papers and notes on methodology

Enhanced detection of lipid transfer inhibitor protein activity by an assay involving only low density lipoprotein

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Abstract Lipid transfer inhibitor protein (LTIP) activity has been typically quantitated by its ability to suppress lipid transfer protein-mediated lipid movement between low density lipoprotein (LDL) and high density lipoprotein (HDL). In an attempt to establish an LTIP activity assay that is more sensitive, we have exploited the reported preference of the inhibitor protein to interact with LDL. A lipid transfer assay was established that involves LDL as both the donor and the acceptor; LDL in one of these two pools was biotinylated to facilitate its removal with immobilized avidin. Compared to the standard LDL to HDL assay, LTIP inhibited lipid transfer from radiolabeled LDL to biotin-LDL 7-fold more. In the absence of LTIP, lipid transfer activity was the same in both assays. An added benefit of this assay was the near linearity (up to 85%) of the inhibitory response, in contrast to the highly curvilinear response of LTIP in LDL to HDL transfer assays. The high sensitivity of the LDL to biotin-LDL transfer assay in measuring LTIP activity could not be duplicated by other transfer assays including assays containing only HDL (HDL to biotin-HDL), assays between liposomes and LDL, or assays between LDL and HDL where the concentration of lipoproteins was reduced 10-fold. Thus, LTIP activity is most effectively measured in homologous lipid transfer assays involving only LDL (and its biotin derivative). This increased sensitivity to LTIP suggests that the inhibitor binds more avidly to the LDL surface than does lipid transfer protein.—**Morton, R. E., and D. J. Greene.** Enhanced detection of lipid transfer inhibitor protein activity by an assay involving only low density lipoprotein. *J. Lipid Res.* 1994. **35:** 2094–2099.

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LTP mediates the partial equilibration of CE and triglyceride during the circulatory lifetime of plasma lipoproteins (1, 2). Although the mechanism of transfer has not been defined, it is known that the transfer process involves the binding of LTP to the surface of lipoproteins (3). Once associated, LTP binds CE or triglyceride in a competitive fashion (4) and subsequently facilitates the transfer of these lipids between lipoproteins by either a shuttle (5) or a ternary complex mechanism (6). The rate of lipid transfer is affected by a number of factors. These include: the concentration of LTP (7), the composition and concentration of lipoproteins (8, 9), the binding affinity of LTP for the lipoprotein surface (10), and the availability of neutral lipids at the lipoprotein surface (11). In addition, LTIP, a plasma glycoprotein, inhibits LTP activity by disrupting the binding of the transfer protein to the surface of lipoprotein substrates (3). LTIP inhibits the LTP-mediated transfer of lipids between any combination of VLDL, LDL, and HDL (12, 13). In the presence of all three lipoprotein classes, however, LTIP is not equally effective in suppressing individual lipid transfer reactions between a given donor and acceptor. LTIP is more effective at inhibiting lipid transfer reactions involving LDL, consistent with the observation that LTIP primarily associates with LDL in whole plasma (14).

Prior reports have illustrated that the recovery of LTIP activity from lipoprotein-deficient plasma during protein purification is highly variable and quantitatively poor (12, 15, 16). In our hands a major impediment to the development of alternative purification schemes to improve this yield has been the relative insensitivity of standard LTIP assays that utilize LDL as donor and HDL as acceptor. In this study, we have exploited the apparently higher reactivity of LTIP with LDL to develop an assay of greater sensitivity for LTIP activity.

Abbreviations: LTP, lipid transfer protein; LTIP, lipid transfer inhibitor protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester; PC, phosphatidylcholine; NHS-biotin, N-hydroxysuccinimide ester of biotin.

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METHODS AND MATERIALS

Materials

[1α,2α(n)-^{3}H]cholesteryl oleate (32 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Radiolabeled CE with a purity of <98% was repurified by thin-layer chromatography in a developing solvent of hexanes–diethyl ether 80:20 (v/v). Avidin-agarose (cat. #A-5150), avidin (cat. #A-9275), CNBr-Sepharose CL-4B, phenyl Sepharose CL-4B, dextran sulfate (M_r = 500,000), and all reagents for salt and buffer solutions were obtained from Sigma Chemical Co. (St. Louis, MO). NHS-biotin and biotin hydrazide were from Pierce (Rockford, IL), and carboxymethyl cellulose (CM-52) was obtained from Whatman LabSales (Hillsboro, OR).

LTP and LTIP purification

Plasma for LTP and LTIP preparations was obtained from the American Red Cross Blood Bank. Lipoprotein-deficient plasma was made by dextran sulfate–Mn^{2+} precipitation (17), and partially purified LTP was isolated from this fraction by hydrophobic and CM-cellulose chromatography as previously described (18). LTP was stored at 4°C in 0.27 mM disodium EDTA, pH 7.4. This fraction of LTP was used in all experiments. 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 (12).

Lipoprotein isolation, biotinylation and radiolabeling

Fresh human plasma from the Blood Bank of the Cleveland Clinic Foundation was the source of LDL and HDL. Lipoproteins were isolated at 4°C by sequential ultracentrifugation (19), extensively dialyzed against 0.9% NaCl, 0.02% EDTA, pH 8.5, and stored at 4°C. Lipoproteins were quantitated based on their total cholesterol content. In some instances, before isolation from plasma, lipoproteins were radiolabeled by a lipid dispersion technique (12). Under these labeling conditions, lipoproteins typically contained ~1.6 × 10^3 dpm [^{3}H]CE/μg cholesterol.

HDL (~3.7 mg cholesterol/ml) and LDL (~7 mg cholesterol/ml) were biotinylated with NHS-biotin (Pierce, Rockford, IL). Equal volumes of lipoprotein and 100 mM $NaHCO_3$, pH 8.0, were combined with sufficient freshly prepared NHS-biotin (1 mg/ml in H_2O) to yield a final biotin concentration of 100 μg/ml. This mixture was incubated at 25°C for 30 min, followed by overnight dialysis in 0.9% NaCl, 0.01% EDTA, 0.02% NaN_3, pH 7.4. The extent of biotinylation was assessed by the ability of excess avidin-agarose to adsorb the derivatized lipoprotein, which typically was >92% for LDL and >71% for HDL. Radiolabeled biotin-LDL was prepared by transferring, via LTP, [^{3}H]CE from radiolabeled HDL into biotinylated LDL, followed by ultracentrifugal re-isolation.

LTIP and LTP assays

Lipid transfer assays between [^{3}H]CE-LDL (donor) and HDL (acceptor) were carried out as previously described (20, 21). [^{3}H]CE-LDL and unlabeled HDL (typically 10 μg cholesterol of each) were incubated with or without LTP and LTIP at 37°C for 1.5 h, then the assay was concluded by selectively precipitating LDL (21). In assays containing less than 10 μg of a particular lipoprotein, unlabeled lipoprotein of the same density class was added at the end of the incubation to bring the assay level to 10 μg.

Homologous transfer assays between [^{3}H]CE-LDL (or [^{3}H]CE-HDL) and unlabeled, biotinylated LDL (or biotinylated HDL) were essentially the same as those described above except that the assays were terminated by affinity adsorption of the biotinylated lipoprotein with avidin-agarose or avidin-Sepharose. After incubation at 37°C, cold immobilized avidin (4°C) was added, samples were mixed end-over-end continuously for 1 h at room temperature, then centrifuged at 750 *g* for 5 min.[2] The pellet was washed once with 0.5 ml of 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, and re-centrifuged as before. After aspirating the supernatant, the lower portion of the tube containing the pellet was removed and its radiolabel content was determined by scintillation counting in 10% aqueous EcoLite (ICN, Costa Mesa, CA). When the assays involved radiolabeled biotinylated LDL as donor and native LDL as acceptor, the supernatant was counted after the initial centrifugation step described above.

PC liposomes containing 0.27 mole% [^{3}H]CE were prepared as previously described (11). Lipid transfer assays from [^{3}H]CE-PC liposomes (190 nmol PC) to LDL (10 μg cholesterol) were performed exactly as previously described (21) and transfer activities were calculated as the difference between samples incubated at 37°C for 0 and 1.5 h.

In all assays, the fraction of radiolabeled donor lipid that was transferred (kt) to the acceptor particle was calculated as described before (20), and reported as percent lipid transfer (%kt). In the absences of LTP, assay blank values were: [^{3}H]CE-LDL to HDL, < 3%; [^{3}H]CE-LDL to biotin-LDL, 6.4%; [^{3}H]CE-biotin-LDL to LDL, 5%; [^{3}H]CE-HDL to biotin-HDL, 9.5%; and [^{3}H]CE-PC liposomes to LDL, 1.7%. Duplicate values generally differed by <10%. LTIP activity was assessed by the ability of samples to suppress the activity of a standard

[2]The LTP-mediated lipid transfer that occurs during this 25°C post-incubation step is less than 5% of the transfer that occurs at 37°C in 90 min.

amount of partially purified LTP. The percentage inhibition was constant with assay time within the linear portion of the lipid transfer activity curve.

Other methods

Protein was quantitated by a modification (22) of the method of Lowry et al. (23), with BSA as standard. Total cholesterol of lipoproteins was assayed by a colorimetric enzymatic method with Reagent-Set (Boehringer-Mannheim). Avidin-Sepharose was prepared by reacting 25 mg (330 units) of affinity-purified avidin (Sigma, A-9275) with 10 ml of CNBr-Sepharose in 0.1 M Na_2CO_3, 0.5 M NaCl, pH 8.3, overnight at 4°C. The gel was blocked with 0.5 M Tris-HCl, pH 8.0, acid- and base-washed to remove unbound ligand, and stored as a 25% slurry in 50 mM Tris-HCl, 150 mM NaCl, 0.01% EDTA, 0.02% NaN_3, pH 7.4.

RESULTS

Following the method of Wade et al. (24), the carbohydrate component of apolipoprotein B in LDL was effectively derivatized by biotin-hydrazide. However, most likely due to the oxidative nature of this derivation method, this biotin-LDL lost its characteristic yellow color and aggregated within 1–2 days of storage in EDTA-containing buffer. Several modifications, including the addition of vitamin E after derivatization, were unsuccessful in stabilizing these preparations. In contrast, NHS-biotin (see Methods and Materials) reacted with lipoproteins to give a reagent with a shelf-life of at least 1 month. This biotinylation method was used in all subsequent studies.

Avidin-Sepharose CL-4B, prepared in our lab, was superior to commercially available avidin bound to 6% agarose. This is most likely due to the larger pore size of the Sepharose CL-4B since one half as much of this avidin derivative was required to adsorb an equivalent amount of biotin-LDL. This resulted in two benefits: *1)* assay blanks, resulting from the nonspecific adsorption of [^{3}H]CE-LDL to the highly positively charged avidin matrix, were reduced by twofold, and *2)* the cost of the immobilized avidin required per assay sample was decreased by a factor of three. Assay blanks, which were ~6% for avidin-Sepharose CL-4B incubated with freshly prepared [^{3}H]CE-LDL, increased with [^{3}H]CE-LDL storage time. This suggests that mild oxidation of LDL, which increases its electronegativity but has no effect on either LTP or LTIP activity (unpublished observations), enhances the nonspecific interaction of [^{3}H]CE-LDL with avidin. Biotin-LDL was bound rapidly by avidin-Sepharose CL-4B, reaching a maximum by 45 min and remaining essentially constant over 90 min. Typically, 88% of biotin-LDL (10 μg) was bound by 25 μl of avidin-Sepharose (settled gel volume) in 60 min; the fraction of biotin-LDL removed varied only ± 1% within a range of 20–30 μl of immobilized avidin.

LTP effectively mediated CE transfer between LDL molecules (**Fig. 1**). Lipid transfer activity was nearly linear for both [^{3}H]CE-LDL to HDL and [^{3}H]CE-LDL to biotin-LDL assays over a range of 25 %kt. LTP activity was very similar in these two assays among eight determinations involving different LTP and lipoprotein preparations (mean = 10.05 vs. 10.41 %kt, respectively). However, one preparation of biotin-LDL consistently gave LTP activities 2-fold greater than that measured in the [^{3}H]CE-LDL to HDL assay. The reason for this elevated LTP activity is unclear; however, it had no apparent effect on the measurement of LTIP activity as described below.

LTIP activity, in contrast to that observed with LTP, was greatly enhanced in the homologous LDL assay compared to the standard [^{3}H]CE-LDL to HDL assay (**Fig. 2**). At lower LTIP concentrations where the inhibitory response in the [^{3}H]CE-LDL to HDL assay is nearly linear, LTIP was 7-fold more active in suppressing LTP activity when only LDL was present in the assay. LTIP added to LTP-containing samples after their incubation at 37°C, but before the addition of immobilized avidin, had no effect on the measured LTP activity. Therefore, LTIP does not affect the separation of donor and acceptor lipoproteins by avidin. Also, LTIP activity was nearly linear with dose over a wide range of inhibitor activity (up to 85% in some studies). The LTIP dose response in [^{3}H]CE-LDL to HDL assays, in contrast, was highly curvilinear above 40% inhibition (12, 14, and see Fig. 4B). LTIP activity in both assays was essentially independent

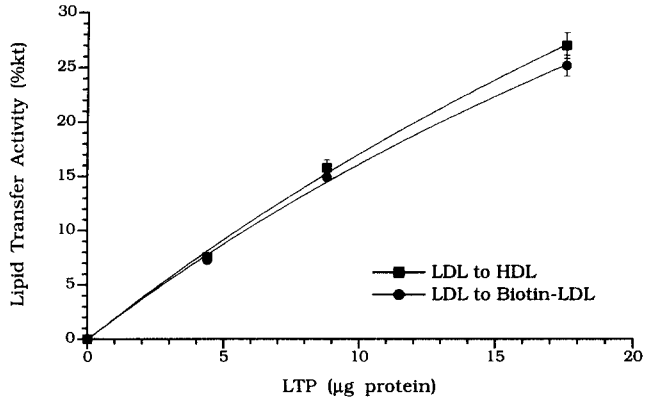

Fig. 1. LTP activity in heterologous and homologous transfer assays. Partially purified LTP was incubated with [^{3}H]CE-LDL and either biotin-LDL (●) or HDL (■) under the conditions described in the Methods and Materials. Values are the mean ± SD of duplicate determinations; the near linear response noted here is representative of three similar experiments. Where not visible, error bars are contained within the symbol.

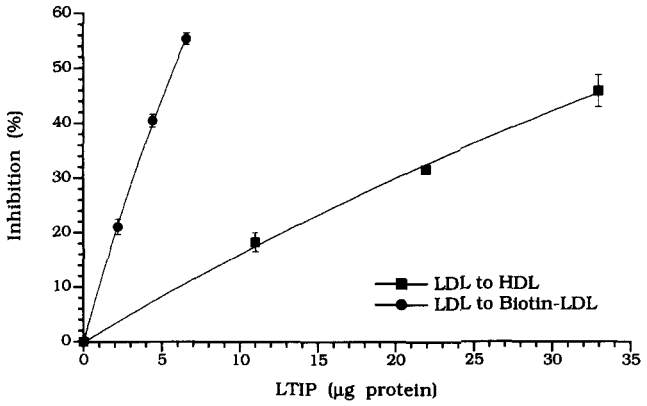

Fig. 2. LTIP activity in heterologous and homologous transfer assays. Partially purified LTIP, in the amounts indicated, was incubated with LTP, [^{3}H]CE-LDL (donor) and either biotin-LDL (●) or HDL (■) as acceptor under the conditions described in the Methods and Materials. LTP activity in the [^{3}H]CE-LDL to biotin-LDL was 16.0 %kt in the absence of LTIP, whereas its activity in [^{3}H]CE-LDL to HDL assays was 17.1 %kt in the absence of LTIP. Values are the mean ± SD of duplicate determinations; these results are representative of three separate experiments. Where not visible, error bars are contained within the symbol.

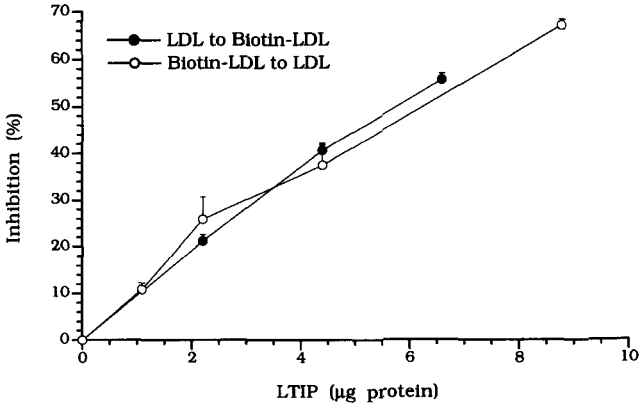

Fig. 3. LTIP activity in homologous LDL assays with biotin-LDL as donor or acceptor. LTIP, in the amounts shown, was incubated with LTP, LDL and biotin-LDL. LTP activity in the absence of LTIP was 16.0% in the [^{3}H]CE-LDL (donor) to biotin-LDL (acceptor) assay (●), and 18.1 %kt in assays with [^{3}H]CE-biotin-LDL as donor and LDL as acceptor (○). All other conditions were as described in the Methods and Materials. Values are the mean ± SD of duplicate determinations; these results are representative of two similar experiments. Where not visible, error bars are contained within the symbol.

of the amount of LTP in the assay (**Table 1**); similar results have been reported for the [^{3}H]CE-LDL to HDL assay (12, 15).

The high sensitivity and near-linearity of the [^{3}H]CE-LDL to biotin-LDL assay was also observed when the transfer reaction was performed in the reverse direction, i.e., from [^{3}H]CE-biotin-LDL to LDL (**Fig. 3**). Although not used routinely in these studies, this reverse assay is technically less difficult and more rapid, once the labeled, biotinylated LDL is prepared, as it greatly simplifies the processing of samples at the end of an experiment (i.e., the washing and re-centrifugation steps for the avidin pellet are eliminated).

TABLE 1. LTIP activity is relatively unaffected by LTP concentration

Transfer Assay (Donor:Acceptor)	LTP Added	LTIP Activity
	µg protein	*% inhibition*
[^{3}H]CE-LDL:HDL	6.6	39.3 ± 2.5
	13.2	47.0 ± 3.0
	19.8	48.2 ± 0.9
[^{3}H]CE-LDL:biotin-LDL	6.6	51.0 ± 12.2
	13.2	44.6 ± 2.9
	19.8	42.9 ± 0.2

Lipid transfer assays from [^{3}H]CE-LDL to HDL or from [^{3}H]CE-LDL to biotin-LDL were carried out as described in Methods and Materials. The level of LTP in these assays was varied as indicated, whereas LTIP concentration was held constant at either 49.4 µg or 4.9 µg protein for the [^{3}H]CE-LDL to HDL or the [^{3}H]CE-LDL to biotin-LDL assay, respectively. Values are mean ± SD (n = 3).

It is well documented that LTIP activity is inversely related to the concentration of lipoproteins in the assay (12, 15). This is consistent with the observation that LTIP binds avidly to the lipoprotein surface and most likely mediates suppression of LTP by displacing the transfer protein from the lipoprotein surface (3). In view of this, it is noteworthy that the markedly enhanced activity of LTIP in [^{3}H]CE-LDL to biotin-LDL assays is not due simply to the reduction in lipoprotein surface that occurs when biotin-LDL is substituted for HDL. LTIP activity in transfer assays containing 10 µg [^{3}H]CE-LDL and only 3.6 µg HDL, which represents the same lipoprotein phospholipid surface area contained in the [^{3}H]CE-LDL to biotin-LDL assay (10 µg each), was similar to assays containing 10 µg of both [^{3}H]CE-LDL and HDL (not shown). The high sensitivity of the homologous LDL assays to LTIP could not be duplicated in [^{3}H]CE-LDL to HDL assays even when the total lipoprotein surface area was reduced 10-fold (**Fig. 4A**). The CE specific activity in [^{3}H]CE-LDL did not permit the use of lower lipoprotein concentrations.

Homologous assays containing only HDL, as opposed to those containing only LDL, were inferior for LTIP activity measurements even compared to the standard [^{3}H]CE-LDL to HDL assay (Fig. 4B). These data suggest that HDL may play a significant role in reducing the measurable LTIP activity. However, substituting PC liposomes for HDL in the transfer assay did not significantly increase LTIP activity (Fig. 4B). Thus, it is not apparent that HDL per se is responsible for the lower LTIP activity in [^{3}H]CE-LDL to HDL assays, rather it is the greater in-

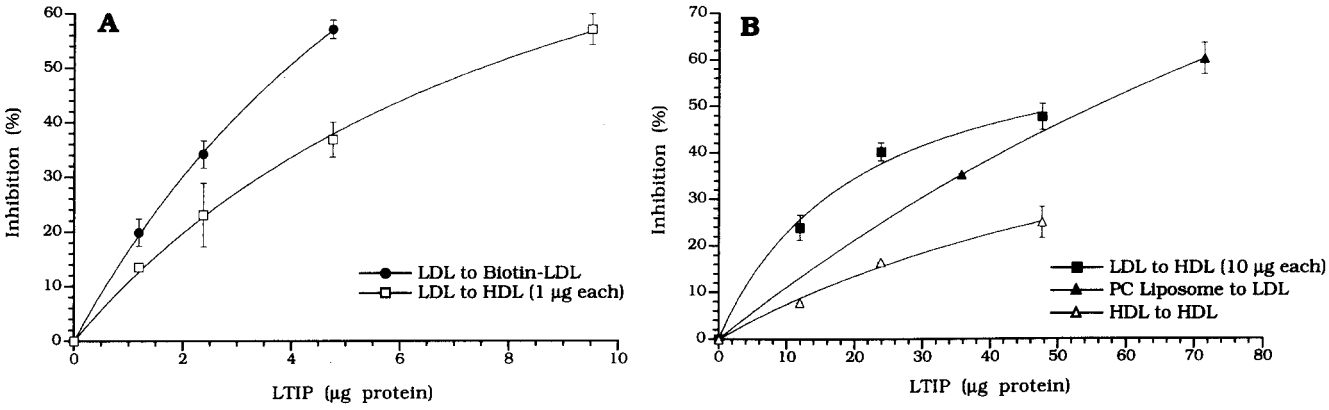

Fig. 4. LTIP activity in various homologous and heterologous assays. LTIP activity was measured in various assays where the donor, acceptor or their concentrations were altered. Panel A: [^{3}H]CE-LDL and HDL (1 μg cholesterol each, □) or [^{3}H]CE-LDL and biotin-LDL (10 μg cholesterol each, ●), LTP and the indicated level of LTIP were incubated as described in the Methods and Materials. Panel B: [^{3}H]CE-LDL and HDL (10 μg cholesterol each, ■), [^{3}H]CE-PC liposomes and LDL (0.2 μmol phospholipid and 10 μg cholesterol, respectively, ▲) or [^{3}H]CE-HDL and biotin-HDL (10 μg cholesterol each, △), LTP and the indicated level of LTIP were incubated as described in the Methods and Materials. LTP was adjusted for each assay to give similar activity levels in the five assays described when LTIP was absent (15.4–21.8 %kt). Values are the mean ± SD of duplicate determinations; these results are representative of at least two similar experiments for each assay shown. Where not visible, error bars are contained within the symbol.

teraction of LTIP with LDL that results in higher LTIP activity when assays contain only LDL.

DISCUSSION

LTIP is more active in suppressing lipid transfer events involving LDL than in those involving VLDL and HDL (14). Based on this selective interaction with LDL, we developed an assay for LTIP activity that is both linear and highly sensitive (7-fold) compared to standard assays involving LDL and HDL. The increased sensitivity of [^{3}H]CE-LDL to biotin-LDL transfer reactions to LTIP was not due to the lower lipoprotein surface area in this assay; [^{3}H]CE-LDL to HDL transfer assays with equivalent or even one-tenth the surface area were not as sensitive.

We have previously demonstrated that LTIP preferentially binds to LDL in plasma (14). Conversely, LTP forms stable complexes with HDL, but not with LDL or VLDL unless they are chemically modified (10, 25). As LTIP inhibits LTP by displacing it from the lipoprotein surface (3), we suggest that the greater activity of LTIP in LDL to LDL transfer assays is due to the relatively higher affinity of LTIP, compared to LTP, for the LDL surface. In transfer assays containing HDL, LTIP may be less active because LTP forms more stable complexes with this lipoprotein. Likewise, the linearity of LTIP activity in homologous LDL assays, in contrast to the curvilinear response of [^{3}H]CE-LDL to HDL assays, may result because the overall inhibition reflects a single event, i.e., the competition of LTIP and LTP for the LDL surface. In LDL to HDL transfer assays, the addition of LTIP probably also induces a redistribution of LTP between LDL and HDL due to the dissimilar binding of LTIP and LTP to these two lipoproteins. This may alter the relative rate of lipid transfers between lipoproteins of the same density class (LDL to LDL and HDL to HDL) and of different density classes (LDL to HDL), which complicates the LTIP dose response.

This assay has been used to measure LTIP activity in partially purified (reported here) and highly purified LTIP preparations (14), and in lipoprotein-deficient plasma (unpublished). In the latter instance, however, the addition of lipoprotein-deficient plasma to the assay more than doubled the nonspecific interaction of [^{3}H]CE-LDL to the avidin matrix, thus necessitating that assay blanks also contain this plasma fraction. This reduction in the signal-to-noise response may limit the use of this assay with lipoprotein-deficient plasma samples containing very low LTIP levels.

This assay differs from the heat-inactivation assay for LTIP (26) we recently reported in several important aspects. In the heat inactivation assay, LTIP activity is determined by the rise in endogenous LTP activity after heat inactivation of LTIP. This assay provides an unique method of determining simultaneously the functional LTIP activity and the maximum LTP activity in lipoprotein-deficient plasma without separation of these two proteins. In contrast, the homologous LDL assay described here, which is based on the ability of samples to suppress exogenously added LTP, provides a sensitive, near-linear assay for LTIP activity that can be applied to various LTIP-containing fractions. This increased detec-

tion of LTIP should aid efforts to purify this protein and to quantitate its activity in various solutions such as cell culture media.

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