Regulation of lipid transfer between lipoproteins by an endogenous plasma protein: selective inhibition among lipoprotein classes

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Abstract Lipid transfer protein (LTP) remodels plasma lipoproteins by promoting mass transfers of cholesteryl ester (CE) and triglyceride between lipoproteins. We have investigated the capacity of an additional plasma protein, lipid transfer inhibitor protein (LTIP) to modify the functional activity of LTP in a complex mixture of lipoproteins. Transfer assays containing isolated LTP, LTIP, and the three major lipoprotein classes, and assays with intact human plasma supplemented with exogenous LTIP were used. In both assays, the inhibition of CE transfer by LTIP varied markedly depending on the lipoproteins involved and was dependent on LTIP concentration. Inhibition of lipid transfer between a given pair of lipoproteins was similar. However, between lipoprotein pairs the extent of inhibition was very different, varying up to 7-fold. Inhibition followed the order of very low density lipoprotein (VLDL)—low density lipoprotein (LDL) transfers > LDL—high density lipoprotein (HDL) transfers > VLDL—HDL transfers. Consistent with the preferential inhibition of transfer events involving LDL, LTIP was shown by gel filtration studies to associate primarily with LDL in plasma. The addition of LTIP to native plasma stimulated the LTP-mediated efflux of CE from HDL to VLDL; this occurred at the expense of LDL CE depletion. Thus, LTIP alters the pattern of lipid transfer reactions in plasma by uniquely affecting the individual transfer events mediated by LTP. By preferentially diminishing transfer events involving LDL, especially those between VLDL and LDL, LTIP enhances the ability of LTP to remove CE from HDL, and thus alters HDL metabolism.—**Morton, R. E., and D. J. Greene.** Regulation of lipid transfer between lipoproteins by an endogenous plasma protein: selective inhibition among lipoprotein classes. *J. Lipid Res.* 1994. **35:** 836–847.

Supplementary key words lipid transfer protein • lipid transfer inhibitor protein • cholesteryl ester • lecithin:cholesterol acyltransferase • *lipoprotein remodeling*

Lipid transfer protein (LTP) mediates the net transfer of triglycerides and cholesteryl esters between plasma lipoproteins (1–3). LTP, in concert with lipoprotein and hepatic lipases, has been implicated as an essential component in the catabolism of VLDL to LDL and in the remodeling of HDL subclasses, the latter process being important in the overall process generally referred to as reverse cholesterol transport (see ref. 4 for review). Roles for LTP other than in intravascular lipoprotein metabolism have been suggested by studies where LTP was shown to promote the efflux of CE from CE-enriched cells in culture (5, 6), and by the observation that adipocytes contain significant intracellular levels of both LTP and its mRNA, suggesting that LTP may participate in lipid metabolism within these cells (7).

The level of LTP activity in both humans and laboratory animals has been shown to vary remarkably under both normal and hyperlipidemic conditions. In some instances, this variable activity seems to reflect changes in the levels or quality of lipoprotein substrates present (8–11). In other cases, the lipoprotein-deficient fraction of plasma is the source of the altered LTP activity (9, 10, 12). In those situations where it has been quantitated, however, variations in the concentration of LTP mass account for only a portion of the change in LTP activity in this fraction. McPherson et al. (13) observed that, among a diverse group of subjects, LTP activities of various lipoprotein-deficient plasmas ranged 2- to 2.5-fold for a given level of LTP mass, suggesting that other factors contribute to the measured LTP activity.

Previously, we reported the presence of a protein in human plasma that inhibits LTP activity in vitro (14). Subsequent reports by Son and Zilversmit (15) and by Nishide, Tollefson, and Albers (16) have confirmed and extended the initial characterization of this factor as a unique ~30–35,000 molecular weight acidic (pI = ~4.0)

Abbreviations: LTP, lipid transfer protein; LTIP, lipid transfer inhibitor protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; CE, cholesteryl ester; PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kt, fraction of radiolabeled donor lipid transferred to an acceptor particle; apo, apolipoprotein.

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glycoprotein. This protein, termed lipid transfer inhibitor protein (LTIP), equally inhibits the LTP-mediated transfer of TG and CE from all lipoprotein classes (14). Although the mechanism of inhibition is not completely understood, binding studies have demonstrated a direct correlation between the suppression of LTP activity by LTIP and the disruption of LTP-lipoprotein binding by this protein (17). Utilizing immobilized lipoproteins as a binding site for LTP, we also demonstrated that LTIP binds to lipoproteins and that LTIP can prevent LTP binding when it is added to lipoproteins first, or it can disrupt preexisting LTP-lipoprotein complexes when added after the transfer protein is bound (17). These observations, plus the finding that the extent of inhibition is dependent on the concentration of lipoproteins in the assay (14, 15) have led to the conclusion that LTIP inhibits LTP by competing with the transfer protein for interaction with the lipoprotein surface.

The role of LTIP in modulating LTP activity in plasma is not known. Although LTIP has been shown to suppress lipid transfer from all lipoproteins in simple binary assay systems (i.e., a donor and an acceptor lipoprotein) (14), its function in the complex lipoprotein environment of plasma has yet to be investigated. In these studies we have attempted to address this question in studies with defined, reconstituted assay systems and in studies with intact human plasma. The capacity of LTIP to alter the profile of lipid transfer reactions in plasma by suppressing certain transfer events over others is also investigated.

METHODS

Materials

Tri[9,10-^{3}H]oleoyl glycerol (26.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA), and cholesteryl [1-^{14}C]oleate (52 mCi/mmol), [4-^{14}C]cholesterol (55 mCi/mmol), and [1α,2α(n)-^{3}H]cholesterol (45.6–48.4 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). [^{14}C]cholesteryl oleate was synthesized from [4-^{14}C]cholesterol and oleoyl chloride (Nu-Chek Prep, Elysian, MN) as described by Pinter, Hamilton, and Muldrey (18). Radiolabeled TG and CE with purities of <98% were repurified by thin-layer chromatography (17).

BSA (fraction V), diethyl *p*-nitrophenyl phosphate, egg PC, butylated hydroxytoluene, 5,5'-dithiobis-(2-nitrobenzoic acid), heparin-Sepharose, all reagents for salt and buffer solutions, and enzymes for fluorometric assays were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol, cholesteryl oleate, and triolein were purchased from Nu-Chek Prep. Lipid solutions were prepared in chloroform containing 10 μg/ml butylated hydroxytoluene and stored under N_2 at −20°C. Phenyl Sepharose CL-4B, dextran sulfate (M_r = 500,000), and reagents for isoelectricfocusing were from Pharmacia Fine Chemicals (Piscataway, NJ), and Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA).

Isolation of LTP and LTIP

Partially purified LTP was isolated from lipoprotein-deficient human plasma by hydrophobic and CM-cellulose chromatography as previously described (1). Partially purified LTP, which was routinely enriched 700- to 900-fold in both TG and CE transfer specific activities, was stored at 4°C in 0.27 mM disodium EDTA, pH 7.4. This fraction of LTP was used in all experiments. Partially purified LTP did not contain detectable lecithin:cholesterol acyltransferase activity (19) and was devoid of the phospholipid-specific transfer protein activity (LTP-II, (20)) based on the absence of significant heat-labile phospholipid transfer activity (21). LTP was also isolated from rabbit plasma (Pel-Freez Biologicals, Rogers, AR) by the same method.

LTIP was isolated by the same chromatographic steps as for LTP, except that the inhibitor was eluted from the hydrophobic column with 15% ethanol after LTP removal. LTIP was further purified on CM-cellulose, equilibrated in 10 mM sodium acetate, 50 mM NaCl, pH 4.5, buffer, where it eluted in the unbound fraction (14). Fractions containing LTIP activity were pooled and concentrated approximately 5-fold by ultrafiltration on YM-10 membranes (Amicon, Danvers, MA). When analyzed by SDS-PAGE, partially purified LTIP preparations consisted of a major broad band migrating at a mean molecular weight of ~32,000, but also contained distinct minor bands of higher and lower M_r (**Fig. 1,** lane 1). A similarly broad band has been reported for highly purified LTIP (15). By densitometric analysis, >90% of the protein was contained within the broad, major band.

Highly purified LTIP was prepared from the CM-cellulose fraction of LTIP by a combination of hydroxyapatite chromatography (15) and preparative flat bed isoelectricfocusing. Hydroxyapatite chromatography was performed exactly as previously described (15); LTIP eluted in the unbound fraction in the presence of 1 mM phosphate buffer, pH 6.8. Fractions containing inhibitor activity from the hydroxyapatite column were pooled, added to a 5% Ampholine mixture (Pharmacia, pH 3.5–5), and focused at 10°C, 8W for 16 h. The gel bed was divided into 20 segments and each was eluted with H_2O. Dialyzed fractions containing LTIP activity (pI 3.7–3.8), as determined by transfer assay, were combined and concentrated as above. Silver-stained SDS-PAGE gels revealed two protein bands; a major band of ~32,000 M_r and a minor contaminant of ~60,000 (Fig. 1, lane 2). By image analysis, the major band accounted for >97% of the protein in this sample.

During the purification of LTP and LTIP, lipid transfer activity was routinely measured by the amount of [^{3}H]TG

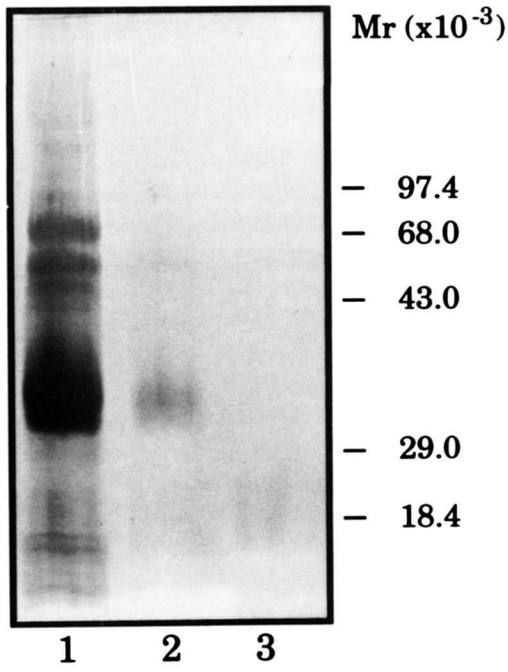

Fig. 1. SDS-PAGE of LTIP. Silver-stained SDS-PAGE gels of: lane 1, CM-cellulose fraction of LTIP (535 ng); lane 2, isoelectricfocusing fraction of LTIP (50 ng); lane 3, buffer blank.

and [^{14}C]CE transferred from LDL to unlabeled HDL (19, 22). Assays contained 10 μg cholesterol of each lipoprotein plus 0.5% BSA in a total volume of 0.7 ml; samples were incubated at 37°C for 1.5 h. These conditions are referred to herein as "standard assay" conditions. LTIP activity was assessed by the ability of samples to suppress the activity of a standard amount of partially purified LTP. Based on the results of seven preparations, after concentration by ultrafiltration, 10 μl of LTIP sample (10–15 μg protein) inhibited LTP activity in the standard assay by 33 ± 7% (mean ± SD). The percentage inhibition was constant with assay time within the linear portion of the transfer activity curve ($\leq$20% kt).

Isolation, radiolabeling, and modification of lipoproteins

Human lipoproteins were radiolabeled by several methods. Most common was the lipid dispersion technique of Morton and Zilversmit (14). Under these labeling conditions, lipoproteins contained [^{3}H]TG and [^{14}C]CE at specific activities of $\sim 1.6 \times 10^3$ and 4.9×10^2 dpm/μg lipoprotein cholesterol, respectively. Alternatively, for some experiments, VLDL or LDL were labeled by radiolabel exchange from reconstituted HDL (20). Labeled and unlabeled lipoproteins were isolated at 4°C by sequential ultracentrifugation (23) at solvent densities of 1.019, 1.063, and 1.21 g/ml to yield VLDL, LDL, and HDL, respectively. All lipoproteins were extensively dialyzed against 0.9% NaCl, 0.01% EDTA, 0.02% NaN_3, pH 7.4, and stored at 4°C. Lipoproteins were quantitated based on their total cholesterol content. LDL (20 mg) was biotinylated with 550 μg NHS-biotin (Pierce, Rockford, IL) in 50 mM $NaHCO_3$, pH 8, at 25°C for 30 min, followed by dialysis.

Assay of LTP activity

Lipid transfer assays were carried out as previously described (19, 22). In most instances, a radiolabeled donor lipoprotein and unlabeled acceptor lipoprotein(s) were incubated with or without LTP at 37°C. Under the standard conditions described above, the assay was terminated by selectively precipitating LDL (donor) by the addition of PO_4^{3-} and Mn^{2+} (22). In other instances, the assay was terminated by cooling the samples to 4°C and then separating the lipoproteins by standard ultracentrifugal methods. In all situations, the extent of transfer was assessed either by determining the radiolabel content of the fraction of interest, or by chemical measurements to determine the extent of mass transfer. The fraction of radiolabeled, donor lipid that was transferred (kt) to an acceptor particle was calculated as described before (19), and is reported either as percent lipid transfer (kt × 100), or as μg lipid transfer, which was calculated by multiplying the kt value times the mass of the lipid in the donor particle. Radiolabeled lipid "transfer" in the absence of LTP, <3% in the standard assay, was subtracted before these calculations. Duplicate values generally differed by <10%.

Measurement of lecithin:cholesterol acyltransferase activity

Human HDL was labeled with [^{3}H]cholesterol by the filter paper method of Nilsson and Zilversmit (24). Samples were incubated with ^{3}H-labeled HDL (15 μg cholesterol) in the presence of 1.67 mM 5,5'-dithiobis-(2-nitrobenzoic acid) for the indicated time and temperature, then adjusted to 11.7 mM 2-mercaptoethanol (25) and incubated at 37°C for 3 h. After incubation, lipids were extracted by the method of Thompson et al. (26) and fractionated by thin-layer chromatography in a developing system of hexanes–diethyl ether 70:30 (v/v). Samples comigrating with an authentic cholesteryl ester standard were scraped and the content of ^{3}H was determined by scintillation counting.

Immunological reagents and methods

Monoclonal antibodies to apolipoprotein D, designated 5H6 and 2B9, and monoclonal antibodies to LTP (TP2) were obtained from Dr. Yves L. Marcel (Montreal, Quebec). The preparation and characterization of these antibodies has been reported (3, 27). Polyclonal rabbit anti-human LTP antisera were prepared as previously described (6); the IgG fraction was isolated by DEAE-cellulose chromatography (28). Sheep antisera against

human apolipoprotein (apo) A-I and apoB were purchased from Boehringer Mannheim (Indianapolis, IN). The apoA-I content of samples was quantitated by electroimmunoassay (29). For this assay, samples were applied to 1.5% agarose gels containing 20% dextran (M_r 70,000) and 7 μl/ml antisera, and electrophoresed for 12 h at 5 V/cm. Samples were depleted of apoA-I by passage over an anti-apoA-I-Sepharose column prepared by coupling the IgG fraction from the sheep antisera to CNBr-Sepharose (Pharmacia LKB Biotech.). Anti-apoB and A-I IgG were isolated by protein G-agarose chromatography.

After separation of protein on 12% SDS-PAGE gels (30), proteins were transferred to nitrocellulose following the method of Towbin, Stachelin, and Gordon (31). Nitrocellulose blots were blocked with 0.4% gelatin and reacted with goat anti-human apoA-I or E (Calbiochem Corp., La Jolla, CA) or with monoclonal mouse anti-human apoD or LTP. Adherent antibodies were reacted with anti-IgG peroxidase conjugates of the appropriate species and visualized by reactivity with 4-chloro-1-naphthol or with a chemiluminescent substrate (ECL, Amersham).

Analytical procedures

Protein was quantitated by the method of Lowry et al. (32) as modified by Peterson (33), with BSA as standard. Total cholesterol of lipoproteins was assayed by a colorimetric, enzymatic method using Reagent-Set (Boehringer-Mannheim). Alternatively, free and total cholesterol levels were quantitated by the fluorometric, enzymatic method of Gamble (34); cholesteryl ester was determined by the difference between these two values, multiplied by a conversion factor of 1.69 to correct for the fatty acid content of the sterol ester. Over an assay range of 142–1065 ng cholesterol, the intra-assay CV was 4.3%. The cholesterol content of organic solutions of lipids was assayed by the method of Zak et al. (35). Triglyceride was measured by either a colorimetric, chemical assay (36) or by the fluorometric, enzymatic method of Mendez, Cabeza, and Hsia (37).

RESULTS

Characterization of LTIP preparations

The LTIP preparations used for these studies contained a single broad protein band plus multiple minor bands (Fig. 1). Immunoblots of SDS-PAGE gels failed to show any reactivity of LTIP preparations with polyclonal antibodies directed against apoE or apoA-I, or with monoclonal antibodies against LTP. Furthermore, LTIP preparations did not contain LTP-II, which alters the expression of LTP (38), as evidenced by the stability of inhibitor activity after heating at 56°C. However, LTIP preparations did react significantly with monoclonal antibodies to apoD; immunoreactivity co-migrated with the major protein band of these preparations (not shown).

Based on several lines of evidence, apoD was not responsible for the inhibitory activity of these preparations nor did it modulate the activity of LTIP. Among six preparations of LTIP, there was no correlation between the apoD content of these fractions as measured by quantitative slot blot analysis and their LTP inhibitory activity ($r = 0.153$, $P = 0.77$). Furthermore, the removal of 54% of the apoD by immunoadsorption with 5H6 and 2B9 monoclonal antibodies and protein A-agarose did not significantly alter the inhibitory activity in this preparation (48.1 vs. 44.2% inhibition/30 μg original protein, without or with antibody, respectively). Further purification of LTIP by a combination of hydroxyapatite chromatography and preparative isoelectricfocusing removed >97% of the immunodetectable apoD from the fraction containing inhibitor activity. The residual anti-apoD immunoreactivity in this preparation may reflect cross-reactivity of these antibodies with other plasma proteins, as previously noted by Weech et al. (27). Negligible inhibitory activity was associated with the apoD-containing fractions from the isoelectricfocusing gel. Unfortunately, the instability and low recovery of inhibitor activity in this purified preparation prevented its routine use in these studies. Collectively, the foregoing studies demonstrate that LTIP preparations do not contain other proteins previously reported to alter LTP activity, including apoA-I (15) and E (39), or LTP-II (38), and that apoD is not responsible for, nor does it appear to alter the expression of, the inhibitory activity of these preparations.

As both LTP and LTIP bind to lipoproteins in an apparently competitive fashion, it was of interest to determine whether the inhibitor protein is a fragment of LTP itself. Rabbit polyclonal IgG (55 μg) directed against human LTP (6) had no effect on the inhibitory activity of LTIP (58.2 vs. 62.0% inhibition/65 μg protein, with vs. without antibody, respectively), whereas much less antibody was able to completely block the activity of human LTP. Also, as noted above, monoclonal antibodies against LTP (TP2), did not react with blots of LTIP. These data suggest that LTIP and LTP are not immunologically related.

Activity of LTIP in lipoprotein mixtures

The activity of LTIP in complex mixtures of lipoproteins was measured to determine whether individual transfer reactions were equally effected by LTIP. Isolated human plasma VLDL, LDL, and HDL were combined at a ratio of 1:4:1.7, respectively, based on cholesterol content, and incubated with LTP and the indicated amount of LTIP. To measure each of the six transfer reactions involving CE transfer between these lipoprotein classes, this lipoprotein mixture contained either radiolabeled VLDL

(e.g., to measure VLDL to LDL and VLDL to HDL transfers), LDL, or HDL. As shown in **Fig. 2,** the extent of LTIP-mediated suppression of CE transfer varied markedly depending on the lipoproteins involved. In all cases, the inhibition of lipid transfer between a given pair of lipoproteins was very similar (e.g., the bidirectional transfer of CE between HDL and LDL, open and filled triangles). However, between lipoprotein pairs the extent of inhibition by the same amount of LTIP was very different. For example, at 100 μg LTIP, LDL to VLDL transfer was inhibited by 40%, whereas HDL to VLDL transfer was reduced by only 5.7%, and the inhibition of HDL to LDL transfer was intermediate at 19%. Overall, the percent inhibition of lipid transfer reactions followed the order of VLDL-LDL > LDL-HDL > VLDL-HDL. The magnitude of this difference in inhibition was greatest at low LTIP concentrations and was as large as 7-fold. In general, transfer events involving LDL were more effectively inhibited than those not involving LDL.

The apparent preferential suppression of some transfer events, when expressed as a percentage, was not due simply to differences in the absolute transfer rates between lipoproteins. This is perhaps best illustrated by comparing the absolute rates of CE transfer from LDL to VLDL and from HDL to VLDL. In both cases, approximately 8 μg of CE was transferred to VLDL from the donor lipoprotein during the assay (**Fig. 3**). However, the addition of LTIP markedly suppressed CE transfer from LDL but not from HDL. Similar comparisons can be made for transfer reactions involving VLDL as the donor, although the magnitude of CE transfer from this CE-poor lipoprotein is much less than that from other lipoproteins.

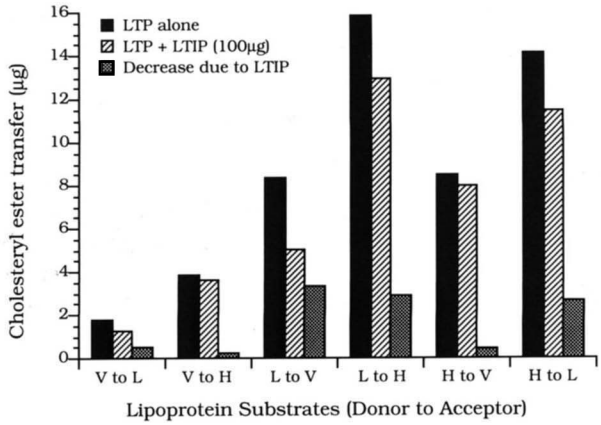

Fig. 3. Effect of LTIP on cholesteryl ester transfer: consideration of pool size. Experimental details are the same as those described in Fig. 2. The cholesteryl ester content of unincubated lipoproteins was determined as described in Methods. To determine cholesteryl ester transfer in μg, kt values (determined by radiolabel transfer) were multiplied by the cholesteryl ester content of the donor lipoprotein. Solid bars show the μg cholesteryl ester transferred in the absence of LTIP; striped bars denote transfer in the presence of 100 μg LTIP; and gray bars denote the net decrease in LTP activity due to LTIP. Abbreviations are: V, VLDL; L, LDL; H, HDL. Values are the mean of two or three determinations.

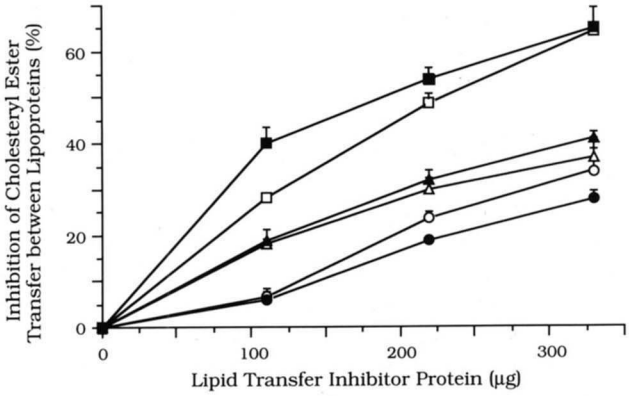

Fig. 2. Selective effects of LTIP on cholesteryl ester transfer between lipoproteins. Plasma lipoproteins were isolated from a normolipemic donor by ultracentrifugation. Isolated lipoproteins were radiolabeled with [^{3}H]triolein and [^{14}C]cholesteryl oleate by co-incubating (5.5 h) a 4-fold excess of unlabeled lipoprotein with LTP and dispersion-labeled lipoproteins of the same density range. These conditions permitted the near equilibration of labeled lipids between the native lipoprotein and that in the dispersion-labeled particle. Subsequently, the radiolabeled lipoprotein-LTP mixture was combined with unlabeled lipoproteins from the other two major lipoprotein classes and incubated for 2 h at 37°C. A typical sample contained VLDL (21 μg cholesterol), LDL (84 μg cholesterol), HDL (35 μg cholesterol), 106 μg partially purified LTP, 0.5% BSA, and the indicated level of LTIP (1100 μg protein/ml) in a total of 0.7 ml. One of these lipoprotein fractions, labeled as described above, contained ≥7,000 cpm ^{3}H and 3,000 cpm ^{14}C. After incubation, samples were fractionated by ultracentrifugation to determine the distribution of radiolabeled lipids. Values obtained from samples incubated at 4°C have been subtracted from those shown. Lipid transfer did not exceed 30% for any lipoprotein donor. Data points shown are the mean ± SD of two to three determinations. When not visible, the error bars are contained within the symbols themselves. These results are representative of three experiments which used lipoproteins from different donors. Symbols are: ■, transfer from LDL to VLDL; □, from VLDL to LDL; ▲, from HDL to LDL; △, from LDL to HDL; ●, from HDL to VLDL; ○, from VLDL to HDL.

The greater activity of LTIP in transfer reactions involving LDL, as noted above, could also be demonstrated by contrasting the effectiveness of LTIP in a standard LDL to HDL assay versus an assay containing only LDL (LDL to biotinylated LDL). LTIP activity was enhanced 5-fold in the latter assay compared to the standard assay. The decrease in lipoprotein surface area that occurred when LDL was substituted for HDL in these assays could explain only a small portion (<2-fold) of this greater activity. Highly purified LTIP (isoelectricfocusing fraction) retained this enhanced reactivity with LDL (4.2-fold higher activity in the LDL only assay), showing that contaminating proteins do not contribute to the preferential suppression of transfer events involving LDL observed with partially purified LTIP.

Effect of LTIP on lipid transfers in whole plasma

To assess the role of LTIP in whole plasma, initial studies were conducted to estimate the amount of LTIP activity in plasma relative to the LTIP preparations. The

capacities of lipoprotein-deficient plasma and isolated LTIP to inhibit LTP activity were contrasted (**Fig. 4**). Based on these data, we estimate that the inhibitory activity of one microliter of lipoprotein-deficient plasma is equivalent to the inhibitory activity contained in ~3 μg of partially purified LTIP. To rule out the possibility that residual apoA-I in lipoprotein-deficient plasma (~80 μg/ml) was responsible for some of its inhibitory activity (15), the apoA-I content of lipoprotein-deficient plasma was diminished by affinity chromatography on an anti-apoA-I–Sepharose column. This procedure reduced the endogenous apoA-I by 50% but had no significant effect on LTIP activity (29 ± 6% vs. 27 ± 3% inhibition for 10 μl aliquots before and after apoA-I depletion, respectively). Consistent with this observation, 5 μg of pure apoA-I, a >6-fold excess over that in 10 μl lipoprotein-deficient plasma, only marginally suppressed LTP activity (27.1 ± 0.6%kt vs. 28.6 ± 0.2 %kt). Thus, in lipoprotein-deficient plasma, little, if any, of the LTIP activity measured is due to residual apoA-I.

When LTIP was added to whole human plasma, the selective effects of the inhibitor seen in Fig. 2 were replicated in large part. As **Table 1** shows, the transfer of radiolabeled TG and CE between lipoproteins was diminished in a dose-dependent manner as plasma was supplemented with LTIP. Based on the data in Fig. 4, the highest level of LTIP added increased the endogenous LTIP activity by ~75%, a level well within the variation in LTIP activities observed between individual normolipemic donors (Morton, R. E. and Steinbrunner, J. V., unpublished observations). The extent of inhibition followed a pattern in which VLDL to LDL and LDL to VLDL transfers were inhibited greater than that of LDL to HDL transfers, and the transfer of lipid from VLDL to HDL was not affected by the levels of LTIP added.

TABLE 1. Effect of increasing plasma LTIP levels on the transfer of radiolabeled cholesteryl ester and triglyceride between lipoproteins

Donor	Inhibitor Added	Acceptor	%kt TG	%kt CE	% Inhibition TG	% Inhibition CE
	μg					
VLDL	0	LDL	9.1 ± 0.1	36.4 ± 0.1		
VLDL	220	LDL	7.5 ± 0.7	31.8 ± 1.4	18	13
VLDL	440	LDL	6.6 ± 0.1	27.9 ± 0.6	27	23
VLDL	0	HDL	6.9 ± 0.2	23.9 ± 2.5		
VLDL	220	HDL	7.4 ± 0.4	27.9 ± 0.4	0	0
VLDL	440	HDL	6.6 ± 0.2	27.3 ± 1.1	4	0
LDL	0	VLDL	12.3 ± 0.2	7.8 ± 0.2		
LDL	220	VLDL	10.1 ± 0.1	6.2 ± 0.1	18	21
LDL	440	VLDL	8.2 ± 0.3	5.4 ± 0.1	33	31
LDL	0	HDL	11.4 ± 0.1	16.0 ± 0.1		
LDL	220	HDL	10.7 ± 0.2	15.9 ± 0.6	6	1
LDL	440	HDL	9.6 ± 0.2	15.2 ± 0.6	16	5

VLDL and LDL from a normolipemic donor were labeled with [^{3}H]triolein and [^{14}C]cholesteryl oleate by transfer from reconstituted HDL as described in Methods. Radiolabeled VLDL (3.4 μg cholesterol, 13,600 cpm ^{3}H and 2,500 cpm ^{14}C) or LDL (9 μg cholesterol, 20,000 cpm ^{3}H and 4,600 cpm ^{14}C) was then added to 200 μl fresh plasma from the same donor, plus LTIP and buffer to give a final volume of 750 μl. After incubation at 37°C for 4 h (VLDL donor) or 6 h (LDL donor), the plasma lipoprotein fractions were isolated by ultracentrifugation. The fraction of lipid transferred to the indicated acceptor (%kt) was calculated as described in Methods. Samples incubated for the same time but at 4°C served as blanks; blank values have been subtracted from the numbers shown. The values shown are the mean ± SD of duplicate determinations.

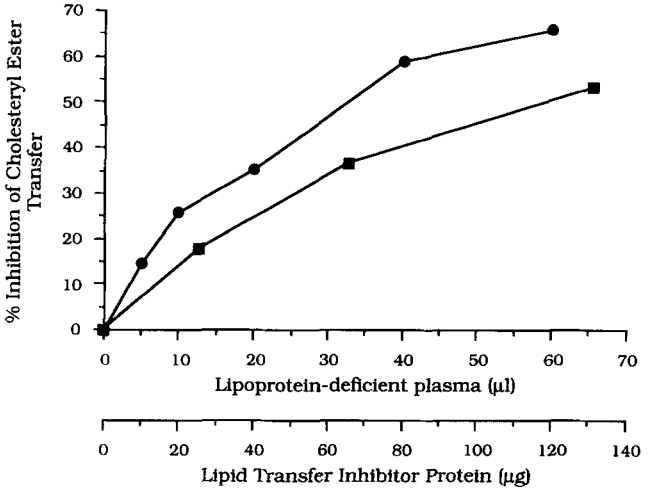

Fig. 4. Inhibition of LTP by lipoprotein-deficient plasma and LTIP. Human LTP (~10 μg) was incubated either alone or with the indicated amounts of LTIP (■) or lipoprotein-deficient plasma (●). Lipid transfer was measured under standard conditions (see Methods) except that the assay contained 40 μg cholesterol of both [^{3}H]TG/[^{14}C]CE-labeled LDL and unlabeled HDL. In the absence of an inhibitor source, transfer was 11.1 %kt and 12.4 %kt for the lipoprotein-deficient plasma and the LTIP assays, respectively. Lipoprotein-deficient plasma was prepared by dextran sulfate/Mn^{2+} precipitation followed by extensive dialysis against 0.9% NaCl, 0.01% NaN_3, and 0.02% EDTA, pH 7.4. For this experiment, a pool of plasmas from eight normolipemic donors was used. The data points are the mean of duplicate determinations. These results are representative of three experiments.

Effect of LTIP on the LTP-mediated equilibration of core lipids

In plasma, LTP mediates the net exchange of CE and TG between TG-rich and CE-rich lipoproteins (4). Specifically, LTP facilitates the net movement of CE from LDL and HDL to VLDL in exchange for TG. Since LTIP selectively reduces lipid transfers between VLDL and LDL, as measured by the exchange of radiolabeled lipids, we investigated whether LTIP may also alter the mass rearrangement of lipid that occurs in plasma during extended incubation in vitro. As expected, after long-term incubation of native plasma, VLDL gained CE resulting in a 2.5-fold enrichment of its CE content (**Fig. 5**). In this plasma sample, most of this CE was derived from LDL. As would be expected for an inhibitor, supplementation of plasma with LTIP resulted in a progressive decline in the

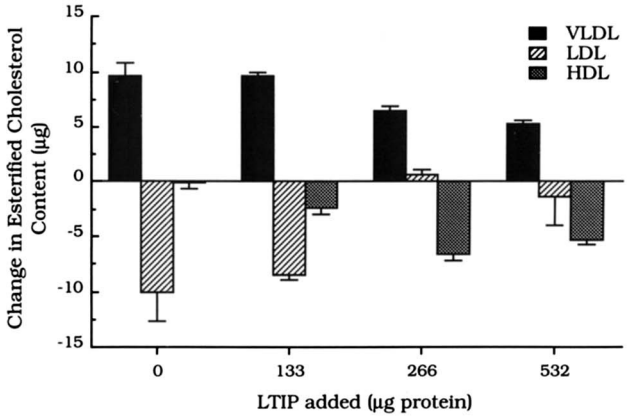

Fig. 5. Effect of LTIP on the distribution of esterified cholesterol among plasma lipoproteins. Fresh human plasma, containing 4 mM Na_2 EDTA, 0.04% NaN_3, and 1 mM diethyl *p*-nitrophenyl phosphate, was incubated for 16 h at 37°C. Each sample contained 100 μl plasma with or without additional LTIP in a total volume of 800 μl. After incubation, the plasma lipoproteins were isolated by sequential ultracentrifugation, dialyzed, and then assayed for total and free cholesterol. Esterified cholesterol (EC) was calculated as the difference between these two values. Shown are the changes in esterified cholesterol content compared to plasma incubated for 4°C under the same conditions. The esterified cholesterol content of 4°C control plasma was 4.7 ± 0.9, 75.0 ± 0.4, and 26.8 ± 0.7 μg for VLDL, LDL, and HDL, respectively. Relative to the 4°C plasma value, the total recovery of esterified cholesterol in incubated samples ranged from 97 to 101%. EC values shown are the mean ± SE of duplicate samples each assayed in duplicate. These results are representative of four similar experiments.

CE enrichment of VLDL. However, in addition to this general inhibitory effect, as the inhibitory activity was increased, the enrichment in VLDL CE that did occur was progressively derived more from HDL. At concentrations of LTIP ≥266 μg of LTIP, which corresponded to at least a 90% increase in plasma inhibitory activity, the enrichment of VLDL with CE was almost completely at the expense of HDL CE. Under these conditions, the CE content of HDL was reduced by as much as 25% by the addition of LTIP compared to that observed in plasma incubated alone. In other plasma samples, the initial contribution of HDL to VLDL CE was greater than that shown in Fig. 5, but the response of these samples to exogenous LTIP followed the same pattern.

Previous studies have suggested that HDL CE is a potent, negative modulator of LCAT activity (40). As LTIP modifies the activity of LTP resulting in a reduction of HDL CE content, we investigated whether this altered lipoprotein composition changes LCAT activity. DTNB-treated plasma was incubated ± additional LTIP under conditions similar to that described above (Fig. 5) to permit alterations in lipoprotein composition. After the addition of 2-mercaptoethanol to reactivate the LCAT enzyme, a dose-dependent increase in LCAT activity was observed as a function of the level of LTIP initially added to the plasma (**Fig. 6,** squares). LCAT activity was stimulated to almost 180% of its basal activity by the addition of 360 μg LTIP, an 80% increase in inhibitory activity. Plasma samples that received LTIP but were not incubated long-term to allow LTP and LTIP to affect changes in lipoprotein composition prior to measuring LCAT did not demonstrate elevated LCAT activities (Fig. 6, circles). Likewise, plasma (150 μl) supplemented with LTIP (162 μg) but incubated long-term (18 h) at 25°C, a temperature not permissive to LTP activity (41), stimulated LCAT activity only 5% above that observed in a native plasma sample incubated under the same conditions. Together, these control data demonstrate that the stimulation of LCAT activity by LTIP was due to altered lipoprotein composition facilitated by the actions of LTP and LTIP, and not due to a stimulatory effect of LTIP itself or other components in this preparation.

Plasma distribution of LTIP

As LTIP and LTP compete for binding to lipoproteins (17), the marked selectivity of LTIP in inhibiting certain LTP-mediated lipid transfer events suggested that this selectivity may arise from its preferential association with particular lipoprotein classes. The plasma distribution of LTIP activity was assessed by determining the inhibitory activity co-eluting with VLDL, LDL, and HDL on gel filtration. **Fig. 7** illustrates that under these column conditions, LTIP activity was associated with the LDL frac-

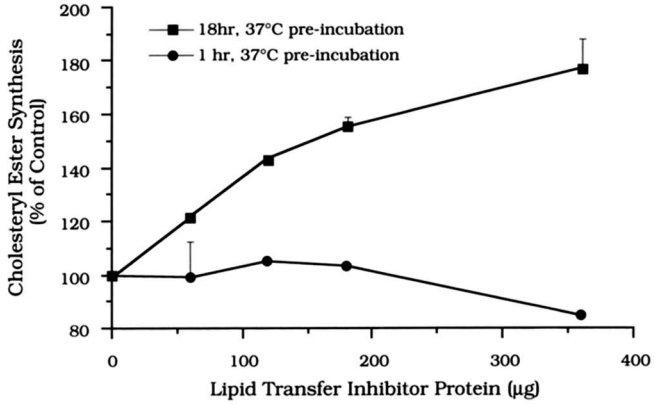

Fig. 6. Effect of LTIP on endogenous LCAT activity. Freshly isolated human plasma (150 μl) containing 1.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid) was combined with VLDL (80 μg cholesterol), [^{3}H]cholesterol-labeled HDL (15 μg cholesterol, 1 × 10^6 cpm ^{3}H) and the indicated amount of LTIP (1440 μg protein/ml) in a total volume of 767 μl. Samples were incubated for either 1 h (●) or 18 h (■) at 37°C, after which samples received 2-mercaptoethanol (11.7 mM final concentration) to activate LCAT activity. After further incubation at 37°C for 3 h, lipids were extracted and separated by TLC as described in Methods. Each curve represents data from a distinct donor. In the absence of exogenous LTIP, the cholesteryl ester synthesis was 9.1 ± 0.1% and 7.4 ± 0.1% for the two plasma samples shown. LCAT activity was linear for up to 6 h. The data points shown are the mean ± SD (n = 2–3); in most instances, the error bars are contained within the symbols themselves. These results are representative of three similar experiments on different plasma sources.

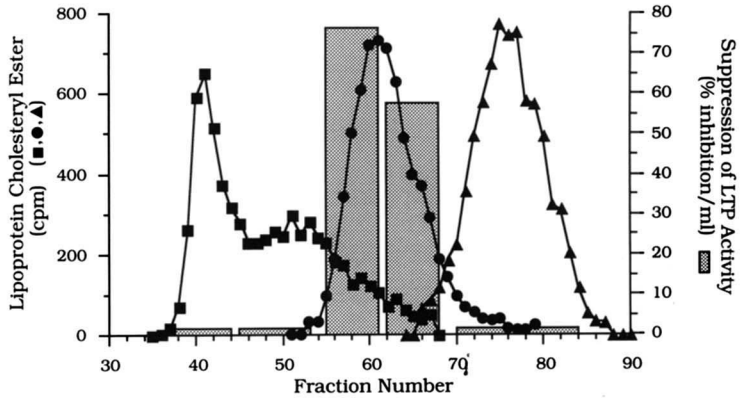

Fig. 7. Plasma distribution of LTIP. Human plasma (1 ml) was applied at 25°C to a Bio-Gel A-5m column (1 × 80 cm) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3 (pH 7.4) buffer containing 0.5% BSA. The column was eluted at 5 ml/h and 0.75-ml fractions were collected. Fractions were assayed for their ability to inhibit rabbit LTP as follows. Aliquots from pooled fractions were assayed in the presence of rabbit anti-human LTP IgG (44 μg), partially purified rabbit LTP (~20% transfer), unlabeled LDL (60 μg cholesterol), and radiolabeled HDL (40 μg cholesterol). All other assay conditions were the same as "standard" conditions except that the assay total volume was 800 μl. Samples were pre-incubated for 1 h at 25°C in the absence of labeled HDL. The assay was started by the addition of the labeled substrate; the assay time was 4.5 h at 37°C. The assay content of LDL and HDL was held constant by adjusting for the lipoprotein content of fractions to be assayed. The results shown are the average data from two plasma samples, each run in duplicate. Recovery of LTIP activity was 87.6 ± 7.5% (mean ± SE, n = 4) compared to the original plasma samples. The distribution of VLDL (■), LDL (●), and HDL (▲) was determined separately by monitoring the elution of radiolabeled lipoproteins individually added to aliquots of plasma.

tion, whereas LTP activity eluted with the HDL peak (not shown). Low levels of inhibitory activity were detected in the VLDL fraction of several plasma samples. The association of LTIP with LDL was supported also by immunoprecipitation studies. The removal of 75% of the apoB-containing lipoproteins from plasma resulted in an equivalent removal of LTIP activity (**Table 2**). There was no detectable cross-reactivity of this antibody with immunoblots of partially purified LTIP (not shown). The association of LTIP with apoB-containing lipoproteins was further illustrated by heparin-Sepharose chromatography (**Table 3**). Both plasma LTIP activity and LDL (as well as VLDL) were bound by the matrix, whereas HDL was not retained. High salt prevented the binding of both lipoproteins and LTIP. Notably, 50 mM $MnCl_2$, which disrupts LTIP-lipoprotein complexes in vitro, blocked the retention of LTIP activity by the column but had no effect on the binding of lipoproteins to the heparin matrix. In the absence of lipoproteins, partially purified LTIP (50 μg) was poorly retained (<15%) by the heparin matrix under the same condition. Collectively, these data show that LTIP activity is associated with LDL (and

TABLE 2. Removal of LTIP by immunoprecipitation of apolipoprotein B-containing lipoproteins

Plasma Sample	Aliquot	Inhibition of Exogenous LTP	% Removal of LTIP Activity
	μl	%	
Native	10	29.3	
Native	20	50.9	
ApoB-depleted	10	8.1	72.4
(75% removal)	20	14.9	70.7

Aliquots of human plasma (1 ml) were incubated with sheep anti-human apoB IgG (10 mg) or an equivalent volume of Tris/NaCl buffer overnight at 4°C. Samples were then centrifuged for 15 min at 16,250 *g* and filtered through 0.22 μm filters. The extent of apoB-containing lipoprotein removal was determined by the amount of exogenously added radiolabeled LDL immunoprecipitated. Lipoprotein-deficient plasma was prepared and assayed for LTIP activity by its ability to suppress the expression of exogenously added LTP activity (see Methods). The values shown are the averages of duplicate determinations.

TABLE 3. Adsorption of plasma lipoproteins by heparin-Sepharose also removes LTIP activity

Addition to Plasma	LDL Bound	LTIP Activity Eluted	LTIP Bound[a]
	%	*units/ml*	*% of control*
None	83.2 ± 4.2	22.2 ± 6.6	81.6
3 M NaCl	21.3 ± 3.7	100.9 ± 2.2	16.3
50 mM $MnCl_2$	86.7 ± 8.1	97.9 ± 1.8	18.8

Aliquots of human plasma (1 ml), with or without the indicated additives, were applied to 1 ml columns of heparin-Sepharose equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 0.01% EDTA, 0.5% BSA, pH 7.4. Columns were eluted with equilibration buffer at 5 ml/h. One-ml fractions were collected and the three fractions of highest protein content were pooled. Lipoprotein-deficient plasma was prepared and assayed for LTIP activity by its ability to suppress exogenously added LTP (see Methods). The values shown (mean ± SD) are the average of results from two experiments, each assayed in duplicate. One unit of LTIP activity equals 10% inhibition of exogenous LTP. LDL binding was determined from the adsorption of radiolabeled LDL added to samples. In control plasma, 9% of HDL and 92% of d < 1.019 g/ml lipoproteins were bound by the columns.

[a]Calculated from the difference between the total units applied (120.6 units/ml) and the LTIP activity recovered in the unbound fraction.

VLDL) in plasma. Additionally, these data are consistent with the hypothesis that plasma inhibitory activity is due to a unique protein and not to the effects of multiple plasma components such as apoA-I (15). These results also further demonstrate that apoD, which does not associate with apoB-containing lipoproteins (42), is not responsible for plasma LTIP activity.

DISCUSSION

Following two rather distinct approaches, Son and Zilversmit (15) and Nishide et al. (16) reported the purification of proteins that inhibit LTP activity in vitro. Based on similarities in the physical characteristics and metabolic activities described for these isolated proteins, they appear to be identical. By molecular weight and isoelectric point criteria (14–16), LTIP appears to be unique among the plasma proteins that have been previously characterized as having an affinity for lipoproteins (43). Furthermore, with the possible exception of apoA-I (15), no other inhibitory fractions of plasma have been observed, strongly suggesting that plasma contains a single suppressor protein for LTP. With the three-step purification procedure used here, LTIP preparations contained a number of minor contaminates as well as immunodetectable apoD which co-migrated with the major protein band in this fraction on SDS-PAGE. This major band also contained LTIP based on its molecular weight determined by gel filtration under dissociating conditions (14). Whether putatively homogeneous LTIP prepared by other investigators (15, 16) contains apoD is not clear. However, the data presented here strongly suggest that LTIP activity is not due to, or dependent on, apoD. This observation is consistent with our earlier studies (22) and those of Albers et al. (44) showing that the removal of apoD from lipoprotein-deficient plasma does not enhance LTP activity.

In these studies, we have demonstrated that LTIP inhibits core lipid transfer between all lipoproteins within a complex mixture of lipoproteins. This was observed in both a reconstituted assay system and when intact plasma was supplemented with exogenous LTIP. Although the addition of LTIP reduced the total lipid transferred (sum of all transfer events), this suppression was not equally distributed among the possible transfer reactions. In both assays, LTIP was much more effective at suppressing lipid transfer between VLDL and LDL that any other transfer reaction measured. Over a range of LTIP concentrations, the inhibition of VLDL-LDL transfers in the reconstituted lipoprotein assay system was 3- to 7-fold greater than that found for VLDL-HDL transfers, with the effect on LDL-HDL transfer reactions being intermediate. It can be generalized that those transfer reactions involving HDL are the least susceptible to LTIP. Conversely, transfer events involving LDL were the most sensitive to LTIP. Even when adjusted for differences in absolute transfer rates, the selective inhibition of VLDL-LDL transfers was evident. In whole plasma, the preferential suppression of VLDL-LDL transfer reactions by exogenous LTIP was also observed.

Our initial attempts to understand the basis for the selective actions of LTIP have focused on the plasma distribution of this protein because the limited information available suggests that LTIP suppresses LTP activity by disrupting its association with lipoprotein substrates. Consistent with its preferential action described above, gel filtration analysis of plasma revealed that LTIP is primarily associated with LDL. This was substantiated by apoB immunoprecipitation and heparin affinity studies. Our observation that little LTIP is associated with HDL is in marked contrast to the findings of Nishide et al. (16). We suggest that the association of LTIP with HDL observed by these investigators may be due to their use of more disruptive methods (i.e., ultracentrifugation and long-term (24 h) electrophoresis at low ionic strength) which may have altered the physiological distribution. We speculate that the greater effectiveness of LTIP in suppressing transfer events involving LDL results from the differing affinities of LTIP and LTP for the individual lipoprotein classes.

As LTIP appears to function by dissociating LTP from lipoproteins and it preferentially associates with LDL, this may explain a long-standing discrepancy concerning the plasma distribution of LTP. Kinetic binding studies indicate that LTP has similar affinities for all lipoproteins (17), and thus should associate with plasma LDL, whereas gel filtration data show that LTP in plasma is primarily associated with HDL and little is bound to LDL (45). Our previous data suggest that the lability of LTP-LDL and LTP-VLDL complexes under nonequilibrium conditions (17) may explain these seemingly disparate results. We now propose that the presence of LTIP on LDL may explain the absence of LTP in this lipoprotein fraction.

Lipid transfer by LTP involves both homoexchange reactions, where like molecules of lipid are exchanged, and heteroexchange reactions, which involve the exchange of triglyceride for cholesteryl ester, resulting in alterations in lipoprotein composition (4). Since the selection of TG versus CE from a lipoprotein is a property of the mass ratio of these two lipids (2), heteroexchange by LTP requires the presence of two lipoproteins differing in TG/CE composition. As the difference in the TG/CE ratio of lipoproteins increases, heteroexchange becomes the more prevalent reaction and the rate at which mass changes occur increases (46, 47). In plasma, the requirement for a TG/CE compositional gradient is met by TG-rich VLDL and CE-rich LDL and HDL. LTP promotes the equilibration of TG and CE between these lipoproteins as illustrated in **Fig. 8A.** Thus, through the actions of LTP, LDL and

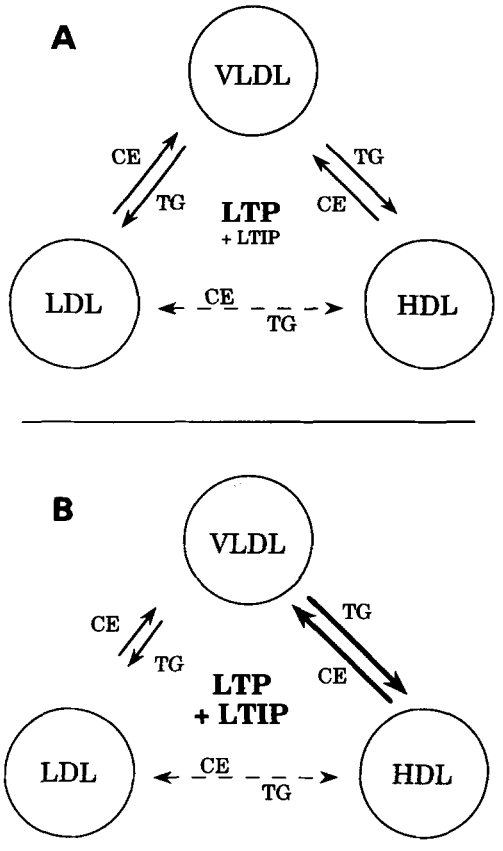

Fig. 8. Mass transfer of triglyceride and cholesteryl ester mediated by LTP in plasma is altered by LTIP. LTP mediates the net transfer of TG from VLDL to HDL and LDL with concomitant CE mass transfer in the reverse direction. Panel A shows the initial transfer rates of these lipids in the presence of a low level of LTIP. Panel B shows lipid transfers in the presence of a relatively higher level of LTIP. The lengths of the arrows for a given transfer reaction in panel A versus B are representative of the relative initial rates of those reactions under the two conditions. The dashed lines between LDL and HDL indicate that there is little, if any, mass transfer of lipids between these lipoproteins. Only heteroexchange reactions, i.e., the net transfer of CE for TG, are shown.

HDL compete for a supply of TG-rich lipoprotein that is limited by both its absolute amount (48) and its brief plasma lifetime, which permits only partial lipid equilibration (49).

In these studies we have observed that LTIP markedly affects this equilibration process by preferentially impeding the transfer of lipids to and from LDL, especially between LDL and VLDL. In transfer studies, we found that increasing the level of LTIP in plasma increased the mass transfer of lipids between HDL and VLDL. Based on radioisotope studies, we propose that this occurs because LTIP minimizes the participation of LDL in the equilibration between TG and CE rich lipoproteins, thus permitting the equilibration process to proceed to a greater extent along the VLDL-HDL pathway. That is, LTIP does not stimulate lipid transfer reactions between VLDL and HDL per se, but rather it allows heteroexchange reactions to occur more frequently by limiting the extent to which the TG/CE gradient is equilibrated along the VLDL-LDL pathway (Fig. 8B). However, pathologically high levels of LTIP, such as the >4-fold increase in inhibitory activity reported in the plasma of uremic patients on maintenance hemodialysis (50), appear to suppress all transfer events resulting in a decrease in CE removal from HDL. Thus, LTIP at certain concentrations may augment LTP-mediated CE efflux from HDL, whereas higher levels may be suppressive.

Numerous studies have concluded that in plasma the principal lipoprotein substrates for LCAT are those within the HDL density fraction (e.g., ref. 51). Among HDL particles, one of the determinants of LCAT activity on a given lipoprotein is its cholesteryl ester content (52). LTP facilitates LCAT activity by removing CE from HDL by mass transfer with other lipoproteins. Yen et al. (53) demonstrated that the addition of monoclonal antibodies against LTP to plasma resulted in a complete loss of LCAT reactivity on HDL after extended incubation. These same studies note the interesting finding that LCAT activity is not inhibited, however, but that LDL becomes an alternative substrate when HDL is no longer usable. The physiological relevance of this observation is questionable but it does suggest that LDL can become modified during in vitro incubations to yield a suitable LCAT substrate. Our results here support the role of LTP in maintaining the substrate quality of the HDL density fraction for the LCAT reaction. LTIP, which stimulated the net removal of cholesteryl esters from HDL by LTP during long-term incubations, markedly augmented LCAT activity on these modified lipoproteins.

In conclusion, LTIP inhibits the transfer of lipids between all lipoproteins in plasma. However, we report here for the first time that LTIP can alter the overall pattern of lipid transfer reactions in plasma by uniquely affecting the individual transfer events mediated by LTP. This selective activity, which preferentially diminishes transfer events involving LDL, especially those between VLDL-LDL, enhances the ability of a fixed level of LTP to promote the net removal of cholesteryl esters from the HDL density fraction during extended incubations. Thus, LTIP regulates LTP function by augmenting its role in events related to reverse cholesterol transport and decreasing its participation in lipid transfer events involving the LDL pool.

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