Determination of Lipid Transfer Inhibitor Protein Activity in Human Lipoprotein-Deficient Plasma

Richard E. Morton, Jeffrey V. Steinbrunner

Lipid transfer protein (LTP) activity is modulated by a distinct plasma protein termed lipid transfer inhibitor protein (LTIP). The objective of this study was to establish an assay for LTIP that could be used to quantify its activity in lipoprotein-deficient plasma. A straightforward heating protocol (56°C for 60 minutes) was found to inactivate more than 90% of LTIP activity. The responses of individual lipoprotein-deficient plasma samples to this heating procedure were unique. Among normolipidemic donors, inactivation of LTIP caused a 230% to 600% increase in LTP activity. Essentially all measurable transfer activity in native and heated samples was inhibited by an antibody to LTP. Whole-plasma samples from these donors were spiked with radiolabeled lipoproteins to measure the rates of lipid transfer among the major lipoprotein classes. In general, plasma lipid transfer rates were negatively correlated with LTIP activity in these samples. However, the decrease in lipid transfers from very-low-density lipoprotein (VLDL) to low-density lipoprotein (LDL) and from LDL to VLDL was from 2.4- to 5.1-fold greater than in the transfers from VLDL to high-density lipoprotein (HDL) or from HDL to VLDL. In these samples, the molecular weight of HDL was negatively correlated with LTIP activity. Thus, LTIP activities among normolipidemic individuals were observed to vary severalfold; compared with other lipoprotein transfers, higher LTIP activities were associated with a relative reduction in LDL-VLDL lipid transfer events. (Arterioscler Thromb. 1993;13:1843-1851.)

KEY WORDS • lipid transfer inhibitor protein • lipid transfer protein • human plasma • lipoprotein metabolism • cholesteryl ester transfer • heat inactivation

Lipid transfer protein (LTP) remodels lipoprotein composition by facilitating the net transfer of triglycerides (TGs) and cholesteryl esters (CEs) between plasma lipoproteins. In concerted action with lipolytic activities, LTP is a key component in the normal intravascular metabolism of lipoproteins in humans. Individuals who are genetically deficient in LTP show marked alterations in their lipoprotein composition, which is characterized by elevated high-density lipoprotein cholesterol (HDL-C) levels and the accumulation of high-density lipoprotein (HDL) enriched in apolipoprotein E, much like the HDL-C noted in cholesterol-fed animals that lack detectable plasma LTP activity.

In the plasma of individuals not genetically deficient in LTP, variations in lipid transfer activity can result from many factors. In some instances, activity appears to reflect changes in the level or quality of lipoprotein substrates present. In other cases, the lipoprotein-free fraction of plasma is the source of altered LTP activity. In these cases, however, variations in the concentration of LTP mass account for only a portion of the change in LTP activity in this fraction. The LTP activity of lipoprotein-deficient plasma can range from 2- to 2.5-fold for a given level of LTP mass, suggesting that other factors contribute to the measured LTP activity.

The existence of an inhibitor of LTP activity in lipoprotein-deficient plasma was first reported by Morton and Zilversmit. This study and others' studies have characterized this factor as a unique ~30,000 to 35,000-molecular-weight acidic glycoprotein. Termed lipid transfer inhibitor protein (LTIP), this protein equally inhibits the LTP-mediated transfer of TGs and CEs from all lipoprotein classes. The only other plasma protein that has been shown to suppress LTP activity in vitro is apolipoprotein A-I. However, this was not confirmed by Nishide et al, and we have found that the apolipoprotein A-I in lipoprotein-deficient plasma can account for only a minor part of the inhibitory activity present in this fraction (R.E. Morton and J.V. Steinbrunner, unpublished data, 1992). Collectively, these data suggest that LTIP is responsible for most, if not all, of the LTP inhibitory activity in lipoprotein-deficient plasma.

Although the mechanism of LTIP-mediated suppression of LTP activity is not completely understood, current evidence indicates that LTIP disrupts the association of LTP and its lipoprotein substrates, thus preventing the transfer process. The importance of LTIP in defining the function of LTP in plasma was
partially illuminated by recent studies from our lab that demonstrated that LTIP is not simply a suppressor of LTP activity but is selective in its actions. R.E. Morton and D.J. Greene, unpublished data, 1993). Lipid transfers between low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) are markedly inhibited by LTIP under conditions in which HDL-VLDL transfers are much less affected.

Widely disparate estimations of LTIP activity in human plasma have been reported. Tollefson et al determined that there was a 1.6- to 2.3-fold increase in LTP activity after chromatography of lipoprotein-deficient plasma on phenyl-Sepharose, which separates LTP and LTIP activities; however, the recoveries of LTP and LTIP after this procedure are not known. In contrast, immunofluorescence chromatography of human plasma over an anti-LTIP column resulted in only a 17% increase in LTP activity in the eluate. This suggests either that LTIP has a relatively minor effect on LTP activity or that this step also removed LTP, perhaps due to a physical association of these two proteins in plasma. The inability to quantify LTIP activity accurately in lipoprotein-deficient plasma confounds attempts to assess the effect of this protein on lipid transfer, to determine the variability of this activity in individuals, or to optimize LTIP purification protocols. In this article we describe an approach for determining the level of LTIP activity in lipoprotein-deficient plasma. This procedure also permits the LTP activity in these samples to be determined in the absence of endogenous inhibitory activity. Variations in the activity of LTIP in fasted normolipidemic individuals are also compared with in vitro measurements of lipoprotein metabolism in whole plasma.

**Materials**

Glycerol tri[9,10-3H]oleate (26.8 Ci/mmol) was obtained from New England Nuclear, Boston, Mass, and cholesterol[1-14C]oleate (52 mCi/mmol), [4-14C]cholesterol (55 mCi/mmol), and [1α,2α(n)-3H]cholesterol (45.6 to 48.4 Ci/mmol) were purchased from Amersham Corp, Arlington Heights, Ill. [14C]Cholesterol oleate was synthesized from [4-14C]cholesterol and oleoyl chloride (NuChek, Elysian, Minn) as described by Pinter et al. Radiolabeled TGs and CEIs with purities of less than 98% were repurified by thin-layer chromatography.

Bovine serum albumin (fraction V), diethyl p-nitrophenyl phosphate, egg phosphatidylcholine, butylated hydroxytoluene, 3,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), all reagents for salt and buffer solutions, phenyl-Sepharose CL-4B, and dextran sulfate (M r=500 000) were obtained from Sigma Chemical Co, St Louis, Mo. Carboxymethyl cellulose (CM-52) was from Whatman LabSales, Hillsboro, Ore.

**Plasma Collection and Processing**

Human plasma was obtained either from the blood bank of the Cleveland Clinic, the American Red Cross Blood Bank, or normolipidemic volunteers from our research facilities. Informed consent, approved by the Institutional Review Board, was obtained from the latter group. Blood bank plasma contained acid-citrate-dextrose and was processed without removal of these reagents. Blood was collected from local volunteers through venous puncture; EDTA and NaN3 were added after collection to give final concentrations of 4 mmol/L and 0.04%, respectively. Plasma was obtained by centrifugation. Lipoprotein-deficient plasma was prepared by using a modification of the method of Burstein et al. Plasma was adjusted to 0.65% dextran sulfate by the addition of a freshly prepared stock solution (6.5%) and mixed for 30 minutes, after which 2 mol/L MnCl2 was added to yield a 0.2 mol/L solution. After 60 minutes, the sample was centrifuged at 20 000 rpm in a Beckman J20.1 rotor for 30 minutes. Supernatants were collected, adjusted to 1.36% BaCl2 with a 15% stock solution, mixed, and, after 30 minutes, centrifuged as above. All steps were carried out at 4°C. Lipoprotein-deficient plasma (≤15 mL) was dialyzed twice for 24 hours at 4°C against 2 L of a buffer containing 50 mmol/L tris(hydroxymethyl)aminomethane-HCl, 150 mmol/L NaCl, 0.02% NaN3, and 0.01% EDTA, pH 6.85. These samples, either with or without subsequent heat treatment (described below), were filtered (0.45 μm, Millipore, Bedford, Mass) before use in transfer assays. LTP and LTIP activities in lipoprotein-deficient plasma were determined immediately after dialysis; LTIP activities began to decline markedly after an additional 24 hours at 4°C.

**Isolation of LTP and LTIP**

Partially purified LTP was isolated from lipoprotein-deficient human plasma by hydrophobic and ion-exchange chromatography as described. Partially purified LTP was stored at 4°C in 0.27 mmol/L disodium EDTA, pH 7.4. This fraction of LTP was used in all experiments. Partially purified LTIP did not contain detectable lecithin:cholesterol acyltransferase activity and was devoid of phospholipid-specific transfer protein activity.

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 LTIP removal and was then further purified on the ion-exchange column, where it eluted at a lower saline concentration than did LTP. Fractions containing LTIP activity were pooled, dialyzed against 150 mmol/L NaCl and 0.01% EDTA, and concentrated approximately fivefold by ultrafiltration on YM-10 membranes (Amicon, Danvers, Mass). When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, LTIP preparations consisted of a major, broad band migrating at a mean molecular weight of ≈32 000, but they also contained several distinct minor bands of greater molecular weight. A similarly broad band is reported for highly purified LTIP. Based on densitometric analysis of Coomassie Blue–stained gels, more than 90% of the protein was contained within the broad, major band. The LTIP activity in these preparations was resistant to extended incubation with trypsin, much like that reported for LTP. LTIP was inactivated in a time-dependent manner by a combination of trypsin and chymotrypsinogen (100 μg LTIP and 10 μg of each protease, pH 7.4, 37°C); activity was completely lost within 2-hour incubation. LTIP was recovered from isoelectric focusing as a single activity peak at an activation constant of approximately 0.4 μmol/L.
isolectric point (pl) of 3.7 to 3.8. In contrast, LTIP eluted at an apparent pl of 4.4 on chromatofocusing, consistent with that reported by Nishide et al.16

During the purification of LTP and LTIP, lipid transfer activity was routinely assayed by determining the extent of radiolabeled transfer from \( [\text{H}]\text{TG} \) and \( [\text{C}]\text{CE} \)-labeled LDL to unlabeled HDL (10 \( \mu \)g cholesterol each) in a total volume of 0.7 mL.21,24 LTIP activity was assessed by the ability of samples to suppress the activity of a standard amount of partially purified LTP.

**Isolation and Radiolabeling of Lipoproteins**

Human lipoproteins were radiolabeled by a lipid dispersion technique.14 Labeled and unlabeled lipoproteins were isolated at 4°C by sequential ultracentrifugation23 at solvent densities of 1.019, 1.063, and 1.21 g/mL to yield VLDL (+intermediate-density lipoprotein), LDL, and HDL, respectively. For these studies, LDL was routinely subjected to a second, extended centrifugation (48 hours at 50,000 rpm at 4°C) at a solvent density of 1.21 g/mL before use. This step reduced the LTP activity coisolated with the HDL fraction by at least 80%; 10 \( \mu \)g of this HDL typically facilitated less than a 2% transfer to or from LDL in 24 hours. All lipoproteins were extensively dialyzed against 0.9% NaCl, 0.01% EDTA, and 0.02% NaN\(_3\), pH 7.4, and were stored at 4°C. Radiolabeled lipoproteins contained \( 1.4 \times 10^3 \) cpm \( \text{H} \) and from 4.8 to \( 8.3 \times 10^2 \) cpm \( \text{C} \) per microgram cholesterol. Lipoproteins were quantified based on their total cholesterol content.

**Assay of LTP and LTIP Activities**

Lipid transfer assays were performed as described.21,24 In most instances, radiolabeled LDL (donor) and unlabeled acceptor lipoprotein(s) were incubated with or without a source of LTP at 37°C. For these experiments, a typical assay contained 40 \( \mu \)g each of \( [\text{H}]\text{TG} \) and \( [\text{C}]\text{CE} \)-labeled LDL and unlabeled HDL. The assay was terminated by selectively precipitating LDL (donor) by the addition of PO\(_4\)\(^{3-}\) and Mn\(^{2+}\).24 The above conditions define the standard assay procedure. 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 by determining the radiolabel content of the fraction of interest. The fraction of radiolabeled donor lipid that was transferred to an acceptor particle was calculated as described.21 It is reported either as the percent lipid transferred (%kt) or as micrograms of lipid transferred, which was calculated by multiplying the fraction of transferred radiolabeled donor lipid times the mass of the lipid in the donor particle. Radiolabeled lipid “transfer” in the absence of LTP, which was less than 2% in the standard assay, was subtracted before these calculations.

LTIP activity in lipoprotein-deficient plasma was determined by two methods. The suppression of endogenous LTP was measured by following the standard assay protocol and calculated as described in the text. The inhibition of exogenously added LTP was determined by measuring transfer activity in the lipoprotein-deficient plasma both in the absence and the presence of a known amount of partially purified LTP. The transfer activity in the presence of exogenous LTP minus the activity in the absence of added LTP was divided by the activity of the exogenously added LTP alone to determine the extent of inhibition.

In some experiments monoclonal antibodies directed against LTP, designated TP2, were added to suppress human LTP activity. Antibodies were isolated from ascites fluid by chromatography on protein A-agarose (Pierce, Rockford, Ill.). Ascites fluid containing TP2 antibodies was obtained from Dr. Yves Marcel, Montreal, Canada; the properties of this antibody have been reported.3

**Determination of Lipoprotein Molecular Weight**

Freshly isolated plasma from fasted individuals was adjusted to a solvent density of 1.20 g/mL with NaBr and centrifuged at 50,000 rpm in a Beckman 50.3 Ti rotor for 15 hours at 17°C. The top fraction was removed, and aliquots were applied to nondenaturing polyacrylamide gradient gels (Isolab, Inc, Akron, Ohio). Gels were electrophoresed as described,26 stained with colloidal Coomassie Blue G-250 (Gradiolite LDL, Pymont, Australia), and imaged. Thyroglobulin was added as an internal standard to each sample. High-molecular-weight standards (Pharmacia LKB Biotech, Piscataway, NJ) of 669, 440, 232, 140, and 69 \( \times 10^3 \) were used to determine particle molecular weight in a manner analogous to that described for determining particle size.26 The lipoprotein fraction corresponding to HDL\(_{1}\) was identified by its comigration with ultracentrifugally isolated HDL\(_{2}\) (1.063 < d < 1.125 g/mL) and its particle size as previously reported.26

**Analytic Procedures**

Protein was quantified by the method of Lowry et al27 as modified by Peterson28 using bovine serum albumin as standard. Lipoprotein cholesterol content was determined by using Cholesterol HP reagent (Boehringer Mannheim, Indianapolis, Ind), and TG content was measured by using the GPO-Trinder method (Sigma Chemical Co).

**Results**

Inactivation of Inhibitor Activity

Our initial approach focused on the identification of a method that could separate LTP and LTIP activities in lipoprotein-deficient plasma. Although effective in separating these two activities, phenyl-Sepharose was not useful, since the recovery of LTIP activity from these columns is highly variable14,15 and quantitatively low.29 The recovery of LTP activity, and presumably LTP mass, was also variable from this matrix. No other chromatographic method that could capitalize on the known differences between these proteins was deemed feasible because of the high protein content of plasma and the amounts of plasma that would need to be applied to permit activity assays.

The second approach was to quantitatively remove one of these two activities in lipoprotein-deficient plasma. The stability of LTP in response to heating is well recognized.30,31 Although we had previously noted14 that partially purified LTIP is stable to 60°C for 20 minutes, Nishide et al16 observe that LTIP activity in an HDL-like fraction from plasma is rapidly and com-
Lipoprotein-deficient human plasma (●) or partially purified lipid transfer inhibitor protein (LTIP) (□) was incubated at 56°C for the indicated times. Endogenous lipid transfer activity of lipoprotein-deficient plasma (75 μL) was determined in 4.5-hour assays as described in “Methods.” The activity of LTIP (=20 μg) was measured in 1.5-hour assays by determining its ability to suppress exogenous lipid transfer protein (LTP). Unheated LTIP suppressed LTP activity by 40.2%, in the absence of LTIP, cholesteryl ester transfer activity was 12.0%. Data points are the mean±SD of duplicate determinations. When not visible, the error bar is contained within the symbol itself.

Heat inactivation of lipoprotein-deficient plasma gave results that were distinct for a given plasma donor. Both before and after heating, LTP activity was nearly linear with the amount assayed (Fig 2). Among the three plasma samples shown, a more than threefold increase in LTP activity after heating was commonly observed throughout the dose response. For a given donor, the heating effect was reproducible. Assays of plasma samples from a single donor collected at four points over a 2-month period demonstrated that LTP activities in lipoprotein-deficient plasma before and after heating ranged about twofold (Fig 3, top). In LTIP-inactivated samples, a more curvilinear inhibition response, perhaps due to residual effects of the heating procedure (Table 2).

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The variability of LTIP activities among normolipidemic individuals was determined in a group of 8 prescreened subjects (5 men and 3 women). After an overnight fast, this group’s total plasma cholesterol and TGs averaged 157 mg/dL (range, 121 to 187 mg/dL) and 92 mg/dL (range, 67 to 139 mg/dL), respectively. Among these 8 samples, LTIP activity in native lipoprotein-deficient plasma was low; overall, native LTP activities ranged about twofold (Fig 3, top). In LTIP-inactivated samples, lipid transfer activities were markedly elevated for all subjects. The increase in LTP activity ranged from 238% to 605% of native sample values; in 7 of 8 subjects, heating caused more than a 300% increase in measurable CE transfer activity. Similar increases were observed for TG transfer (not shown). The addition of monoclonal antibody against human LTP (TP2) decreased the measured LTP activity in response, it was apparent that more than 90% of the inhibitory activity of lipoprotein-deficient plasma was removed by the heating procedure (Table 2).

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**Table 1. Inactivation of Exogenously Added LTIP in Lipoprotein-Deficient Plasma**

<table>
<thead>
<tr>
<th>Treatment of Lp-Deficient Plasma</th>
<th>LTIP Added</th>
<th>CE Transfer, %kt</th>
<th>Inhibition, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>...</td>
<td>2.97</td>
<td>56.6</td>
</tr>
<tr>
<td>Heated</td>
<td>0</td>
<td>6.85</td>
<td>...</td>
</tr>
<tr>
<td>Heated</td>
<td>43</td>
<td>5.43</td>
<td>20.7</td>
</tr>
<tr>
<td>Heated</td>
<td>86</td>
<td>4.87</td>
<td>28.9</td>
</tr>
<tr>
<td>Heated twice</td>
<td>0</td>
<td>6.84</td>
<td>0.2</td>
</tr>
<tr>
<td>Heated twice</td>
<td>43</td>
<td>6.35</td>
<td>7.3</td>
</tr>
<tr>
<td>Heated twice</td>
<td>86</td>
<td>6.72</td>
<td>1.9</td>
</tr>
</tbody>
</table>

LTIP indicates lipid transfer inhibitor protein; Lp, lipoprotein; CE, cholesteryl ester; and kt, fraction of radiolabeled donor lipid that was transferred. Human lipoprotein-deficient plasma was subjected to the treatments or additions noted and then assayed for lipid transfer activity as described in “Methods.” Heated samples were incubated for 60 minutes at 56°C. Heated samples received the indicated levels of partially purified LTIP and were either assayed directly or subjected to a second, identical heat treatment before assay. Values are the averages of duplicate determinations. These data are representative of three similar experiments.

*Inhibition of lipid transfer protein activity relative to heat-treated lipoprotein-deficient plasma.
defined by the equation $y = \frac{m \times x}{n + x}$, where $m = 115.1$ and was inhibited by an average of 84% by the antibody (not of endogenous lipid transfer activity. LTIP activity, samples. Data were fitted by nonlinear regression to a hyperbola curves were observed for at least three different lipoprotein- inhibition shown for each sample. Very similar dose-response lent amount of lipoprotein-deficient plasma that would elicit the inhibition shown for each sample. Very similar dose-response curves were observed for at least three different lipoprotein-deficient plasma samples, demonstrating that individual plasma samples respond in a dose-dependent fashion analogous to that seen with the plasma pool above. $t_1$ (equivalent inhibitory activity of heated samples divided by the equivalent inhibitor activity of native samples) times 100. Mean±SEM removal of LTIP activity was 90.7±2.3%. heated samples by an average of 89% (Fig 3, top). LTP activity in native lipoprotein-deficient plasma samples was inhibited by an average of 84% by the antibody (not shown). Thus, essentially all of the lipid transfer activity measured in native and heat-inactivated lipoprotein-deficient plasma is due to LTP.

Previous studies\textsuperscript{14,15} as well as our unpublished studies show that the percent inhibition caused by a given level of lipoprotein-deficient plasma or partially purified LTIP is constant for LTP concentrations ranging at least fourfold. These studies indicate that LTIP activity is independent of LTP concentration under standard assay conditions. Thus, the inhibitory activity in plasma samples containing different endogenous levels of LTP can be directly compared by determining the inhibition of endogenous lipid transfer activity. LTIP activity, expressed in units (1 unit=10% inhibition), was calculated as $1 - \left(\frac{nLTA}{hLTA}\right) \times 10$, where nLTA and hLTA are the lipid transfer activities in native (inhibited LTP) samples. Data were fitted by nonlinear regression to a hyperbola. Assay conditions were identical to those used for the samples. Data were fitted by nonlinear regression to a hyperbola defined by the equation $y = \frac{m \times x}{n + x}$, where $m = 115.1$ and $n = 40.1$. This standard curve was used to calculate the equivalent amount of lipoprotein-deficient plasma that would elicit the inhibition shown for each sample. Very similar dose-response curves were observed for at least three different lipoprotein-deficient plasma samples, demonstrating that individual plasma samples respond in a dose-dependent fashion analogous to that seen with the plasma pool above.

Correlation of LTIP Activity With Lipoprotein Metabolism

The effect of variable LTIP activity on lipoprotein metabolism was studied in normolipidemic donors. To simplify the comparison of absolute lipid transfer rates between individuals, a subgroup of 4 male subjects from the 8 individuals described above was selected based on their similar plasma TG and total cholesterol values. For these 4 subjects, the plasma TG and total cholesterol values were 76±14 mg/dL and 160±19 mg/dL, respectively (mean±SD). Fresh, DTNB-inhibited whole plasma from these individuals was supplemented with radiolabeled VLDL, LDL, or HDL and incubated at 37°C to determine the initial rate of individual lipid transfer reactions. As seen in Fig 4, the extent of CE transfer from VLDL to LDL decreased with increasing LTIP activity (slope, $r = 0.10$, $P = 0.0001$). Similarly, lipid transfer from VLDL to HDL also correlated well with this variable ($r = 0.99$, $P = 0.0088$). However, the lipid transfer from VLDL to HDL decreased with a 2.4-fold greater slope than did the VLDL to HDL transfer event (slope, $r = -0.214$ versus $-0.089$, respectively). At lower LTIP levels, the transfer rates for these two reactions were different by twofold, but they were almost equivalent at higher LTIP levels. Very similar results were obtained for TG transfer from VLDL to these lipoproteins (data not shown). Likewise, CE transfer from LDL to VLDL was also markedly dependent on LTIP activity (slope, $r = -0.83$; $r = 0.966$, $P = 0.0342$), whereas lipid transfer from HDL to VLDL was relatively unaffected (slope, $r = 0.162$; $r = 0.861$, $P = 0.1387$) by changes in LTIP activity (Fig 5).

**TABLE 2. Effectiveness of Heating Protocol in Removing Inhibitor Activity in Lipoprotein-Deficient Plasma**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heat</th>
<th>Inhibition of Added LTP, %</th>
<th>Equivalent Inhibitory Activity, μL *</th>
<th>Removal of LTIP Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>98</td>
<td>229</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>26</td>
<td>11.7</td>
<td>94.9</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>79</td>
<td>87.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12</td>
<td>4.7</td>
<td>94.6</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>66</td>
<td>53.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12</td>
<td>4.7</td>
<td>91.3</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>77</td>
<td>80.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>7.4</td>
<td>90.9</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>84</td>
<td>108.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>19</td>
<td>7.9</td>
<td>92.7</td>
</tr>
<tr>
<td>6</td>
<td>−</td>
<td>55</td>
<td>36.6</td>
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<tr>
<td></td>
<td>+</td>
<td>18</td>
<td>7.4</td>
<td>79.8</td>
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</tbody>
</table>

LTIP indicates lipid transfer protein; LTP, lipid transfer inhibitor protein. Lipoprotein-deficient plasma from six separate donors was prepared and assayed for its ability to inhibit exogenously added LTP. Inhibitory activity was determined in both native samples (+) and samples heated for 60 minutes at 56°C (+). Inhibition was calculated as described in "Methods." Values are the mean of duplicate determinations for 75-μL samples. *Equivalent inhibitory activity was determined from an inhibition dose-response curve of lipoprotein-deficient plasma (pool of eight donors) vs the observed percent inhibition of exogenously added LTP. Assay conditions were identical to those used for the samples. Data were fitted by nonlinear regression to a hyperbola defined by the equation $y = \frac{m \times x}{n + x}$, where $m = 115.1$ and $n = 40.1$. This standard curve was used to calculate the equivalent amount of lipoprotein-deficient plasma that would elicit the inhibition shown for each sample. Very similar dose-response curves were observed for at least three different lipoprotein-deficient plasma samples, demonstrating that individual plasma samples respond in a dose-dependent fashion analogous to that seen with the plasma pool above.

**FIG 2.** Line graph showing dose response of lipoprotein-deficient plasma from three blood bank donors before and after heat treatment. Lipoprotein-deficient plasma was assayed for endogenous lipid transfer protein activity either before (open symbols) or after (closed symbols) heating at 56°C for 60 minutes. Open and closed symbols of the same shape are for the same donor. Shown are the cholesteryl ester transfer activities from low-density lipoprotein to high-density lipoprotein (4.5 hours); triglyceride transfer activities (not shown) showed similar trends. Each point represents the mean of duplicate determinations. The data for these three donors are representative of at least six donors assayed in the same manner.

and heat-inactivated (uninhibited LTP) samples, respectively. For the 8 normolipidemic individuals shown in Fig 3, top, LTIP activities ranged between 5.8 and 8.6 units (Fig 3, bottom).
FIG 3. Bar graphs showing variability of lipid transfer inhibitor protein (LTIP) activity in the lipoprotein-deficient (Lp-def) plasma of eight normolipidemic subjects. Plasma was obtained after an overnight fast. Top, Endogenous cholesteryl ester transfer activity was measured as described in "Methods" with 75-μL aliquots of lipoprotein-deficient plasma before and after heating at 56°C for 60 minutes. For these assays, lipoprotein-deficient plasma and all assay components either with or without anti-lipid transfer protein (LTP) monoclonal antibody (TP2, 10 μg) were combined and preincubated at 25°C for 2 hours. Transfer activity was subsequently measured at 37°C for 4.5 hours. Bottom, Units of LTIP activity were calculated from the data in the top panel. 1 Unit = 10% inhibition of endogenous LTP activity. Units were determined by the formula 1 - (transfer activity in native samples/transfer activity in heated samples) x 10. For both panels, each bar shows the mean±SD of duplicate determinations. When not visible, the error bar is contained within the graph bar itself. Subjects A, B, C, G, and H are male.

This apparent preferential suppression, as assessed by the slope, of lipid transfers involving LDL and VLDL was not simply due to the fact that the rate of lipid transfer was greater between these lipoproteins compared with HDL and VLDL. Even the relative inhibition of transfers between LDL and VLDL, calculated as the reduction in transfer rates between the lowest and highest LTIP level divided by the transfer rate at the lowest LTIP level, was 42% to 55% greater for these reactions than for the HDL-VLDL transfer reactions. The lipoprotein specificity of LTIP was further illustrated by contrasting the effect of increased plasma LTIP activity on LDL to VLDL versus LDL to HDL transfers. The LDL to VLDL reaction was reduced by 55% by the overall increment in LTIP content (Fig 4), whereas the LDL to HDL transfer, which had a fourfold higher absolute transfer rate, was inhibited by only 7% over the same LTIP range (not shown).

Correlation of LTIP Activity With Lipoprotein Size

The role of lipid transfer in modulating lipoprotein size and composition has been clearly demonstrated. Since the foregoing data suggest that LTIP alters the LTP-mediated flux of lipid between lipoproteins, we investigated the relation between LTIP levels and lipoprotein particle size. The molecular weights of HDL and LDL were determined by nondenaturing polyacrylamide gradient gel electrophoresis. The modal molecular weight of the HDL₂ fraction of HDL from the 4 subjects in Figs 4 and 5 correlated negatively with the LTIP levels in these subjects (Fig 6). HDL₂ molecular
of these, heat treatment of lipoprotein-deficient plasma with LTIP levels in this limited number of subjects (not
but this increased particle size did not correlate well
nature of LTIP,14,16 it seems most likely that heating
plasma was inactivated by a subsequent heating step.
And finally, LTIP added to lipoprotein-deficient
fraction to suppress exogenously added LTP; this assay
resulted in the nearly complete loss of the ability of this
activity based on several lines of evidence. Most notable
weight was decreased by 8% (18,000 molecular weight)
over this LTIP range. LDL size, on the other hand,
increased slightly (4%) from low to high LTIP levels,
but this increased particle size did not correlate well
with LTIP levels in this limited number of subjects (not
shown).

Discussion
In this study we demonstrated that the endogenous
lipid transfer activity of lipoprotein-deficient plasma can be
markedly and maximally increased when this plasma fraction is preincubated at elevated temperatures (56°C) for at least 30 minutes. The rise in LTIP activity was attributed to the inactivation of LTIP activity based on several lines of evidence. Most notable of these, heat treatment of lipoprotein-deficient plasma resulted in the nearly complete loss of the ability of this fraction to suppress exogenously added LTP; this assay is a hallmark measurement for the presence of inhibitor activity in lipoprotein-deficient plasma.14 As discussed above, existing data strongly support the conclusion that LTIP is responsible for most, if not all, of the inhibitory activity in this plasma fraction. Additionally, the rise in lipid transfer activity that was elicited by heating was completely inhibited by an antibody against LTIP, indicating that endogenous LTP was being expressed more fully. And finally, LTIP added to lipoprotein-deficient plasma was inactivated by a subsequent heating step. Since partially purified LTIP did not show the same heat instability, heat treatment did not appear to cause the denaturation of LTIP per se, but required the presence of other proteins. Given the hydrophobic nature of LTIP,14-16 it seems most likely that heating
induces either the precipitation or denaturation of LTIP
due to its association with other plasma components that are heat labile. Collectively, these data indicate that heat treatment of lipoprotein-deficient plasma facilitates a rise in endogenous LTIP activity that results from the heat-mediated inactivation of LTIP.

Current assays of LTIP are based on its activity. These measurements require the removal of endogenous lipoproteins before the assay. Therefore, quantification of the recovery of plasma LTIP in lipoprotein-depleted plasma will require the development of other methods, such as an immunoassay for LTIP. The data presented, while not determining the recovery of LTIP activity in lipoprotein-deficient plasma, do indicate that this recovery is reproducible. Furthermore, it appears that the recovery of LTIP activity in lipoprotein-deficient plasma prepared by polyanion–divalent cation precipitation, such as that used here, is greater than that obtained by ultracentrifugation.18 Consistent with this finding, we have observed that the binding of LTIP to lipoproteins is disrupted by the use of at least 50 mmol/L MnCl2 (R.E. Morton and J.V. Steinbrunner, unpublished data, 1992), which is fourfold lower than the MnCl2 concentration used to generate lipoprotein-
deficient plasma by the precipitation method used here. In the absence of Mn²⁺, LTIP may be slowly dissociated from plasma lipoproteins by the shear force of ultracentrifugation.

The increase in LTP activity after heat inactivation of LTIP was at least 2.8-fold in all normolipidemic subjects studied, but the magnitude of this rise differed by as much as 2.5-fold among these individuals. Expressed as the extent to which endogenous LTP activity was suppressed by LTIP in these standardized assays, LTP activity among these individuals differed by as much as 50%. Even given the limited number of individuals studied, a negative correlation between LTIP activity and the initial rates of lipid transfer events in whole plasma was observed. Over the range of LTIP activities observed, increasing LTIP activity was associated with a decrease in the rate of CE transfers from VLDL to LDL (61%) and from LDL to VLDL (55%). In contrast, lipid transfers from VLDL to HDL and from HDL to VLDL were much less affected by changes in this activity. On average, the former transfer reactions were suppressed 49% more by this range of LTIP than were the latter transfer reactions. Thus, with this limited study, an association is observed between LTIP activity measurements made on lipoprotein-deficient plasma and the rates of lipid transfer events between lipoproteins in whole plasma.

This study supports our findings (R.E. Morton and D.J. Greene, unpublished data, 1992) that LTIP preferentially suppresses lipid transfer events involving LDL, especially those between LDL and VLDL. We speculate that the action of LTIP could result in the formation of larger, potentially less atherogenic LDL. Such a mechanism may explain the negative correlation of LTIP and HDL₂ molecular weight. Thus, HTIP may act as an inhibitor of LTP in its role in converting HDL₂ to HDL₃.

In summary, we have described a method of suppressing LTP activity in lipoprotein-deficient human plasma that allows both the maximum LTP activity and the effective activity of LTIP to be determined. Variations in LTIP activity among normolipidemic individuals were significant; higher LTIP activities correlated with reduced lipid transfers between all lipoproteins in whole plasma, but transfers between VLDL and LDL were preferentially suppressed.

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References


Determination of lipid transfer inhibitor protein activity in human lipoprotein-deficient plasma.

R E Morton and J V Steinbrunner

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