Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein

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Abstract We have isolated from human plasma a unique subclass of the high density lipoproteins (HDL) which contains a potent lipid transfer inhibitor protein (LTIP) that inhibited cholesteryl ester, triglyceride, and phospholipid transfer mediated by the lipid transfer protein, LTP-I, and phospholipid transfer mediated by the phospholipid transfer protein, LTP-II. This HDL subclass not only inhibited cholesteryl ester transfer from HDL to LDL or VLDL, but also inhibited cholesteryl ester transfer from HDL to HDL. The inhibitor protein was isolated by sequential chromatography of human whole plasma on dextran sulfate-cellulose, phenyl-Sepharose, and chromatofocusing chromatography. Isolated LTIP had the following characteristics: an apparent molecular weight of 29,000 ± 1,000, (n = 10) by sodium dodecyl sulfate gel electrophoresis, and an isoelectric point of 4.6 as determined by chromatofocusing. LTIP remained functional following delipidation with organic solvents. Antibody to LTIP was produced, and an immunoaffinity column of the anti-LTIP was prepared. Passage of human, rat, or pig whole plasma over the anti-LTIP column enhanced cholesteryl ester transfer activity in human (17%), pig (200%), and rat plasma (125%). The HDL subclass containing LTIP was isolated from whole human HDL (d 1.063–1.21 g/ml) by immunoaffinity chromatography. The isolated LTIP-HDL complex was shown to: *i*) contain about 60% protein and 40% lipid, *ii*) have α and pre-β electrophoretic mobility, *iii*) have particle size distribution somewhat smaller than whole HDL, about 100,000 daltons, as determined by gradient gel electrophoresis, and *iv*) contain only a small amount of apoA-I (less than 5%) and a trace amount of apoA-II. Assay of ultracentrifugally obtained lipoprotein fractions revealed that approximately 85% of the total functional LTIP activity was in the d 1.063–1.21 g/ml HDL fraction. Furthermore, immunoblot analysis of whole plasma by nondenaturing gradient gel electrophoresis revealed that LTIP was found predominantly in particles in the size range of HDL. This unique HDL subclass may play an important role in the regulation of plasma lipid transfer and metabolism.—**Nishide, T., J. H. Tollefson, and J. J. Albers.** Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein. *J. Lipid Res.* 1989. **30:** 149–158.

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Most of the plasma cholesteryl esters (CE) are generated on the high density lipoprotein (HDL) fraction by lecithin:cholesterol acyltransferase (LCAT) (1) and are redistributed to very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (2, 3). This redistribution of lipids is mediated by specific lipid transfer proteins, LTP (4, 5). We have previously reported that the cholesteryl ester transfer protein, designated LTP-I, mediates the exchange and net mass transfer of CE, triglycerides (TG), and phospholipids (PL) between the plasma lipoproteins, and that a second transfer protein, designated as LTP-II, has no affinity for neutral lipids (CE, TG), but transfers PL between the plasma lipoproteins (5, 6). We have also reported that pig and rat plasma have very little neutral lipid transfer activity compared to human plasma, but passage of d > 1.21 g/ml plasma of these animals through phenyl-Sepharose reveals significant neutral lipid transfer activity (7). These data suggest that some inhibitory component in rat and pig plasma, most likely a protein, can significantly modulate neutral lipid transfer. In the present study, we have isolated a specific lipid transfer inhibitor protein (LTIP) from human plasma and developed antibody to LTIP, designated anti-LTIP. A unique HDL subclass containing an inhibitor protein was isolated from HDL by anti-LTIP immunoaffinity chromatography. This unique HDL subclass containing the inhibitor protein was characterized with regard to its effects on lipid transfer between the plasma lipoproteins mediated by LTP-I and LTP-II. A preliminary report of this work has been presented (8).

Abbreviations: HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; LTP, lipid transfer protein; LTIP, lipid transfer inhibitor protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids.

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METHODS

All isotopically labeled lipids were purchased from New England Nuclear (Boston, MA). Most column matrices were from Pharmacia (Piscataway, NJ). Dextran sulfate-cellulose was a generous gift from Kaneka Co. (Osaka, Japan). Cyanogen bromide-activated Sepharose 4B used for immunoaffinity purification of goat and rabbit anti-human LTIP was purchased from Sigma (St. Louis, MO). All centrifugation materials were from Beckman Instruments (Palo Alto, CA).

Preparation of labeled lipoprotein substrates

The [^{14}C]cholesteryl ester (CE)-HDL_3, [^{3}H]triglyceride(TG)-HDL_3, and [^{3}H]phosphatidylcholine (PC)-HDL_3 donors were prepared as described previously (4). The approximate specific activities of these labeled lipoproteins were: [^{14}C]cholesteryl ester(CE)-HDL_3: ~2500 dpm/μg CE (assuming an average mol wt of 650 for cholesteryl ester); [^{3}H]triglyceride(TG)-HDL_3: ~18,000 dpm/μg TG; and [^{3}H]phosphatidylcholine(PC)-HDL_3: ~8,000 dpm/μg PL, respectively.

Preparation of human plasma LTP-I and LTP-II

LTP-I and LTP-II were purified from human plasma by sequential chromatography on phenyl-Sepharose, DEAE-Sepharose, CM-cellulose, heparin-Sepharose, and hydroxyapatite as previously described (4, 5). When necessary, the final preparations were further purified by electrophoresis on a 2 M urea polyacrylamide slab gel, and the bands of interest were excised from the gel and recovered by incubating the gel pieces in 0.15 M NaCl/10 mM Tris, pH 7.4, overnight at 4°C, with approximately 70–80% recovery of lipid transfer activity.

Radioassays of lipid transfer activity

All assays were performed at the optimal lipoprotein donor:lipoprotein acceptor ratio (1:5 mass ratio) for each HDL_3 donor and d < 1.063 g/ml acceptor lipoprotein to yield a maximal transfer activity (4). Briefly, labeled HDL_3 donor and unlabeled d < 1.063 g/ml lipoprotein acceptor were incubated with test sample to be assayed. Donor and acceptor lipoproteins were then separated by dextran sulfate-$MgCl_2$ precipitation, and an aliquot of the supernatant was counted in a Packard scintillation counter. Transfer activity is expressed as the percentage of labeled substrate transferred to the d < 1.063 g/ml acceptor lipoprotein relative to a control incubation without added lipid transfer activity. All assays were within the linear range (the assay was linear to approximately 30% transfer of labeled substrate per incubation).

Solid-phase radiolabeled lipid transfer assays. Five ml of whole HDL (d 1.063–1.21 g/ml) was labeled with 50 μCi of [^{14}C]cholesteryl ester and coupled to 10 ml (settled volume) of cyanogen bromide-activated Sepharose 4B as described by Sparks et al. (9). Briefly, solid phase (bound) labeled HDL was incubated with nonlabeled lipoproteins with and without the additions of LTP and LTIP. Following an incubation, the labeled solid phase donor lipoprotein was separated from acceptor lipoproteins by a brief (5 min) low speed centrifugation, and the radioactivity of the supernate was determined.

Radioassay of the lipid transfer inhibitor protein

A source of lipid transfer activity, usually the water-eluted lipid transfer proteins obtained from phenyl-Sepharose (4, 5), was added to the LTP activity assay (see radioassays of lipid transfer activity, above) to produce approximately 10–20% transfer/18 hr incubation. A test sample to be tested for LTIP activity was added to this mixture in varying amounts. Inhibition was calculated as the percent decrease in radiolabel transfer from HDL_3 donor lipoproteins to d < 1.063 g/ml acceptor lipoproteins. Alternatively, the inhibition of cholesteryl ester transfer from HDL_3 donor to either HDL, LDL, or VLDL was assessed.

Chromatography

Dextran sulfate-cellulose chromatography. Fifty ml of fresh whole plasma (ca. 65 mg protein/ml) was applied to a 2.6 × 7 cm column of dextran sulfate-cellulose previously equilibrated with 150 mM NaCl, 10 mM Tris, pH 7.4, buffer (hereafter referred to as Tris-saline buffer). The sample was loaded at a flow rate of approximately 30 ml/hr, then washed with Tris-saline buffer until the absorbance (A_{280}) was less than 0.05. The bound material was eluted with a linear gradient (500 ml total volume) of 150 mM NaCl to 1.0 M NaCl in 10 mM Tris, pH 7.4. The column was regenerated by washing with several column volumes of 2.0 M NaCl, 10 mM Tris, pH 7.4, then re-equilibrated with Tris-saline buffer.

Hydrophobic matrix chromatography. Hydrophobic interaction chromatography was performed using phenyl-Sepharose as described (4). Briefly, the inhibitor fraction obtained from the dextran sulfate-cellulose column was adjusted to 2 M NaCl/10 mM Tris, pH 7.4, then applied to a 1.5 × 5 cm column of phenyl-Sepharose CL-6B equlibrated with 2 M NaCl, 10 mM Tris, pH 7.4, at a flow rate of 20 ml/hr. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.05. The eluant was changed to Tris-saline buffer to lower the ionic strength of the column prior to elution of the inhibitor fraction with distilled water. The column was then washed with a 0–100% ethanol in water gradient.

Chromatofocusing. The inhibitor fraction obtained from phenyl-Sepharose was dialyzed against 25 mM imidazole, pH 7.4, then applied to a 0.9 × 10 cm column of PBE 94 polybuffer exchanger equilibrated with the same buffer at

a flow rate of approximately 10 ml/hr. The pH gradient was generated with 300 ml of polybuffer PBE 74 diluted 1:8 with deionized water. The pH of each fraction was taken and aliquots were dialyzed against Tris-saline buffer then assayed for LTIP activity.

Preparation of LTIP column. Ten mg of partially purified LTIP (through the dextran sulfate-cellulose and phenyl-Sepharose steps) was coupled to 10 ml (settled volume) of cyanogen bromide-activated Sepharose 4-B according to the manufacturers' recommendations.

Preparation of polyclonal anti-LTIP antibody. Two hundred μg of isolated LTIP was used for the initial inoculation of a goat and rabbit, with a booster injection of 200 μg of LTIP administered 3 weeks later. Bleedings were taken 1 week after the booster injection. The whole blood was centrifuged at low speed (3000 rpm × 30 min), and the plasma was applied to the LTIP-Sepharose column described above. Affinity-purified antibody was obtained by elution with 3 M KSCN, followed by immediate dialysis against Tris-saline buffer.

Preparation of anti-LTIP column. Twenty mg of goat anti-human (or 10 mg of rabbit anti-human) LTIP antibody was affinity-purified by passage over the LTIP-Sepharose column described above. The affinity-purified antibody was then coupled to 5 ml of cyanogen bromide-activated Sepharose 4B.

Gradient gel electrophoresis. Gradient gel electrophoresis was performed using precast 4–30% gradient gels (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) including a high molecular weight calibration protein mixture (Pharmacia) for each run. Electrophoresis was carried out in 0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA buffer, pH 8.35, at 10°C, 125 V for 24 hr. Gels were stained overnight with 0.04% Coomassie Brilliant Blue G-250 in 3.5% perchloric acid, destained in 7% acetic acid until the background was clear, and scanned by a laser densitometer (Pharmacia). Particle size was calculated using the LKB 2400 Gelscan XL™ software. Analysis of non-denaturing gradient gels by Western blotting was performed essentially as described (10, 11).

Animal plasma. Fresh plasma from fasted male Sprague-Dawley rats (approximately 250 g each) was obtained from the abdominal aorta under light ether anesthesia. For each experiment, plasma from four to six rats was pooled for subsequent column chromatography. Pig plasma was obtained from a local abattoir (Mel's Meats, Sumner, WA) and was processed within 4 hr. Plasma from two to three pigs was pooled for subsequent analysis.

Other methods. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (12). Agarose gel electrophoresis was from Beckman (Palo Alto, CA). Isoelectric focusing gel electrophoresis (IEF) was performed by a modification of the method of Warnick et al. (13). Amino acid analysis was kindly performed by Dr. H. Charbonneau, R. Wade, and S. Kumar, Dept. of Biochemistry, University of Washington, by PTC-amino acid analysis as previously described (14). Protein mass was estimated by the method of Lowry et al. (15).

RESULTS

Isolation and characterization of LTIP preparation used for immunization

Fifty ml of fresh plasma was passed through the dextran sulfate-cellulose column and washed with Tris-saline buffer until the absorbance at 280 nm was negligible. The bound protein was eluted with a sodium chloride gradient and each fraction was pooled separately and dialyzed against Tris-saline buffer. A fraction that inhibited cholesteryl ester transfer was recovered in the bound fraction and eluted at approximately 250 mM NaCl (**Fig. 1A**). The fraction containing the lipid transfer inhibitor was adjusted to 2 M NaCl, 10 mM Tris, pH 7.4, and applied to phenyl-Sepharose. The column was washed with 2 M NaCl, Tris-saline, water, and finally an ethanol gradient. The LTIP activity was consistently recovered in the water-

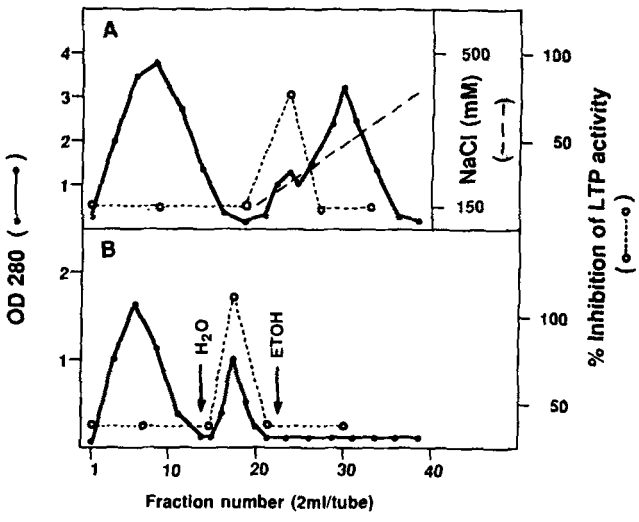

Fig. 1. Elution of LTIP off dextran sulfate-cellulose (top) and phenyl-Sepharose (bottom). Fifty ml of whole plasma was applied to a 2.6 × 7 cm column of dextran sulfate-cellulose previously equilibrated with Tris-saline buffer (top panel) at a flow rate of 30 ml/hr. The column was then washed with Tris-saline buffer until the absorbance (closed circles) at 280 nm was negligible. A linear sodium chloride gradient (150–1000 mM, 10 mM Tris, pH 7.4, 500 ml total volume) was applied to the column, and the indicated fractions were dialyzed and assayed for LTIP activity as described in Methods. LTIP eluted during the first quarter of the gradient. This material was adjusted to 2 M NaCl and applied to a 1.5 × 5 cm column of phenyl-Sepharose (bottom panel) previously equilibrated with 2.0 M NaCl, 10 mM Tris, pH 7.4. The column was washed with Tris-saline buffer until the absorbance at A_{280} was negligible. LTIP was eluted with water as described in Methods (open circles).

eluted material (Fig. 1B); occasionally some inhibitory material could be recovered with the ethanol gradient.

Chromatofocusing of the LTIP fraction obtained from phenyl-Sepharose was carried out on a 0.9 × 10 cm column of PBE 94. Inhibition of lipid transfer was observed in the region of pH 4.6 (**Fig. 2**).

Analysis of the isolated LTIP fraction by SDS-PAGE (7–20% gradient gel) exhibited a major band with an apparent molecular weight of 29,000 ± 1,000 (n = 10), and a slightly slower mobility than apoA-I (**Fig. 3**). In the absence of reducing agents such as β-mercaptoethanol, by electrophoresis of LTIP on SDS-PAGE (7–20%) we occasionally observed two bands, one migrating near 29,000 daltons, and another at 60,000 daltons (data not shown), suggesting that isolated LTIP can form a dimer in the absence of a reducing agent. The estimated molecular weight by gel filtration on Sephadex G-100 in the presence of 8 M urea was 28,000 (range of 26,000 and 30,000; n = 2).

Amino acid analysis. Analysis of four preparations of LTIP revealed a unique amino acid composition as compared to the other known apolipoproteins. LTIP was relatively enriched in serine (10.1 mol%) and isoleucine (5.0 mol%), and relatively deficient in leucine (8.3 mol%), lysine (3.5 mol%), and tyrosine (0.7 mol%).

Preparation and characterization of LTIP antibody

One week after the booster injection of the 29,000- dalton component excised from the SDS-PAGE gel, the goat and rabbit were bled and the antisera was passed over the LTIP-Sepharose column. Affinity-purified LTIP antibody was tested for its immunoreactivity. One μg of the above LTIP preparations was preincubated with varying amounts (5 to 20 μg) of affinity-purified LTIP antibody at 37°C for 1 hr, followed by an 18-hr, 4°C incubation. Immune complexes were removed by low speed centrifugation (3000 rpm, 10 min) before adding aliquots of the supernatant to the LTIP inhibition assay described above. Anti-LTIP blocked the observed LTIP inhibition of CE transfer, and LTIP had no effect on lipid transfer in the presence of 15 μg of anti-LTIP (**Fig. 4**).

The anti-LTIP column was also evaluated for the ability to remove endogenous inhibitors from the plasma of two animals reported to have low levels of CE transfer activity, the rat and the pig (7, 27). Comparing the unfractionated whole plasma versus the flow-through fraction obtained from the anti-LTIP column, we observed a 200% increase in whole plasma CE transfer activity for the pig, and approximately a 125% increase in whole plasma CE transfer activity in the rat. There appears to be significantly less LTIP in normal human plasma, as the CE transfer activity in the post-anti-LTIP fraction increased by an average of only 17% (15% and 19%, n = 2).

Isolation and characterization of an HDL subclass containing LTIP (LTIP-HDL)

Fifty ml of ultracentrifugally separated HDL (d 1.063–1.21 g/ml), dialyzed against Tris-saline buffer, was loaded on the anti-LTIP column and washed extensively with Tris-saline buffer until the absorbance at 280 nm was less than 0.05. The material bound to the column was eluted with 3 M KSCN and immediately dialyzed against Tris-saline buffer. Under these conditions, the anti-LTIP column removed approximately 50% of the inhibitory material bound to HDL. This material isolated from whole HDL was designated LTIP-HDL. The LTIP-HDL complex was analyzed for protein and lipid content, and was shown to be approximately 60% protein, 40% lipid

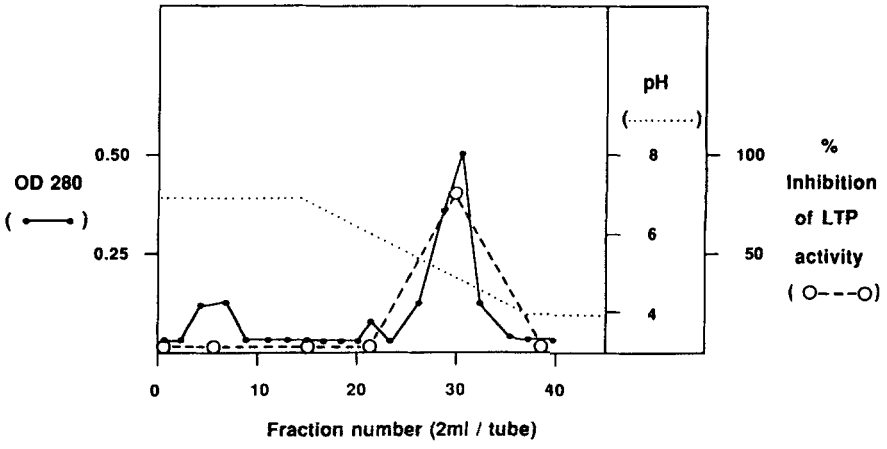

Fig. 2. Chromatofocusing of partially purified LTIP. The inhibitor fraction eluted from the phenyl-Sepharose column was pooled and dialyzed against 25 mM imidazole, pH 7.4, and applied to a 0.9 × 10 cm column of PBE 94 polybuffer exchanger as described in Methods. Following elution, the pH (dotted line) and A_{280} (closed circle) of each fraction was measured. The indicated fractions were then assayed for LTIP activity as described in Methods (open circles).

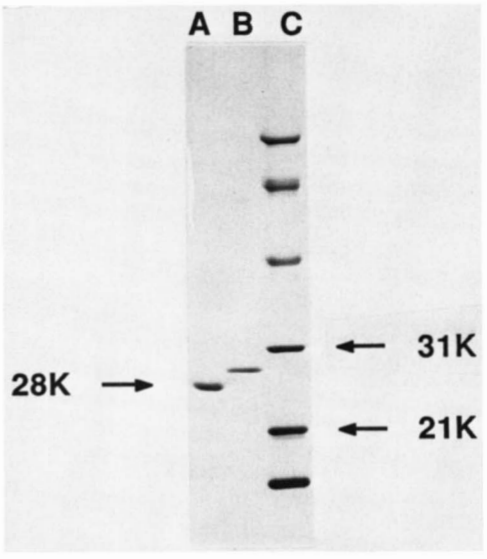

Fig. 3. Sodium dodecyl sulfate gel electrophoresis of isolated LTIP. Aliquots of the inhibitor fraction eluted from the chromatofocusing column were dialyzed against 5 mM ammonium bicarbonate and then lyophilized. Samples were dissolved in 0.0625 M Tris-HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. The samples were heated at 100°C for 2 min, then electrophoresed at 100 V on a gradient gel of 7–20%. The gel was stained overnight in 0.035% Coomassie R250, 25% isopropyl alcohol, 10% acetic acid, and then destained in 25% isopropyl alcohol, 10% acetic acid. Lane A: 10 μg apoA-I; lane B: 5 μg LTIP; lane C: molecular weight standards, from top to bottom: phosphorylase B, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31.5 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

(47.9% PL, 31.3% CE, 12.5% FC, and 8.3% TG, n = 3). The LTIP-HDL complex had only small amounts of apoA-I (less than 5%) and only trace amounts of apoA-II or apoB as determined by specific radioimmunoassays. In contrast, the protein mass of whole HDL contains on the average 66% A-I and 20% A-II. We cannot, however, rule out the possibility that the anti-LTIP column dissociates an LTIP-lipid complex from an LTIP-A-I complex in HDL.

As shown in **Fig. 5,** LTIP-HDL is approximately 100,000 daltons (range 90,000–110,000), somewhat smaller than HDL (150,000–300,000). The electrophoretic mobility of the LTIP-HDL complex was determined next and is shown in **Fig. 6.** When stained for either protein or lipid, LTIP-HDL was a mixture of both α and pre-β particles. In contrast, the starting whole (d 1.063–1.21 g/ml) HDL, and the nonretained fraction recovered from the anti-LTIP column had only α mobility.

As shown in **Fig. 7,** 10 μg of the LTIP-HDL eluted from the anti-LTIP affinity column completely inhibited the cholesteryl ester transfer of partially purified cholesteryl ester transfer protein (through the phenyl-Sepharose step), LTP-I. Fifty percent inhibition was obtained with about 3 μg of LTIP-HDL. Up to 5 μg of whole HDL had no effect on cholesteryl ester transfer activity, while 10 μg of whole HDL inhibited CE transfer by approximately 10%. Complete inhibition of cholesteryl ester transfer activity required about 40 μg of whole HDL. Furthermore, whole HDL that had been passed through the anti-LTIP column three times was less inhibitory after each exposure to the antibody (data not shown). ApoA-I was also tested for the ability to inhibit cholesteryl ester transfer, and, as shown in Fig. 7, pure apoA-I does not appear to inhibit cholesteryl ester transfer. However, we did observe two preparations of apoA-I that did inhibit cholesteryl ester transfer activity (although less than one-tenth as potent as LTIP-HDL). These preparations of apoA-I were most likely contaminated with traces of LTIP, as passage of these apoA-I preparations over the anti-LTIP column removed the observed inhibitory effects of these particular preparations of apoA-I. The effect of the lipid content of LTIP-HDL on inhibition was evaluated by chloroform–methanol 2:1 (v:v) delipidation of either whole LTIP-HDL or lyophilized LTIP-HDL. Following lipid extraction, apoLTIP was reconstituted to its original volume with Tris-saline buffer and assayed for inhibition as described in Methods. LTIP-HDL exhibited similar inhibitory properties before and after delipidation (Fig. 7).

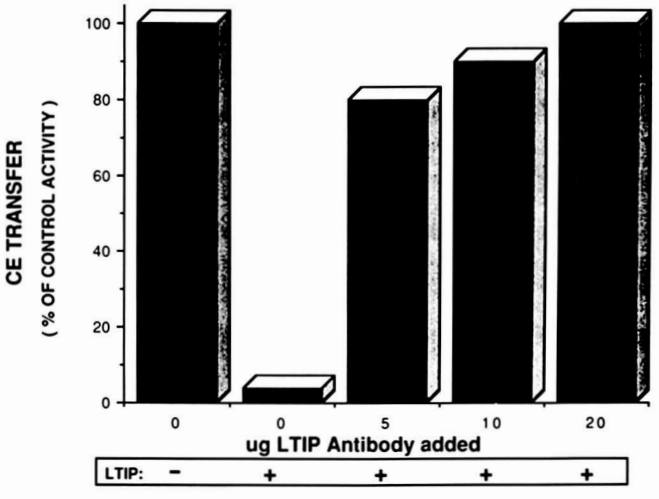

Fig. 4. Effect of goat anti-human LTIP immunoglobulin on LTIP-HDL inhibition of cholesteryl ester transfer activity. LTIP-HDL (5 μg) was incubated for 1 hr at 37°C with 0 to 20 μg of affinity-purified goat anti-human LTIP immunoglobulin, and then the mixture was incubated overnight at 4°C. Immune complexes were pelleted by low speed centrifugation (3000 rpm × 30 min). Aliquots of the supernatant were added to tubes that contained a known amount of cholesteryl ester transfer activity. Results are expressed as a % of a control incubation to which no LTIP was added.

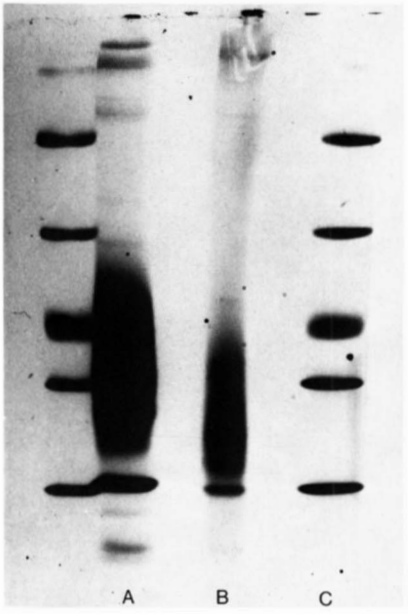

Fig. 5. Nondenaturing gradient gel electrophoresis of LTIP-HDL. LTIP-HDL was isolated from whole (d 1.063–1.21 g/ml) HDL by immunoaffinity chromatography as described in Methods. For comparison, whole HDL (lane A) and calibration proteins (1: thyroglobulin, 669 kDa; 2: apoferritin, 440 kDa; 3: catalase, 232 kDa; 4: lactate dehydrogenase, 140 kDa; 5: bovine albumin, 67 kDa) lane C were included with LTIP-HDL, lane B. Electrophoresis was performed in Tris/borate/EDTA buffer (0.09 M Tris, 0.08 M borate, 0.003 M EDTA, pH 8.35) at 10°C, 125 V for 24 hr. The gel was stained and destained as described in Methods.

LTIP (LTIP-HDL that was further purified by electroelution from an isoelectric focusing gel, then chromatographed on Sephadex G-100 in 8 M urea) was assayed for its ability to inhibit each of the reported lipid transfer activities (cholesteryl ester, triglyceride, and phospholipid) of purified preparations of the plasma lipid transfer proteins, LTP-I, and LTP-II. As shown in **Fig. 8,** 1 μg of LTIP completely inhibited the CE, TG, and PC transfer mediated by LTP-I, with 50% inhibition achieved with approximately 0.25 μg LTIP. LTIP also inhibited the plasma phospholipid transfer protein, LTP-II, but to a lesser extent; 50% inhibition was achieved with 1 μg LTIP, and complete inhibition of LTP-II phospholipid transfer activity required 3 μg of LTIP.

The ability of LTIP-HDL to inhibit intra- as well as inter-lipoprotein lipid transfer was evaluated using solid phase, Sepharose-bound labeled HDL lipoprotein donor, and ultracentrifugally isolated unlabeled VLDL, LDL, and HDL as acceptors. **Fig. 9** shows the observed facilitated transfer rate of solid phase [^{14}C]cholesteryl ester-HDL to HDL (nonbinding fraction from the anti-LTIP column, essentially inhibitor-free HDL), LDL, and VLDL at equal lipoprotein cholesterol concentrations in the absence of added LTIP-HDL. There appears to be a similar rate of cholesteryl ester transfer when each acceptor lipoprotein is assayed at 20 μg total cholesterol. However, at higher levels of acceptor cholesterol concentration, the rate of cholesteryl ester transfer to HDL exceeded that of VLDL and LDL. When the above assays were repeated at 20 μg of acceptor lipoprotein cholesterol in the presence of both LTP-I (transfer protein) and LTIP-HDL (0–10 μg), LTIP-HDL inhibited CE transfer to HDL, LDL, and VLDL, **Fig. 10.** A typical dose-response revealed near-complete inhibition of all lipid transfer, regardless of the lipoprotein pairing, with 7–10 μg of LTIP-HDL, Fig. 10.

The lipid transfer proteins LTP-I, and LTP-II have been shown to be distinct by virtue of their differences in thermal stability (4, 5); LTP-I (cholesteryl ester transfer protein) is relatively stable to a 1-hr, 56°C incubation (less than 10% loss of activity), whereas LTP-II (a phospholipid transfer protein) loses >90% of its activity when exposed to the above 56°C, 1-hr incubation. We examined the thermal stability of LTIP-HDL, and, as shown in **Fig. 11,** most of the inhibitory properties of LTIP-HDL are rapidly lost upon exposure to elevated temperatures.

To estimate the total amount of LTIP-HDL present within whole HDL (d 1.063–1.21 g/ml), 5 mg of whole HDL was passed over the anti-LTIP immunoaffinity column under conditions of antibody excess. After thorough washing of the column with Tris-saline buffer (A_{280} < 0.05), LTIP-HDL was eluted from the column with 3 M KSCN. This material was dialyzed, and protein was determined (15) to quantitate the amount of protein removed

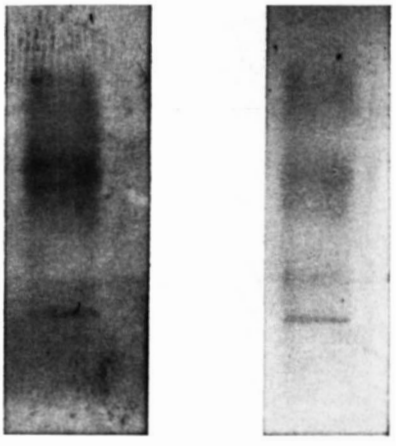

Fig. 6. Electrophoretic mobility of immunoaffinity-isolated LTIP-HDL particles on agarose gel electrophoresis. Aliquots (5 μl) of LTIP-HDL were applied to two separate Paragon™ Lipo Gels (Beckman) and electrophoresis was performed for 25 min. At the conclusion of the run, the gels were stained for lipid with Sudan Red 7B (left panel) or for protein with Coomassie Brilliant Blue G-250 (right panel). The bottom band on each gel represents the origin, the broad band at approximately R_f = 0.5 is the pre-β position, and the top band (R_f approximately 0.8) is the α-migrating material.

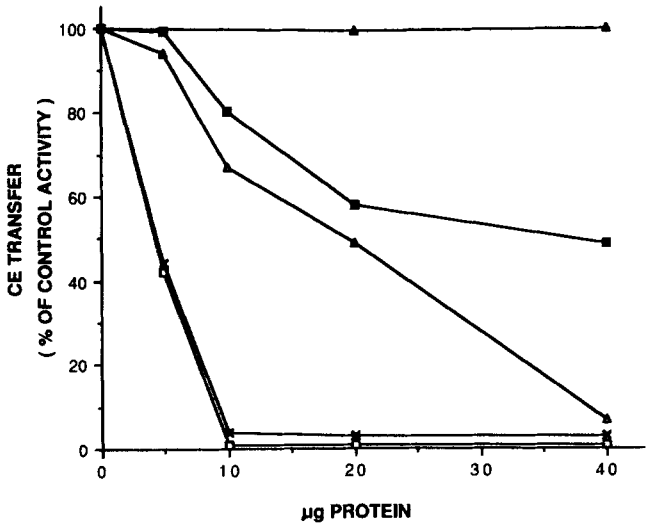

Fig. 7. Inhibition of partially purified cholesteryl ester transfer protein by LTIP-HDL, apoLTIP-HDL, HDL, and apoA-I. LTIP-HDL was isolated from whole HDL by immunoaffinity chromatography as described in Methods, and an aliquot of the isolated LTIP-HDL was delipidated with chloroform-methanol 2:1 (v/v). Aliquots of whole LTIP-HDL (open squares); apoLTIP-HDL (x—x); whole HDL (open triangles); apoA-I (closed squares, closed triangles) were added at the indicated protein concentration to a known amount of active cholesteryl ester transfer protein (~20% transfer/15 µl phenyl-Sepharose pool/18 hr incubation). Note that some preparations of apoA-I were inhibitory; however, when these preparations were passed through the anti-LTIP immunoaffinity column, all inhibitory properties were removed.

by the anti-LTIP column. We observed (n = 5) that LTIP-HDL represents approximately 1% of the total HDL mass (range = 0.8 to 1.2%). The proportion of total plasma LTIP associated with HDL was assessed by two techniques. Firstly, aliquots of fresh normolipidemic plasma were adjusted to d 1.006, 1.019, 1.063, and 1.21 g/ml and ultracentrifuged for 48 hr at 45,000 rpm. The floating lipoprotein fractions plus the d > 1.21 g/ml bottom fraction were harvested by tube slicing, dialyzed, lyophilized, and then delipidated with either chloroform-methanol 2:1 (v/v), or isopropyl ether-butanol 60:40 (v/v). Each fraction was then resuspended in its original starting volume (40 ml) with Tris-saline buffer, pH 7.4. Each fraction was then assayed for functional LTIP activity as described in Methods. There was no detectable LTIP activity in any of the d < 1.063 g/ml lipoprotein fractions. Approximately 85% of the total LTIP was recovered in the d 1.063-1.21 g/ml fraction (HDL), with the remainder found in the d > 1.21 g/ml plasma fraction. These results were corroborated by Western blot analysis with goat anti-LTIP antibody of nondenaturing gradient gels of normolipidemic plasma (**Fig. 12**). Most of the particles recognized by anti-LTIP were of HDL size; however, a small proportion were either large (VLDL/LDL size) or very small (approximately 30 kD). It should be noted, however, that preliminary results with hyperlipidemic plasma suggest that significant amounts of LTIP activity can be recovered from d < 1.030 g/ml plasma, and we observed considerably more apoB-containing particles that were recognized by anti-LTIP during Western blot analysis of hyperlipidemic plasma on nondenaturing gradient gels.

DISCUSSION

Together with LCAT, hepatic and lipoprotein lipase, and the plasma lipid transfer proteins LTP-I and LTP-II, the lipid transfer inhibitor protein, LTIP, may play a key role in the regulation and distribution of lipids in human plasma (8). It is apparent that lipid transfer activity is regulated by the mass of the lipid transfer protein, amount and composition of donor and acceptor lipoproteins, and the amount of LTIP.

The isolated LTIP in the present study has an isoelectric point, as determined by chromatofocusing, of approximately 4.6, with an estimated molecular weight of 28,000 as determined by gel filtration on Sephadex G-100 in the presence of 8 M urea. The estimated molecular weight on SDS-PAGE was 29,000 ± 1,000 (n = 10) in the presence of reducing agents. ApoA-I does not appear to play a role in lipid transfer inhibition. The instances when inhibition of CE transfer by apoA-I was observed could be explained by a minor contamination of the apoA-I preparation with LTIP since passage of these inhibitory preparations of apoA-I over the anti-LTIP affinity column removed the inhibitory component. Whole HDL also inhibited lipid

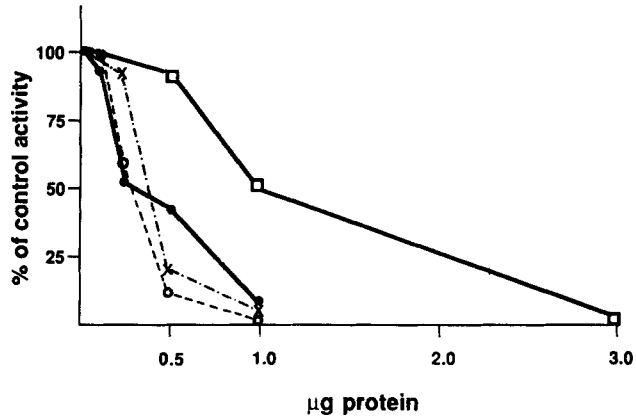

Fig. 8. Inhibition of purified LTP-I (CE, TG, and PC) and LTP-II (PC) lipid transfer by isolated lipid transfer inhibitor protein (LTIP). LTIP was isolated as described in Methods, (LTIP-HDL that was further purified by electroelution from an isoelectric focusing gel, then chromatographed on Sephadex G-100 in 8 M urea) and added at the indicated concentrations to assays of LTP-I-mediated cholesteryl ester (solid circle), triglyceride (open circle), and phospholipid (x---x); and to LTP-II-mediated phospholipid transfer (open squares). Each assay was optimized to yield approximately 20% transfer/incubation in the absence of added LTIP.

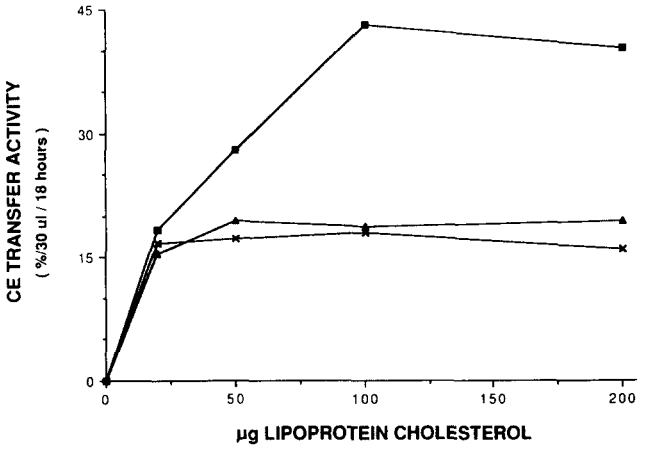

Fig. 9. Transfer of solid-phase HDL cholesteryl esters mediated by LTP-I to soluble VLDL, LDL, and inhibitor-free HDL. Whole (d 1.063–1.21 g/ml) HDL was isolated, radiolabeled, and coupled to Sepharose as described in Methods. Fifty µl of solid-phase [^{14}C]cholesteryl ester-HDL was incubated with 30 µl of partially purified cholesteryl ester transfer protein (phenyl-Sepharose pool, ref. 4) plus the indicated amounts of lipoprotein cholesterol from VLDL (closed triangle), LDL (x—x), and HDL (closed square) that had been exposed to the anti-LTIP Sepharose column.

transfer (Fig. 7) but was 40–80 times less potent (on a protein mass basis) than LTIP. A significant proportion of the observed inhibition of whole HDL could be attributed to the presence of LTIP-HDL.

The mechanism of lipid transfer in unknown, and even less is known about the possible mechanisms of lipid transfer inhibition. It is possible that LTIP-HDL prevents the binding of and/or dissociates the cholesteryl ester transfer protein from the surface of the lipid donor and/or acceptor lipoprotein. Morton (16) has shown that increases in lipid transfer inhibition were correlated with decreased LTP binding to lipoproteins.

Analysis of the LTIP-HDL complex revealed some similarity to whole (d 1.063–1.21 g/ml) HDL. However, LTIP-HDL had a protein and lipid composition and particle size more similar to a dense HDL_3 fraction. One may speculate as to how a particle so deficient in apoA-I (the primary LCAT activator) may acquire near normal amounts of cholesteryl ester. Others have shown with artificial substrates that apoC-I (17) or apoE-2, E-3, and A-IV (18) can activate LCAT. Furthermore, it has been recently demonstrated that significant cholesterol esterification occurs in A-I-free plasma (19). Alternatively, there may be intraplasmic remodeling of these plasma lipoproteins, involving the exchange of surface apolipoproteins. LTIP may displace apoA-I from the surface of more mature, cholesteryl ester-rich HDL particles, thus allowing the displaced apoA-I to bind to other HDL particles.

Unlike HDL, LTIP-HDL contained particles of both α and pre-β mobility on agarose gel electrophoresis (Fig. 6). The basis for this heterogeneity is not known. Kunitake, La Sala, and Kane (20) identified a subpopulation of HDL that had pre-β mobility, yet unlike LTIP-HDL, the protein constituted 90% of the particle's mass, and apoA-I was the predominant apoprotein. It appears that an HDL subclass with properties similar to LTIP-HDL has not yet been described. Koren, McConathy, and Alaupovic (21) reported the isolation of a minor lipoprotein subclass by immunoaffinity chromatography containing apoprotein F. ApoF has an apparent molecular weight by SDS gel electrophoresis of 28,000 (22) similar to LTIP. However, unlike LTIP-HDL, lipoprotein F had β electrophoretic mobility and lipid composition that differed significantly from LTIP-HDL.

It was reported many years ago by Shore and Shore (23) that some isoelectric forms of apoA-I contain up to a mole of isoleucine. We also reported that the apparent isoleucine content of apoA-I increases with decreasing isoelectric point (24). This observation was attributed to an unknown contaminating protein in the A-I preparations. In view of the relatively high content of isoleucine in LTIP, and our finding that LTIP is often found in A-I preparations, we speculate that LTIP is the major source of isoleucine in A-I preparations.

Human LTIP was first reported by Morton and Zilversmit (25), followed by Son and Zilversmit (26). They reported that there may exist several proteins that inhibit lipid transfer activity. The major protein component of their work does have some properties similar to the inhibitor protein of the present study, in that it had an apparent

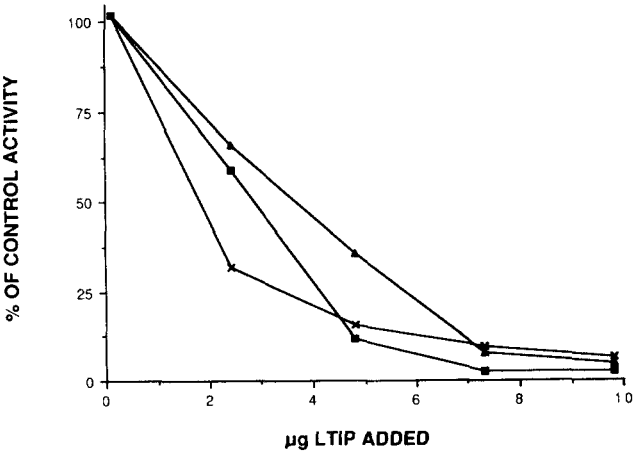

Fig. 10. Inhibition of LTP-I-mediated solid-phase HDL cholesteryl ester transfer to soluble VLDL, LDL, and HDL by LTIP-HDL. Fifty µl of solid-phase [^{14}C]cholesteryl ester HDL was incubated with 20 µg of VLDL (solid triangle), LDL (x—x), or HDL (closed square) cholesterol, plus 30 µl of partially purified cholesteryl ester transfer protein (phenyl-Sepharose pool, ref. 4) to yield approximately 20% transfer/30 µl per 18-hr incubation. Aliquots of LTIP-HDL (0–10 µg) were added to each assay (HDL:HDL; HDL:LDL; HDL:VLDL). Control activity, in the absence of added LTIP-HDL, is expressed as 100%.

molecular mass of 32–35 kDa with an acidic isoelectric point of 3.9–4.3. However, our estimated M_r for LTIP is 29 kDa, with a less acidic isoelectric point of 4.6 versus approximately 4.0. One difference between their study and the current study is the source of starting material for the isolation of the inhibitor. Their isolation of inhibitor began with lipoprotein-deficient plasma. Furthermore, they eluted the inhibitor from phenyl-Sepharose with 5 to 15% ethanol in water. In our laboratory, when d > 1.21 g/ml plasma was used as starting material for inhibitor isolations, our recovery of inhibitor using 5 or 15% ethanol in water was very low. We therefore used a 0–100% ethanol in water gradient which slightly improved recovery of the inhibitor from phenyl-Sepharose. However, when the d > 1.063 g/ml plasma fraction was used as starting material, the recovery of inhibitor off phenyl-Sepharose improved dramatically. Therefore, we selected HDL as our starting material as it is a very rich source of inhibitor, and the specific activity of the starting material is far greater than lipoprotein-free plasma. Additional differences between the current study and earlier reports are: *i*) this study provides immunological and chemical data that distinguishes LTIP from any of the other reported apolipoproteins of HDL; and *ii*) the inhibitory fraction eluted from phenyl-Sepharose was recovered in the water elution and not the ethanol washes.

It has been reported that cholesteryl ester transfer activity differs between certain animal species (7, 27). For example, the rat and pig have been shown to have little cholesteryl ester transfer activity. However, passage of either rat or pig plasma over our anti-LTIP affinity column removes the endogenous inhibitor, and reveals

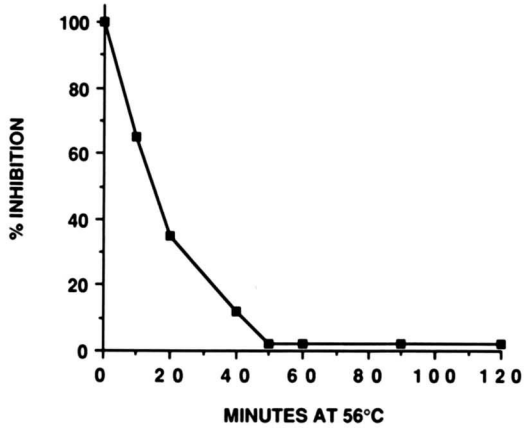

Fig. 11. Thermal stability profile of LTIP-HDL. LTIP-HDL was isolated by immunoaffinity chromatography as described in Methods. Duplicate aliquots (10 μg) of LTIP-HDL were preincubated at 56°C for 0–120 min. Following the preincubation, material at each time point (0, 10, 20, 30, 45, 60, 90, and 120 min) was assayed for inhibition of cholesteryl ester transfer as described in Methods. The nonheated sample (t = 0) completely inhibited all cholesteryl ester transfer.

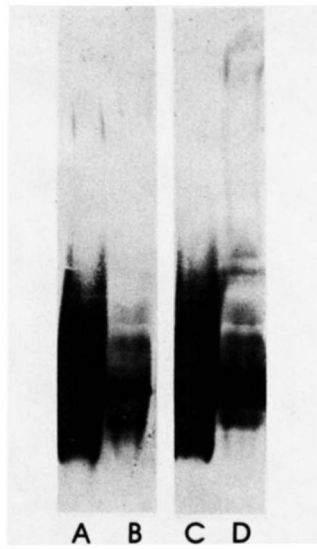

Fig. 12. Western blot analysis of LTIP particles from whole plasma separated by nondenaturing gradient gel electrophoresis. Two μl of fresh whole plasma plus freshly isolated high density lipoprotein was electrophoresed as described in Methods. The gel was then transblotted as described and reacted with goat anti-human LTIP antibody (lanes A and B), or mouse monoclonal anti-A-I (a generous gift from Dr. Linda Curtiss), lanes C and D. The samples are: from left to right, A, HDL; B, whole plasma; C, HDL; and D, whole plasma.

significant cholesteryl ester transfer activity in both species. It is of interest that the rat and pig are mammals with high levels of HDL, and both are relatively resistant to the development of atherosclerosis, given a normal diet. Low lipid transfer activity or high LTIP activity, or both, may be anti-atherogenic agents. LTP activity may contribute to the development of atherosclerosis by facilitating cholesteryl ester transfer of newly synthesized cholesteryl esters to particles that are deposited in tissues ultimately leading to plaque formation (28). In this context, LTIP could decrease lipid transfer activity, and thus be classified as an anti-atherogenic agent. Conversely, the lipid transfer protein, LTP-I, presumably also plays a role in removing cholesteryl esters from HDL for transport to the liver (29–31).

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