^{14}C]PC from HDL$_3$ to LDL. $PT_{HDL_3 \rightarrow LDL}$ activity was significantly higher in incubation mixtures containing both CETP and PTP than in mixtures containing only CETP or only PTP. After deduction of TBS control values, the transfer rates measured by incubating [^{14}C]PC-HDL$_3$ and LDL in the presence of both CETP and PTP agreed well with the sum of transfer rates that were measured parallely by incubating [^{14}C]PC-HDL$_3$ and LDL in the sole presence of either CETP or PTP (Fig. 4C).

Comparative effects of oleic acid on lipid transfer activities of PTP and CETP

The ability of nonesterified fatty acids to modulate CET, $PT_{liposome \rightarrow HDL_3}$, AND $PT_{HDL_3 \rightarrow LDL}$ activities was investigated by measuring lipid transfer activities in the presence of oleic acid at final concentrations ranging from 0 to about 80 μmol/l.

Whereas oleic acid was able to significantly increase the CETP-mediated transfer of radiolabeled cholesteryl esters from HDL$_3$ to LDL (**Fig. 5A**), confirming data from previous studies (16–18), it altered neither the spontaneous nor the PTP-mediated transfer of [^{14}C]phosphatidyl-

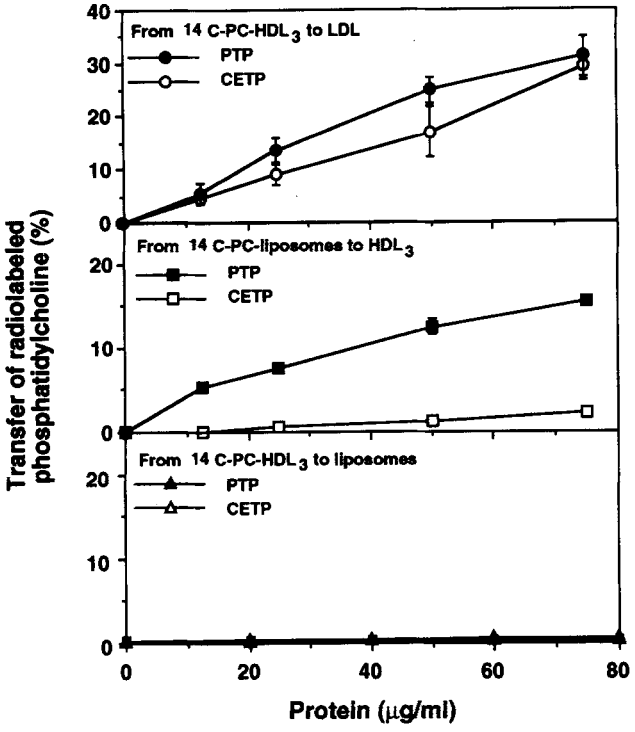

Fig. 3. Concentration-dependent effect of PTP and CETP on phospholipid transfer activity as measured either from HDL_3 to LDL (upper panel), from liposomes to HDL_3 (middle panel), or from HDL_3 to liposomes (lower panel). $PT_{HDL3 \rightarrow LDL}$ activity was measured by incubating for 3 h at 37°C various concentrations of either PTP (closed circles) or CETP (open circles) fractions in the presence of [^{14}C]PC-HDL_3 (phospholipid concentration, 21.5 μmol/l) and LDL (phospholipid concentration, 107.5 μmol/l) in a final volume of 600 μl. $PT_{liposome \rightarrow HDL3}$ activity was measured by incubating for 20 min at 37°C various concentrations of either PTP (closed squares) or CETP (open squares) fractions in the presence of [^{14}C]PC-liposomes (phospholipid concentration, 312.5 μmol/l) and HDL_3 (phospholipid concentration, 61.0 μmol/l) in a final volume of 400 μl. $PT_{HDL3 \rightarrow liposome}$ activity was measured by incubating for 20 min at 37°C various concentrations of either PTP (closed triangles) or CETP (open triangles) fractions in the presence of [^{14}C]PC-HDL_3 (phospholipid concentration, 61.0 μmol/l) and PC-liposomes (phospholipid concentration, 313.0 μmol/l) in a final volume of 400 μl. Values represent mean ± SD of three determinations.

choline from liposomes to HDL_3 (Fig. 5B). By contrast, oleic acid alone progressively enhanced the transfer of [^{14}C]phosphatidylcholine from HDL_3 to LDL and a parallel increase of $PT_{HDL_3 \rightarrow LDL}$ activity was observed in the presence of CETP and PTP (Fig. 5C). These observations suggest that oleic acid could induce phospholipid transfers directly, without affecting the CETP- and PTP-mediated $PT_{HDL_3 \rightarrow LDL}$ activity.

Nonesterified fatty acid (NEFA) assay revealed that small, but significant, amounts of nonesterified fatty acids were found associated with CETP and PTP preparations. Indeed, CETP and PTP preparations contained 33.0 and 2.2 nmol of NEFA per mg of protein, respectively. In addition, substantial amounts of nonesterified fatty acids, 18.0 and 76.0 nmol per mg of protein, were associated with HDL_3 and LDL fractions, respectively. In order to determine whether nonesterified fatty acids could account, at least in part, for the $PT_{HDL_3 \rightarrow LDL}$ activity of PTP and CETP, rates of [^{14}C]phosphatidylcholine transferred from HDL_3 to LDL were determined in the presence of increasing concentrations of fatty acid-free albumin. As shown in **Fig. 6,** CETP-mediated phospholipid transfer rates decreased from 16.7 to 9.8% as albumin concentration increased from 0 to 20 g/l. Under the same conditions, CETP-mediated cholesteryl ester

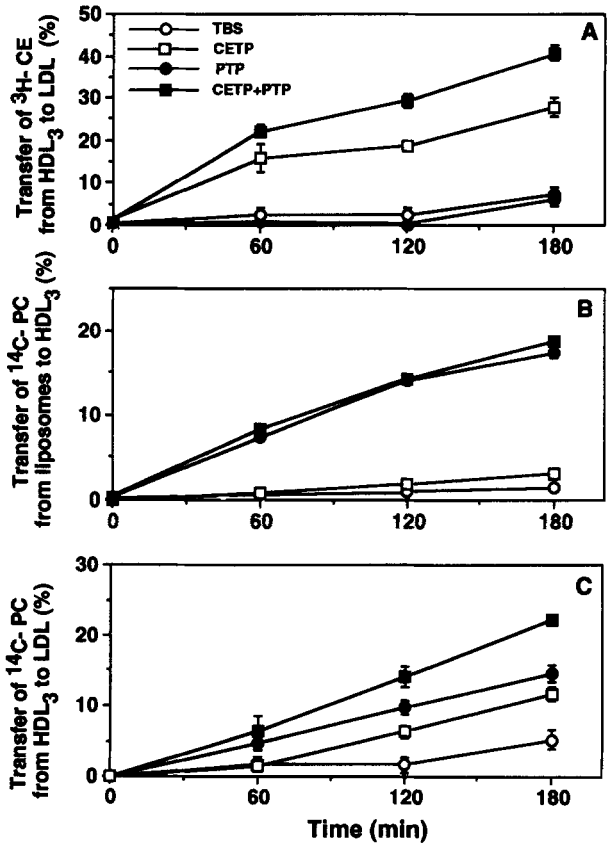

Fig. 4. Time-dependent effects of either CETP, PTP, or a combination of CETP and PTP on cholesteryl ester and phospholipid transfer activities. Lipid transfer activities were determined by incubating labeled and unlabeled substrates in the absence (TBS control, open circles) or in the presence of either CETP (open squares), PTP (closed circles) or both CETP and PTP (closed squares). In CET activity measurements (panel A), [^{3}H]CE-HDL_3 (cholesterol concentration, 50 μmol/l), LDL (cholesterol concentration, 200 μmol/l), and lipid transfer protein fractions were incubated 37°C in a final volume of 50 μl. In $PT_{liposome \rightarrow HDL3}$ activity measurements (panel B), [^{14}C]PC-liposomes (phospholipid concentration, 312.5 μmol/l), HDL_3 (phospholipid concentration, 61.0 μmol/l), and lipid transfer protein fractions were incubated at 37°C in a final volume of 400 μl. In $PT_{HDL3 \rightarrow LDL}$ activity measurements (panel C), [^{14}C]PC-HDL_3 (phospholipid concentration, 21.5 μmol/l), LDL (phospholipid concentration, 107.5 μmol/l), and lipid transfer protein fractions were incubated at 37°C in a final volume of 600 μl. When present, the final protein concentration of each of the two lipid transfer protein fractions was either 30 mg/l (panel A) or 20 mg/l (panels B and C). Values represent mean ± SD of three determinations.

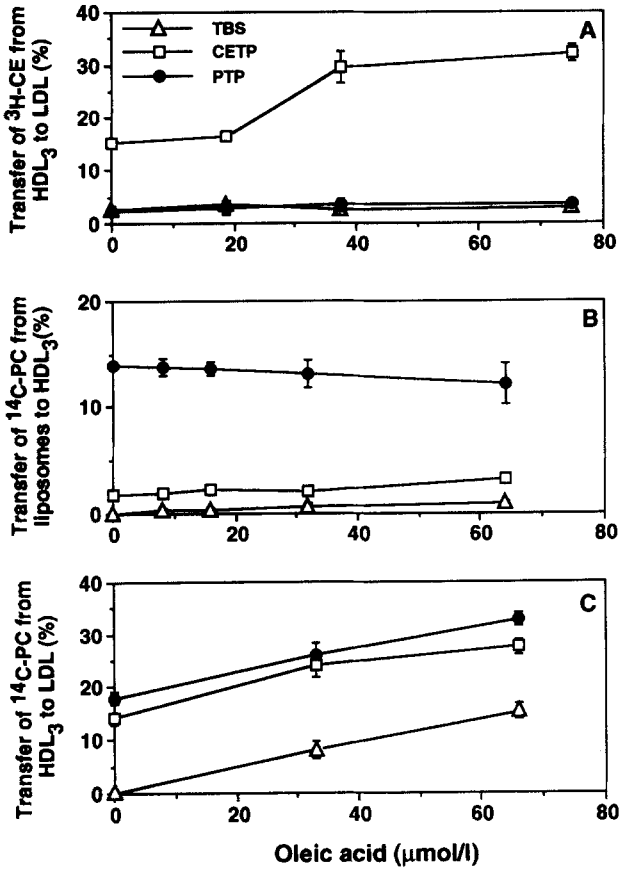

Fig. 5. Concentration-dependent effect of oleic acid on lipid transfer activities of CETP and PTP. CET (panel A), $PT_{liposome \rightarrow HDL3}$ (panel B), and $PT_{HDL3 \rightarrow LDL}$ (panel C) activities were determined as described in Fig. 4 except that the final protein concentration of each of the two lipid transfer protein fractions was either 30 mg/l (panels A and B) or 15 mg/l (panel C). Mixtures were incubated in the presence of various concentrations of oleic acid at 37°C for either 90 min (panel A), 20 min (panel B), or 180 min (panel C). Values represent mean ± SD of three determinations.

transfer rates decreased from 38.2 to 21.0% (results not shown). By contrast, fatty acid-free albumin did not significantly affect the PTP-mediated transfer of radiolabeled phospholipids (Fig. 6).

DISCUSSION

The aim of the present study was to compare the ability of plasma lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PTP), to transfer phospholipids between various lipoprotein substrates. To this end, we first set up a sequential chromatographic procedure to obtain simultaneously purified CETP and PTP fractions, both of which were deprived of lecithin:cholesterol acyltransferase (LCAT), a factor that may alter lipid transfer processes (25). The comparative study of CETP and PTP clearly revealed that the two proteins catalyzed different phospholipid transfer activities and suggested further that they would function with distinct mechanisms.

Whereas cholesteryl ester transfer activity has been attributed to CETP but not to PTP, both lipid transfer proteins have been shown to facilitate phospholipid transfers (1). However, the ability of CETP and PTP to transfer phospholipids was dependent on the type of assay used and some discrepancies appeared among results from different laboratories. In an early study, Damen, Regts, and Scherphof (21) showed that a heat-labile, heparin-binding phospholipid transfer protein, partially purified from human plasma, can promote the transfer of radiolabeled phosphatidylcholine from unilamellar liposomes to human HDL. While Tall, Abreu, and Shuman (9) confirmed that PTP promotes the net transfer of phospholipids from phosphatidylcholine vesicles into plasma HDL, they did not observe any PTP-mediated exchange of phosphatidylcholine between two different plasma lipoprotein fractions, i.e., HDL and LDL. Conversely, they observed that CETP could facilitate the transfer of phosphatidylcholine between HDL and LDL but not from phospholipid vesicles into HDL (9), suggesting that phospholipid transfer activities of PTP and CETP are in fact different. While similar observations were reported by Sweeny and Jonas (26), subsequent studies revealed in fact that, like CETP, PTP was able to promote the transfer of phospholipids between native lipoprotein fractions

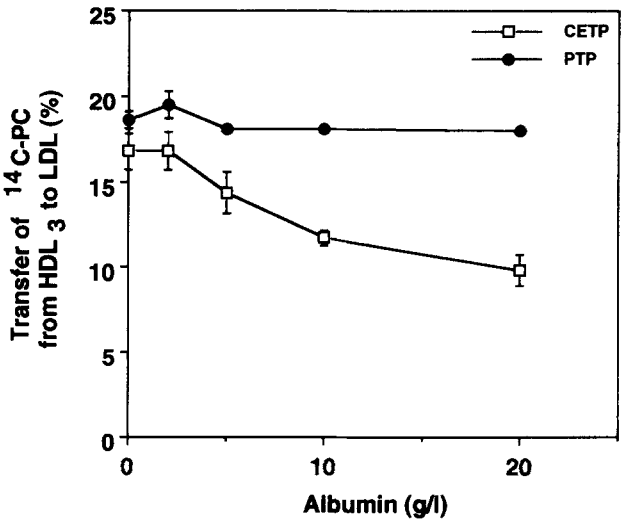

Fig. 6. Concentration-dependent effect of fatty acid-free albumin on $PT_{HDL3 \rightarrow LDL}$ activity of CETP and PTP. $PT_{HDL3 \rightarrow LDL}$ activity of either CETP (open squares) or PTP (closed circles) was measured by incubating for 3 h at 37°C [^{14}C]PC-HDL_3 (phospholipid concentration, 21.5 μmol/l), LDL (phospholipid concentration, 107.5 μmol/l), and lipid transfer proteins (protein concentration, 20 mg/l) in a final volume of 600 μl. In addition, incubation mixtures were supplemented with increasing concentrations of fatty acid-free human serum albumin. Values represent mean ± SD of three determinations.

(10, 27). Recently, Speijer and coworkers (6) demonstrated by using anti-human CETP antibodies that phospholipid transfer activity in human plasma, as measured with a radiolabeled phospholipid vesicle/HDL assay system, results mainly from the activity of PTP.

In the present study, we observed that only PTP facilitated phospholipid transfers from [^{14}C]PC-liposomes to HDL_3 ($PT_{liposome \rightarrow HDL_3}$), while both CETP and PTP were able to mediate phospholipid transfers from [^{14}C]PC-HDL_3 to LDL ($PT_{HDL_3 \rightarrow LDL}$). By contrast, neither PTP nor CETP facilitated phospholipid transfers from HDL_3 to [^{14}C]PC-liposomes ($PT_{HDL_3 \rightarrow liposome}$), indicating that PTP is a phospholipid transfer rather than a phospholipid exchange protein. This hypothesis is consistent with the ability of PTP to promote the net mass transfer of phosphatidylcholine from PC-liposomes to HDL_3. While PTP presented no intrinsic cholesteryl ester transfer (CET) activity, it was able to significantly increase CETP-mediated cholesteryl ester transfers from HDL_3 to LDL. By contrast, CETP did not influence the $PT_{liposome \rightarrow HDL_3}$ activity of PTP. An additional finding of the present report was that nonesterified fatty acids (NEFA) have a differential effect on CETP and PTP. Indeed, we observed that oleic acid was able to significantly increase CET activity of CETP, but not $PT_{liposome \rightarrow HDL_3}$ activity of PTP. The NEFA-binding capacity of fatty acid-free albumin revealed further that $PT_{HDL_3 \rightarrow LDL}$ activity of purified CETP was, for a large part, NEFA-dependent.

One of the hypotheses to explain differences in phospholipid transfer activity of CETP and PTP might relate to variations in their ability to interact with either phospholipid liposomes or lipoprotein particles. Indeed, binding of CETP to lipoprotein particles, a key step of the lipid transfer process, has been shown to relate directly to the density of negative charges on lipoprotein surface (28, 29). As apolipoproteins constitute one of the factors that can confer negative charges in lipoprotein structure (30), interaction of CETP with phospholipid liposomes could be relatively low as compared with plasma lipoprotein particles. This hypothesis is sustained by recent data of Nishikawa et al. (31) who demonstrated that introduction of either apolipoprotein A-I, A-II, C-II, C-III, or E in triolein particles, coated with an egg phosphatidylcholine monolayer, considerably increased the CETP-mediated transfer from plasma LDL fraction. By contrast, PTP has been shown to bind strongly to phosphatidylcholine vesicles that were devoid of apolipoproteins (9). We postulate that a higher affinity of PTP for lipid emulsions, as compared with CETP, might explain why PTP, but not CETP, can catalyze $PT_{liposome \rightarrow HDL_3}$ activity. The inability of CETP to interact with phospholipid liposomes was sustained by some of the present experiments in which the combined effects of CETP and PTP on $PT_{liposome \rightarrow HDL_3}$ activity were determined. In particular, the absence of CETP-induced alteration in the $PT_{liposome \rightarrow HDL_3}$ activity of PTP, as described in the present report, has probably something to do with the previously described incapacity of CETP to significantly modify the cholesteryl ester distribution in a lipid emulsion:lipoprotein assay system (31). By contrast, we observed that PTP stimulates the CETP-mediated transfer of radiolabeled cholesteryl esters between HDL_3 and LDL. This observation extended data from previous studies that demonstrated that PTP can significantly increase the CETP-mediated transfer cholesteryl esters between HDL_3 and VLDL (10). Because phospholipids may constitute one of the primary sites for interaction of CETP with lipoprotein particles (29), we postulate that the ability of PTP to modulate CETP activity could relate to variations in phospholipid content that PTP induces in lipoprotein surface. Finally, we noted neither cooperativity nor competition in the ability of CETP and PTP to catalyse $PT_{HDL_3 \rightarrow LDL}$ activity, suggesting that the two lipid transfer proteins transferred phospholipids through independent processes.

In the present study, we also searched for potential alterations of CET, $PT_{HDL_3 \rightarrow LDL}$, and $PT_{liposome \rightarrow HDL_3}$ activities by nonesterified fatty acids, one of the major lipolytic products that are formed during triglyceride-rich lipoprotein hydrolysis. The present experiments were stimulated by previous data that demonstrated that lipolysis can enhance the transfer of phospholipids from triglyceride-rich lipoproteins into HDL (32) while it can favor the movement of cholesteryl esters in the reverse direction, from HDL into triglyceride-rich lipoproteins (33). In accordance with previous data (16, 17), we observed that oleic acid was able to significantly increase CET activity. By contrast, oleic acid did not modify the PTP-mediated transfer of radiolabeled phospholipids from liposomes towards HDL_3. These results suggest, therefore, that the previously observed stimulation of PTP-mediated phospholipid exchange, or transfer, during lipolysis (32) was not directly linked to the concomitant generation of NEFA. Interestingly, we observed that oleic acid alone, in the absence of lipid transfer proteins, induced a concentration-dependent redistribution of radiolabeled phospholipids from HDL_3 towards LDL. This latter observation could relate to the ability of NEFA to mediate directly the dissociation of surface lipids from lipoprotein substrates, as recently described (34). The magnitude of NEFA-induced variations in $PT_{HDL_3 \rightarrow LDL}$ activity observed in the sole presence of oleic acid was similar to that measured in incubation mixtures containing, in addition, either PTP or CETP. These data suggested, therefore, that oleic acid itself can induce phospholipid transfers. In other words, $PT_{HDL_3 \rightarrow LDL}$ activity of purified CETP and PTP preparations might relate to small amounts of NEFA that were brought into the incubation mixtures as components of lipid transfer protein preparations and lipoprotein fractions. In order to test this hypothesis, $PT_{HDL_3 \rightarrow LDL}$ activity of purified CETP

and PTP preparations was determined in the presence of fatty acid-free albumin. Whereas $PT_{HDL_3 \rightarrow LDL}$ activity of purified PTP remained virtually unchanged, fatty acid-free albumin was able to considerably reduce the $PT_{HDL_3 \rightarrow LDL}$ activity of purified CETP. Consequently, the CETP-mediated phospholipid transfer reaction, unlike that catalyzed by PTP, appeared to be at least partially NEFA-dependent. These observations can be put together with previous data that demonstrated in vitro that the cholesteryl ester transfer reaction (16, 17) as well as the HDL conversion process (15, 35) induced by CETP were largely influenced by the presence of nonesterified fatty acids in incubation mixtures.

In conclusion, results of the present study established that phospholipid transfer activities of PTP and CETP are different. In particular, the modulation by NEFA of the CETP-mediated, but not the PTP-mediated, phospholipid transfers and the absence of interdependence of phospholipid transfer activities catalyzed by the two lipid transfer proteins indicated that phospholipid transfer activity of CETP and PTP might rely on distinct mechanisms. In vitro alteration of the cholesteryl ester transfer reaction by phospholipid transfers suggests that PTP might be a determinant factor in modulating in vivo the CETP-mediated redistribution of cholesteryl esters among various plasma lipoprotein fractions.

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