Lipid transfer inhibitor protein (apolipoprotein F) concentration in normolipidemic and hyperlipidemic subjects

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Abstract **Lipid transfer inhibitor protein (LTIP) is an important regulator of cholesteryl ester transfer protein function. We report the development of an immunoassay for LTIP and its use to quantify LTIP in plasma of varying lipid contents. A rabbit antibody against bacterially produced recombinant LTIP detected two LTIP isoforms in plasma differing in carbohydrate content. This antibody was used in a competitive, enzyme-linked immunoassay that uses partially purified LTIP bound to microtiter plates. To optimize LTIP immunoreactivity, plasma samples required preincubation in 1% Tween-20 and 0.5% Nonidet P-40. In normolipidemic plasma, LTIP averaged 83.5 μg/ml. LTIP was 31% higher in males than in females. LTIP was positively associated with HDL cholesterol in normolipidemic males but not in females. In hypertriglyceridemic males, LTIP was only 56% of control values, whereas in hypertriglyceridemic females, LTIP tended to increase. Additionally, in males with normal cholesterol and triglyceride (TG) ≤ 200 mg/dl, LTIP varied inversely with plasma TG. Overall, we have confirmed the negative association between plasma TG levels and LTIP previously suggested by a small data set, but now we demonstrate that this effect is seen only in males. The mechanisms underlying this gender-specific response to TG, and why LTIP and HDL levels correlate in males but not in females, remain to be determined.**—Morton, R. E., H. M. Gnizak, D. J. Greene, K-H. Cho, and V. M. Paromov. **Lipid transfer inhibitor protein (apolipoprotein F) concentration in normolipidemic and hyperlipidemic subjects.** ***J. Lipid Res.*** **2008.** 49: **127–135.**

Supplementary key words cholesteryl ester transfer protein • cholesteryl ester transfer protein inhibition • glycoprotein • enzyme-linked immunosorbent assay

In plasma, cholesteryl ester transfer protein (CETP) mediates the net transfer of cholesteryl ester (CE) from LDL and HDL to VLDL in return for triglyceride (TG) (1, 2). Several lines of evidence suggest that lipid transfer inhibitor protein (LTIP), also known as apolipoprotein F (3), alters this process and influences the capacity of CETP to remodel lipoproteins. We have shown that among normolipidemic subjects, LTIP activity levels in whole plasma associate negatively with the rate of lipid transfer between VLDL and LDL (4). The addition of exogenous LTIP to native plasma reduces the participation of LDL in lipid transfer events but leads to a dose-dependent increase in the efflux of CE from HDL to VLDL, resulting in HDLs that are markedly better substrates for lecithin:cholesterol acyltransferase (5). We have proposed that this increased CETP activity on HDL happens because plasma TG, not CETP, is rate-limiting for net lipid transfer in normal plasma (6, 7). Thus, in the presence of LTIP, less VLDL TG is expended by transfer to LDL, allowing the transfer from VLDL to HDL to be increased.

We substantiated the foregoing observations through studies of patients undergoing continuous ambulatory peritoneal dialysis, who have very low LTIP activity (8). In these patients, the 2-fold preference of CETP for HDL as a lipid donor (compared with LDL) seen in normal plasma is absent. In separate studies, we determined that CETP actually displays no preference for total HDL as a substrate in assays with isolated lipoproteins; however, the addition of plasma levels of LTIP to these assays completely reconstitutes the 2-fold preference of CETP for HDL that exists in native plasma (9). These data strongly suggest that the enhanced lipid transfer from HDL in control plasma, which generally has been attributed to a preferential interaction between CETP and HDL, is the consequence of LTIP activity. Recently, we reported that this stimula-

Manuscript received 4 June 2007 and in revised form 25 September 2007.

Published, JLR Papers in Press, September 27, 2007.
DOI 10.1194/jlr.M700258-JLR200

Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; LTIP, lipid transfer inhibitor protein; NANase, *N*-acetylneuraminidase; rLTIP, recombinant lipid transfer inhibitor protein; TG, triglyceride.

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tion of CETP-mediated lipid transfer from HDL by LTIP occurs almost exclusively on HDL_3 particles (10). In aggregate, these data show that LTIP can alter the overall pattern of lipid transfer events between lipoproteins mediated by CETP.

Despite its potentially important role as a regulator of CETP function, the physiological concentration of LTIP in normolipidemic subjects is not known, nor is it known how these levels may vary in various hyperlipidemic states. We previously observed in a small study that LTIP activity is increased in hyperlipidemia and that this increase is mitigated when plasma TG levels are high (11). In this study, we report the development of an immunoassay for LTIP quantification and its application to plasma of varying lipoprotein contents. We have used this assay to further examine the relationship between LTIP and plasma TG levels in a larger, better defined patient population. We observed that LTIP levels, and their change in the presence of hyperlipidemia, are gender-specific.

METHODS

Materials

N-Acetylneuraminidase (NANase) and *O*-glycanase were purchased from Glyko, Inc. (Novato, CA). Human LDL and HDL were isolated by sequential ultracentrifugation (12). LDL was radiolabeled with [^{3}H]CE by a dispersion transfer method (13). CETP and LTIP were partially purified from frozen plasma as described previously (14). CETP and LTIP activities were measured by their ability to stimulate CE transfer from LDL to HDL and to inhibit CETP-mediated CE transfer from LDL to HDL, respectively, as described previously (13, 15, 16).

Expression of recombinant LTIP in bacteria and antibody production

Human LTIP (apolipoprotein F), originally cloned as described and inserted into pCR3.1 (3), was excised with *Eco*RI and the purified fragment was ligated into pGEX-4T-1 (GE Healthcare, Piscataway, NJ). After transformation of SoloPack Gold *Escherichia coli* (Stratagene, La Jolla, CA) with this construct, proper insert orientation was confirmed by *Bam*HI and *Xba*I restriction. For fusion protein production, BL21(DE3)pLysS *E. coli* (Novagen, Madison, WI) was transformed with this construct. Log-phase cells were grown in broth containing ampicillin (100 μg/ml) and 2% glucose. After a 2 h induction with 1 mM isopropylthio-β-galactoside, bacteria were harvested by centrifugation. The cell pellet was resuspended in PBS containing a protease inhibitor cocktail (Roche), sonicated, and incubated for 30 min at 4°C after the addition of 1% Triton X-100. After centrifugation, the supernatant was incubated with glutathione-Sepharose, the matrix was washed extensively with PBS, and then LTIP-GST (for glutathione *S*-transferase) fusion protein was eluted with 50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0. As determined from Coomassie blue-stained electrophoretic gels, three protein bands of similar intensity were present: a band of 44.3 kDa, consistent with intact LTIP-GST fusion protein, and bands at 28.5 and 26 kDa. All three bands were reactive with anti-GST antibody, indicating that the two smaller bands are degradation products, which, because they retain the ability to bind to glutathione-Sepharose, presumably contain GST and only a portion of the LTIP sequence. Polyclonal antibody against LTIP was produced in rabbits using this mixture of LTIP-GST proteins (Biosynthesis, Inc., Lewisville, TX).

Expression of recombinant LTIP in Sf21cells

The purified *Eco*RI fragment of LTIP cDNA, as described above, was ligated into pBlueBacHis2B (Invitrogen). Correct orientation and sequence were confirmed by PCR. This construct was inserted into Bac-N-Blue AcMNPV DNA by homologous recombination, and the resulting recombinant virus was purified by plaque assay and amplified to obtain P3 high-titer stocks. Sf21 cells were seeded into 500 ml spinner flasks and grown to 50% confluence. Cells were infected with recombinant baculovirus and harvested after 4 days. After low-speed centrifugation to remove cells and debris, conditioned medium was stored at −80°C.

Recombinant lipid transfer inhibitor protein (rLTIP) was precipitated from conditioned medium by ammonium sulfate (40% final saturation). After 5 h at 4°C, the medium was centrifuged and the pellet was resuspended in PBS followed by extensive dialysis against PBS. The ammonium sulfate fraction was adjusted to 6 M guanidine-HCl, 10 mM imidazole, and 20 mM β-mercaptoethanol, pH 7.4, and incubated with Ni-NTA (Qiagen, Inc., Valencia, CA) at 25°C for 5 h. The agarose matrix was packed into a column and washed extensively with 8 M urea, 10 mM imidazole, and 20 mM β-mercaptoethanol in PBS. rLTIP was eluted with the same buffer adjusted to contain 100 mM imidazole.

Western blots

Samples containing 63 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.5 mg/ml bromophenyl blue, pH 8.3, were heated for 5 min at 100°C and then applied to 7.5% or 4–20% polyacrylamide gels (Cambrex BioScience, Rockland, ME). Subsequently, proteins were electrotransferred to polyvinylidene fluoride membranes (17), and membranes were blocked with 5% dry milk and 1% calf serum in PBS and then reacted with the indicated antibody in the same buffer. Bound primary antibodies were reacted with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1,000) (Calbiochem, San Diego, CA), and complexes were detected by enhanced chemiluminesence-mediated (GE Healthcare) autoradiography.

LTIP immunoassay (ELISA)

Partially purified LTIP was diluted in 15 mM Na_2CO_3 and 0.02% NaN_3, pH 9.6, binding buffer to yield 6 μg protein/ml. Diluted LTIP (100 μl) was added to each well of 96-well microtiter plates (Nunc Maxisorp; Nalge Nunc International, Rochester, NY) and incubated for 4 h. This and all subsequent steps were performed at room temperature. Wells were washed twice with PBS and blocked with 200 μl of 3% BSA, 2 mM EDTA, and 0.02% NaN_3 in PBS overnight. Samples were diluted and adjusted to contain 1% Tween-20 in PBS or 1% Tween-20 and 0.5% Nonidet P-40 in PBS, as indicated, and incubated overnight. The next day, 200 μl of anti-LTIP antiserum (1:500 dilution in 1% BSA and 0.02% EDTA in PBS) was combined with 200 μl of treated samples, and 100 μl aliquots were added to each of three wells. This resulted in a final detergent concentration of 0.5% Tween-20 and 0.25% Nonidet P-40 (when present). Plates were incubated for 3 h, then washed four times with PBS containing 0.05% Tween-20. Goat anti-rabbit IgG horseradish peroxidase conjugate (1:2,000 dilution in 1% BSA and 0.02% EDTA in PBS) was added (100 μl/well) and reacted for 2 h. After three washes with PBS containing 0.05% Tween-20 and three washes with PBS alone, wells received 100 μl of *o*-phenylenediamine substrate solution (1 mg of *o*-phenylenediamine in 25 mM citric acid and

50 mM Na_2HPO_4 containing 0.03% H_2O_2). Color development was stopped with 50 μl of 10 N H_2SO_4, and the absorbance at 490 nm was read.

Because we have been unable to isolate sufficient pure LTIP from plasma or from cells expressing rLTIP, we developed an alternative strategy to calibrate the ELISA. rLTIP was partially purified (~80% pure as assessed by silver staining of PAGE gels) from baculovirus-infected Sf21 cells as described above. This construct contains a HisG tag epitope identical to that contained in Positope (Invitrogen). Positope is a 53 kDa recombinant protein whose concentration was determined by a standard protein assay (per the manufacture). The molar concentration of rLTIP was calculated from multiple quantitative Western blots with varying concentrations of Positope and rLTIP using anti-HisG monoclonal antibody (Invitrogen). Subsequently, in the same manner but using anti-LTIP Western blots, calibrated rLTIP was used to determine the LTIP content of plasma pooled from four normolipidemic subjects. Using a molecular weight of 17,425 for the protein portion of LTIP (18), a final concentration was assigned. This standard pool was divided into aliquots and stored at −80°C.

Clinical samples

All plasma specimens were obtained from subjects who enrolled in GeneBank, a large (n = ~10,000) and well-characterized tissue repository from subjects undergoing elective diagnostic left heart catheterization. Coronary vessel stenosis was reported on a scale of 0–2, where 0 = normal, 1 = diffuse, moderate (<50% stenosis), and 2 = >50% stenosis for the most affected vessel. In all groups, the vessel stenosis score was greater in males than in females and averaged >1.7, except for normolipidemic (1.20) and hypercholesterolemic (1.42) females. Plasma samples used in this study were from individuals with no reported renal disease, acute hepatitis, or history of cirrhosis and normal creatinine values. Diabetes was an exclusion criterion for hyperlipidemic subjects. Furthermore, normolipidemic subjects taking lipid-lowering medication were excluded to eliminate the possibility that some hyperlipidemic subjects fall in the normolipidemic range as a result of pharmacologic intervention. All GeneBank participants gave written informed consent, and the Institutional Review Board of the Cleveland Clinic approved the study protocol. Plasma was isolated from fasting whole blood collected in EDTA tubes. Samples were maintained at 4°C immediately after phlebotomy, processed within 4 h of blood draw, and stored at −80°C until use. Lipoprotein/lipid measurements were performed as described previously (19). Samples were randomized and assayed for LTIP content as described above and for CETP mass by a commercially available ELISA kit (Wako Diagnostics, Richmond, VA).

RESULTS

Characterization of anti-LTIP antibody

Rabbit anti-LTIP raised against the recombinant bacterial LTIP-GST fusion protein recognized three proteins in plasma, with molecular masses of 133, 34.5, and 29.2 kDa (**Fig. 1A**). Preimmune rabbit serum or mouse anti-GST detected none of these three plasma bands, whereas two bands, of molecular masses consistent with albumin and IgG heavy chain, were detected on all blots. The two lower molecular mass immunoreactive bands in plasma bracketed the single band (31.7 kDa) detected in partially purified LTIP. Upon ultracentrifugation at d = 1.21 to remove plasma lipoproteins, the 133 kDa protein was quantitatively recovered in the lipoprotein-free fraction, whereas the lower immunoreactive bands were recovered in the lipoprotein-containing fraction (Fig. 1B). Although the identity of the 133 kDa band is unknown, it appears unlikely that this band contains LTIP, because this band is not detected with previously described (3) anti-LTIP peptide antibodies (unpublished observations).

LTIP is heavily glycosylated, resulting in an aberrantly high molecular mass on SDS-PAGE gels compared with its core protein of ~17.4 kDa (3, 18). We considered that the two low molecular mass immunoreactive bands in plasma might be LTIP with different carbohydrate con-

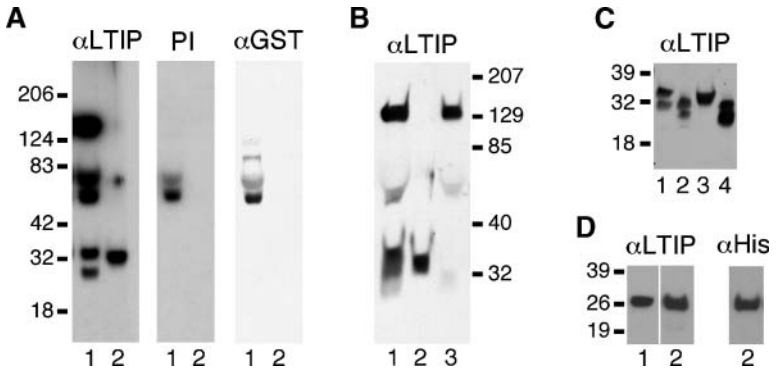

Fig. 1. Characterization of anti-lipid transfer inhibitor protein (LTIP) antibody. A: Western blot of anti-LTIP, preimmune serum (PI), or anti-glutathione *S*-transferase (GST) antibody against human plasma (lane 1) and partially purified LTIP (lane 2). B: Western blot of anti-LTIP reactivity with plasma (lane 1) and the d < 1.21 g/ml (lane 2) and d > 1.21 g/ml (lane 3) density fractions of human plasma. C: Anti-LTIP Western blot of plasma incubated without (lane 1) or with *N*-acetylneuraminidase (NANase) (lane 2) (1 μl of plasma ± 10 mU of NANase) and partially purified LTIP incubated without (lane 3) or with NANase (6 μg of LTIP ± 10 mU of NANase) (lane 4). D: Western blot of partially purified LTIP (lane 1) or recombinant LTIP (lane 2) with anti-LTIP and anti-HisG antibodies. Samples were electrophoresed on 7.5% polyacrylamide gels in A–C and on 4–20% gels in D. LTIP has a lower apparent molecular mass on gradient gels. Values of the molecular mass markers shown are in kDa.

tent. When plasma was treated with NANase to remove sialic acid residues, these two bands persisted but shifted ~3 kDa smaller (Fig. 1C). However, treatment of partially purified LTIP with this enzyme yielded two proteins that comigrated with the bands present in enzyme-treated plasma (Fig. 1C). These results indicate that differences in Western blot band patterns between plasma and partially purified LTIP are accounted for by differences in sialic acid content. We conclude that both low molecular mass immunoreactive bands in plasma are LTIP. Additional proof that the immunoreactive band(s) detected with this antibody are LTIP was obtained with recombinant LTIP isolated from baculovirus-infected Sf21 insect cells. Anti-LTIP reacted with partially purified rLTIP, revealing a molecular mass similar to that for plasma-derived LTIP (Fig. 1D). Identification of this band as rLTIP is based on coreactivity with anti-HisG, the epitope tag in this construct.

With partially purified LTIP, we further examined its carbohydrate content and evaluated the consequences of carbohydrate removal on LTIP activity. When treated with NANase, there was a small but significant ($P < 0.03$) reduction in LTIP activity (**Fig. 2**). Treatment with NANase followed by removal of serine/threonine-linked carbohydrate chains with *O*-glycanase markedly suppressed LTIP activity. Treatment with these two enzymes also resulted in the production of a single LTIP band with a molecular mass of ~20 kDa, slightly larger than the expected size for the protein core of LTIP (Fig. 2, inset). This suggests that the single predicted *N*-glycosylation site at asparagine 103 might be occupied. Although treatment of LTIP with *N*-glycanase had no effect on LTIP molecular mass (data not shown), this negative result is not definitive given that *N*-glycanase does not cleave all asparagine-linked carbohydrates. For LTIP contained in plasma, NANase plus *O*-glycanase resulted in the complete loss of the lower molecular mass form of LTIP and about half of the upper band, concomitant with the appearance of a single 20 kDa product, as seen with partially purified LTIP (data not shown). Complete digestion of the upper band has not been accomplished in the limited studies performed. Like isolated LTIP, plasma LTIP was resistant to *N*-glycanase.

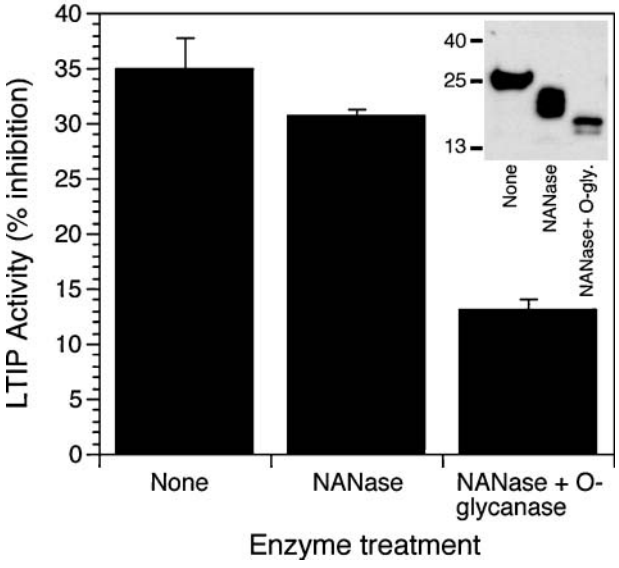

Fig. 2. Effects of enzymatic deglycosylation on LTIP activity. Partially purified LTIP (40 μg) was treated with NANase (20 mU, 1 h at 37°C) or NANase followed by *O*-glycanase (4 mU, 3 h at 37°C), and then LTIP activity was measured in a LDL-to-HDL lipid transfer assay (see Methods). Values are means ± SD of n = 3 (control), n = 4 (NANase), or n = 2 (NANase + *O*-glycanase) replicates. Inset: LTIP Western blot of treated samples. Electrophoresis was performed on 4–20% gradient gels, resulting in lower apparent molecular mass for native LTIP and poor resolution of the two bands produced by NANase treatment compared with Fig. 1.

Development of an LTIP ELISA

For LTIP quantitation by an ELISA, partially purified LTIP was bound to microtiter plates and then reacted with anti-LTIP in the presence or absence of competing soluble antigen. Multiple variables were examined. Among the most critical was establishing conditions for adequate solubilization of LTIP contained in plasma. We examined the effects of Tween-20 (0.1–2.0%), Nonidet P-40 (0.5%), and Triton X-100 (0.1–0.5%) on the reactivity of LTIP in plasma. Overall, Tween-20 was the most effective. However, low Tween-20 concentrations resulted in a flat response at higher plasma concentrations (low dilutions), suggesting that Tween-20 concentrations of <0.5% are not adequate to solubilize plasma LTIP over the entire dose range. Conversely, Tween-20 concentrations of ⩾2% tended to flatten the overall dose-response curve, indicating that antigen-antibody interactions were being compromised. One percent Tween-20 was chosen because it avoided both of these problems. Fresh and frozen/thawed plasma behaved identically in the ELISA, showing that freezing does not measurably alter LTIP immunoreactivity.

The specificity of the ELISA for LTIP is demonstrated in **Fig. 3**. Despite containing different contaminating proteins, parallel response curves were obtained with plasma and multiple chromatographic fractions obtained during LTIP purification. The phenyl-Sepharose unbound fraction, which contained very little LTIP but high levels of the 133 kDa protein by Western blot (Fig. 1B), gave no response in the ELISA.

This configuration of the ELISA has been used in multiple applications to quantify LTIP in plasma and plasma fractions. However, when used to measure LTIP in hyperlipidemic plasma, we observed that this detergent treatment was not adequate when cholesterol or TG concentrations were increased markedly, as shown by the failure of different dilutions to yield similar values for LTIP. We subsequently determined that the addition of 0.5% Nonidet P-40 to the 1% Tween-20 pretreatment step was sufficient to overcome these effects without significantly affecting the shape or slope of the standard curve generated with normolipidemic plasma. When plasma of varying lipid contents was pretreated with this detergent mixture, nearly parallel dose-response curves were observed (**Fig. 4A**). Only when plasma TG levels were very high (400 mg/dl) did a slight shift in the dose-response

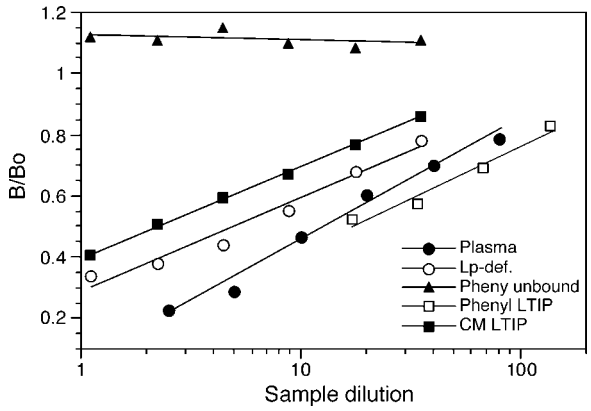

Fig. 3. ELISA for LTIP in plasma and plasma fractions. LTIP was purified from plasma as described in Methods. Then, plasma was chemically delipidated, yielding lipoprotein-deficient (Lp-def.) plasma. Lipoprotein-deficient plasma was applied to phenyl-Sepharose, resulting in an unbound fraction (Phenyl unbound) and an eluted fraction containing LTIP (Phenyl LTIP). This fraction was subsequently applied to carboxymethyl-cellulose, and LTIP was eluted (CM LTIP). Samples were diluted as indicated and assayed for LTIP by ELISA. Values are means of triplicate determinations. B/Bo, where B = binding of anti-LTIP in the presence of sample (competitor) and Bo = binding of anti-LTIP in the absence of competitor.

curve occur. Typically, the ELISA was log-linear for normolipidemic plasma dilutions from 5- to 80-fold, which corresponds to 49–778 ng of LTIP per well (0.97–15.56 μg LTIP/ml). A representative standard curve generated with pooled normolipidemic plasma is shown in Fig. 4B. Under these final conditions, the interassay and intra-assay coefficients of variance were 7.6% and 6.8%, respectively. Typically, unknown plasma samples were diluted 20-fold for analysis.

LTIP in plasma from subjects with normal and high lipid levels

The lipid and lipoprotein levels of study subjects are shown in **Table 1**. Among the four study groups, there were no differences in subject age. Within a study group, male and female groups were comparable in mean lipid and lipoprotein levels except for hypertriglyceridemic subjects, in which group males tended to have lower total cholesterol levels (−12%) and consequently lower LDL (−23%) and HDL (−16%) cholesterol levels. Males with combined hypercholesterolemia and hypertriglyceridemia had a slightly lower mean age compared with females in this group. The use of lipid-lowering drugs was common for hyperlipidemic subjects. Hormone replacement therapy was very low in normolipidemic females (2 of 42) and moderate in hypercholesterolemic females (4 of 12), hypertriglyceridemic females (4 of 10), and those with increased plasma cholesterol and TG (3 of 12).

LTIP values for the study groups described in Table 1 are shown in **Table 2**. Among normolipidemic subjects, males had significantly more LTIP than females. Up to 27% of normolipidemic subjects were diabetic, although diabetes did not appear to have an effect on LTIP levels. LTIP levels in nondiabetic versus diabetic normolipidemic females were 73.8 versus 70.2 μg/ml, and in males these values were 94.3 versus 98.6 μg/ml. The trend for higher LTIP levels in normolipidemic males was also seen in hypercholesterolemic subjects, but this difference did not quite reach significance ($P = 0.06$). In hypertriglyceridemic subjects, the converse was observed. Male hypertriglyceridemic subjects had markedly reduced LTIP content, equivalent to only 56% of male normolipidemic subjects. On the other hand, LTIP levels tended to increase in females with high TG levels compared with normolipidemic females, but these did not reach significance ($P = 0.065$). LTIP levels in male versus female hypertriglyceridemic subjects were highly significantly different. Within a lipid phenotype, we did not observe any significant differences in LTIP values between those taking versus not taking lipid-lowering medications. In contrast to the observations for LTIP, there were no differences in CETP levels for males versus females in any lipid phenotype group or between lipid phenotypes. CETP tended to be higher in groups with high cholesterol, but this did not reach significance.

In addition to normolipidemic subjects and hypertriglyceridemic subjects (TG > 200 mg/dl), we also examined whether the changes in LTIP noted between these two groups also existed in individuals with mildly increased TG levels (150 < TG < 200 mg/dl). In males in whom

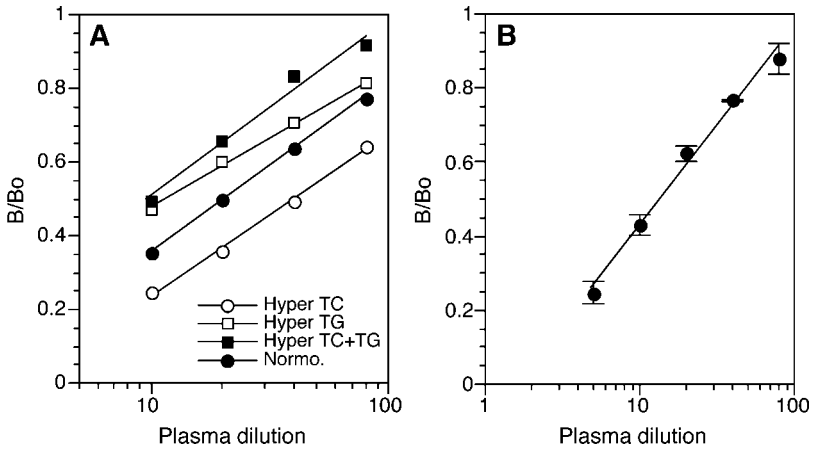

Fig. 4. ELISA response to plasma LTIP in samples treated with Tween-20 and Nonidet P-40. A: LTIP dose-response curves for plasma from hypercholesterolemic (hyper TC; plasma cholesterol = 223 mg/dl), hypertriglyceridemic [hyper TG; plasma triglyceride (TG) = 400 mg/dl], combined hypercholesterolemic/hypertriglyceridemic (hyper TC+TG; cholesterol = 203 mg/dl, TG = 212 mg/dl), and normolipidemic (normo.) subjects. B: A representative standard curve generated with the normolipidemic standard plasma pool. Values are means ± SD (n = 3). B/Bo, where B = binding of anti-LTIP in the presence of sample (competitor) and Bo = binding of anti-LTIP in the absence of competitor.

TABLE 1. Patient population statistics

Lipid Phenotype	Group	No.	Age	TC	TG	HDL Cholesterol	LDL Cholesterol	Percentage L-l Rx[a]	Percentage Diabetic[b]
			years	*mg/dl*					
Normolipidemic	Total	76	65.1 ± 1.5	160 ± 3	93 ± 3	44.8 ± 1.4	96.7 ± 2.7	0	21
	Female	42	64.0 ± 2.1	165 ± 4	91 ± 4	46.0 ± 2.1	100.7 ± 3.2	0	17
	Male	34	66.5 ± 2.1	154 ± 5	94 ± 5	43.4 ± 1.8	91.7 ± 4.4	0	27
Hypercholesterolemic	Total	25	64.1 ± 2.6	228 ± 5	127 ± 10	52.0 ± 3.2	150.3 ± 5.1	28	0
	Female	12	67.4 ± 4.3	225 ± 5	128 ± 14	56.9 ± 4.9	142.2 ± 6.1	50	0
	Male	13	61.2 ± 3.1	230 ± 8	126 ± 15	47.5 ± 3.9	157.7 ± 7.6	8	0
Hypertriglyceridemic	Total	25	62.0 ± 2.1	168 ± 4	301 ± 18	36.0 ± 1.9	78.3 ± 3.4	84	0
	Female	10	62.8 ± 3.2	181 ± 6	285 ± 20	41.8 ± 2.8	86.1 ± 4.4	80	0
	Male	15	61.4 ± 2.9	160 ± 4[c]	311 ± 27	32.1 ± 2.0[c]	72.4 ± 4.3[d]	87	0
Combined hypercholesterolemic + hypertriglyceridemic	Total	25	64.7 ± 2.4	248 ± 8	357 ± 36	39.4 ± 1.8	150.2 ± 8.0	44	0
	Female	12	70.0 ± 3.3	252 ± 11	403 ± 64	42.0 ± 2.8	143.9 ± 9.9	58	0
	Male	13	60.2 ± 3.0[d]	244 ± 13	315 ± 35	37.1 ± 2.3	154.8 ± 11.9	31	0

TC, total cholesterol; TG, triglyceride. Patients were categorized as follows: normolipidemic, TC ⩽ 200 mg/dl, TG ⩽ 150 mg/dl; hypercholesterolemic, TC > 200 mg/dl, TG ⩽ 200 mg/dl; hypertriglyceridemic, TC ⩽ 200 mg/dl, TG > 200 mg/dl; combined hypercholesterolemic + hypertriglyceridemic, TC > 200 mg/dl, TG > 200 mg/dl. Values are means ± SEM.

[a] L-l Rx, percentage of study subjects taking lipid-lowering medications.

[b] Percentage of study subjects who are diabetic. All such subjects were actively taking insulin and/or oral hypoglycemic medications.

[c] $P < 0.01$ (male vs. female of the same lipid phenotype).

[d] $P < 0.05$ (male vs. female of the same lipid phenotype).

hypertriglyceridemia resulted in markedly reduced LTIP levels, there was a tendency toward reduced LTIP levels in mildly hypertriglyceridemic subjects (**Fig. 5**), although this trend was not significant. In contrast, like that seen in female hypertriglyceridemic subjects, even a modest increase in plasma TG in females was associated with higher LTIP levels ($P = 0.038$) (Fig. 5). When data for all females with increased TG were combined (i.e., those with TG > 150 mg/dl), there was a highly significant increase in LTIP levels compared with normolipidemic females ($P < 0.01$). Together, these data indicate that increased plasma TG levels result in marked, but opposite, effects on LTIP levels in male and female subjects.

Within a given lipid group, correlation analysis did not reveal any statistically significant associations between LTIP levels and subject age, plasma cholesterol or TG concentration, LDL or HDL cholesterol level, or the ratio of LDL to HDL. When male and female subjects were evaluated separately, the only demonstrable correlation was between LTIP and HDL cholesterol levels in male subjects with normal lipid levels (**Fig. 6**). HDL levels were positively correlated with LTIP levels. As would be expected, CETP levels were significantly and positively associated with plasma cholesterol levels in normolipidemic ($r = 0.311$, $P = 0.007$) and in combined normolipidemic/hypercholesterolemic ($r = 0.264$, $P = 0.008$) groups.

DISCUSSION

We report here the development of a quantitative assay for LTIP in human plasma that uses partially purified LTIP bound to the solid phase. By Western blot, we observed that LTIP exists as two molecular mass forms. Removal of sialic acids shifted both bands to lower molecular mass, whereas *O*-glycanase treatment converted these two forms to a single protein band still larger than that predicted for carbohydrate-free LTIP. Differences in the band pattern between plasma LTIP and partially purified LTIP could be explained by variances in sialic acid content, suggesting

TABLE 2. LTIP and CETP levels in the study populations

Lipid Phenotype	LTIP			CETP
	All Subjects	Male	Female	All Subjects
		μg/ml		
Normolipidemic	83.5 ± 4.8	96.1 ± 8.2[a]	73.2 ± 5.1	1.71 ± 0.06
Hypercholesterolemic	89.5 ± 7.6	102.8 ± 10.7	75.0 ± 9.3	1.87 ± 0.10
Hypertriglyceridemic	70.0 ± 6.3	53.4 ± 4.8[b,c]	94.9 ± 9.8	1.55 ± 0.12
Combined hypercholesterolemic + hypertriglyceridemic	79.1 ± 8.4	83.7 ± 14.9	74.1 ± 7.6	1.99 ± 0.24

CETP, cholesteryl ester transfer protein; LTIP, lipid transfer inhibitor protein. Patients were categorized into lipid phenotypes as described in Table 1. Plasma was diluted 1:20 into buffer containing 1% Tween-20 and 0.5% Nonidet P-40 and incubated overnight, and then LTIP was quantified by ELISA. Plasma CETP was measured by a commercially available immunoassay. Values are means ± SEM. Group sizes are given in Table 1.

[a] $P < 0.02$ (normolipidemic males vs. females).

[b] $P < 0.0001$ (hypertriglyceridemic vs. normolipidemic males).

[c] $P < 0.0005$ (hypertriglyceridemic males vs. females).

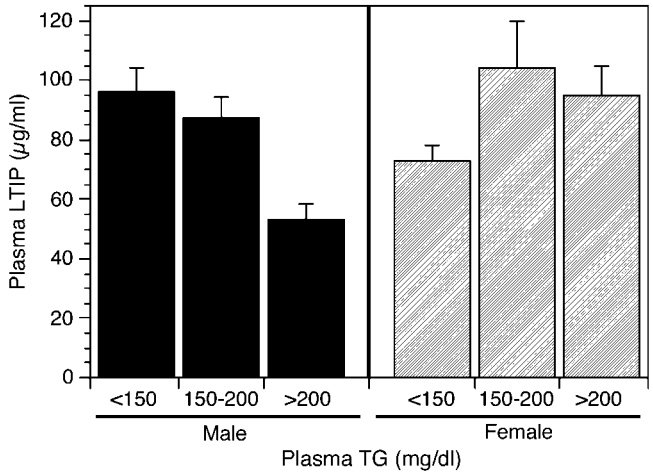

Fig. 5. Variation in LTIP mass as a function of plasma TG. LTIP levels are shown for normocholesterolemic subjects with normal (<150 mg/dl), moderately increased (150–200 mg/dl), and high (>200 mg/dl) TG. Values are means ± SEM. Numbers of male and female subjects with moderately increased TG were 16 and 7, respectively. Other group sizes are shown in Table 1.

that there is a loss of some sialic acid during purification. Enzymatic removal of sialic acid from LTIP resulted in a very small change in LTIP activity, suggesting that the conformation of LTIP is not altered significantly by the removal of these charged carbohydrate groups, and further indicating that the immunoreactivity of partially purified LTIP and native plasma LTIP is not likely to be different. Thus, like CETP, variable glycosylation results in the presence of two plasma forms of LTIP. Unlike CETP, in which this variation is accounted for by the presence or absence of *N*-linked carbohydrate on asparagine 341 (20), LTIP forms arise from variable *O*-linked carbohydrate content. Variable glycosylation of CETP alters its specific activity; whether this also occurs with LTIP is unknown.

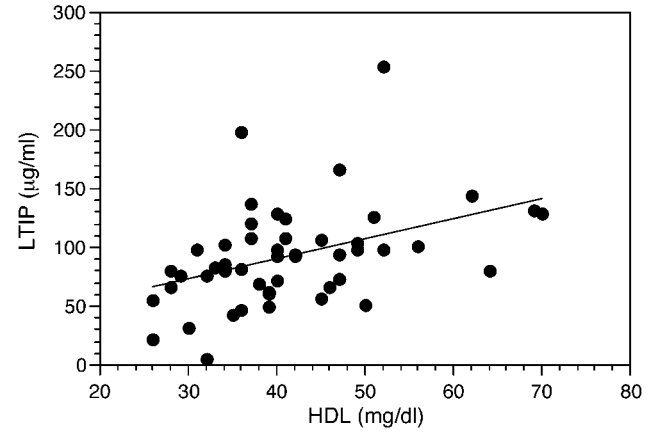

Fig. 6. Correlation of plasma LTIP levels in normocholesterolemic males with HDL cholesterol levels. Data are for males with normal cholesterol levels and TG < 200 mg/dl (i.e., normal + moderately increased TG). $r = 0.422$, $P = 0.0023$.

The presence of two LTIP-sized bands in plasma was surprising because we previously observed a single band (11), which comigrated with the lower of the two bands detected here, when blots were developed with antibodies raised against LTIP peptides (designated F1 and F2) (3). This is likely explained by the presence of carbohydrate chains on these peptide sequences in natural LTIP, which reduces antibody recognition of these peptides. For example, the sequence for peptide antibody F1 contains two of three predicted *O*-linked mucin-type carbohydrate sites, and the sequence for peptide antibody F2 contains the sole predicted *N*-linked carbohydrate site. We suggest that the upper LTIP band in plasma, which appears to be the most extensively glycosylated form based on our enzymatic studies, is a poor substrate for these peptide antibodies.

We determined here that LTIP levels in normolipidemic subjects are similar to the 70 μg/ml estimate we calculated previously (3), but considerably higher than the 27 μg/ml value estimated by others using an electroimmunoassay (21). To calibrate the ELISA, we used an alternative approach that is based on comparative immunoreactivities of insect cell-derived rLTIP with a known protein containing the same epitope tag and, subsequently, of this calibrated rLTIP standard with a plasma pool. This approach was taken because of our inability to produce sufficient pure LTIP or rLTIP that could have served as a primary standard. However, we believe this approach to be valid for several reasons. First, rLTIP and the reference protein, Positope, contain the identical HisG epitope that is recognized by a commercially available monoclonal antibody. Both proteins also contain a second tag, Xpress, and Western blots with anti-Xpress monoclonal antibody reported a very similar quantitative relationship between these two proteins. Second, although insect-derived rLTIP lacks sialic acid residues, removal of these groups from plasma LTIP did not alter its immunoreactivity and had little effect on its activity. However, other glycosylation of rLTIP, which dramatically alters LTIP immunoreactivity and activity, appears intact because this rLTIP displays the same anomalous behavior as plasma LTIP by 7.5% SDS-PAGE (i.e., shows an apparent molecular mass ~10–12 kDa larger than expected) and its inhibitory specific activity is similar to that of plasma-derived LTIP (data not shown).

Little is known about the variability of LTIP levels in normolipidemic and hyperlipidemic individuals. Previously, we observed in a small group of hyperlipidemic subjects (all hypercholesterolemic, some hypertriglyceridemic) that LTIP activity is increased by 3-fold over that in normolipidemic subjects (11). Additionally, within this group, there was a strong negative correlation between LTIP activity and plasma TG levels. These trends were substantiated by changes in LTIP immunoreactivity, as detected on Western blots of plasma using an anti-LTIP peptide antiserum (11). Here, we have reexamined these relationships in a larger number of subjects with more discrete lipid abnormalities.

In normolipidemic subjects, LTIP averaged 83.5 μg/ml, indicating that LTIP (apolipoprotein F) has a plasma con-

centration similar to that of apolipoproteins C-I, C-III, D, and E (22). We report for the first time that LTIP mass is higher in male versus female normolipidemic subjects. Whether these differences exist in a younger adult population remains to be examined. Among normolipidemic males, we observed a significant positive correlation between LTIP levels and HDL cholesterol. This association is consistent with the proposed role of LTIP in regulating lipid flux among lipoproteins in normolipidemic subjects. That is, not only does high LTIP reduce lipid transfer to LDL, it also inhibits CE transfer from HDL_2, which could result in a net increase in HDL cholesterol levels even though CE transfer out of HDL_3 is stimulated (9, 10).

Our previously reported marked increase in LTIP in hypercholesterolemic subjects (11) was not substantiated here in hypercholesterolemic subjects with or without hypertriglyceridemia. A possible explanation is that in the previous study, plasma cholesterol levels in the study subjects ranged from 203 to 314 mg/dl, whereas here the range was only from 201 to 238 mg/dl. Thus, with this mild hypercholesterolemia, changes in LTIP may be too small to detect. Alternatively, because we did not find a correlation between LTIP and plasma cholesterol levels in the earlier study, it is possible that increased LTIP levels occur only at higher plasma cholesterol levels. The fact that we also failed to see an increase in CETP in hypercholesterolemic subjects, as has been commonly reported, is consistent with the initial suggestion above that the magnitude of hypercholesterolemia in this study group may simply be too low to detect significant effects on LTIP or CETP.

In contrast to the foregoing, we did observe a correlation between LTIP and plasma TG levels, as noted before (11), but the effect was unique for each gender. In males, we found that hypertriglyceridemia was associated with a marked reduction in LTIP levels. Among normolipidemic and hypertriglyceridemic male subjects, there was a significant negative correlation between LTIP and plasma TG ($r = -0.406$, $P = 0.005$). We previously suggested that this negative correlation may be attributable to the redistribution of LTIP from LDL to VLDL, resulting in increased plasma clearance of LTIP when TG levels are increased (11). Contrary to that found in males, we were surprised to find in females that LTIP levels tended to increase in hypertriglyceridemia. Even females with moderate increases in TG showed increased LTIP. The mechanisms underlying this gender difference are unclear, but it does not appear to result from an effect of hormone replacement therapy in this older female population, because only a minority of subjects received such treatment and there was no difference between LTIP levels in hypertriglyceridemic females receiving versus not receiving therapy.

In conclusion, we show that LTIP levels are different in normolipidemic males and females. We also provide strong evidence of an association between LTIP levels and plasma TG levels, suggesting a link with VLDL metabolism. Notably, this correlation of LTIP and plasma TG was opposite for males and females. We also show that CETP and LTIP have different responses to changes in plasma lipid levels, resulting in unique changes in the LTIP/CETP ratio in various dyslipidemic conditions, especially hypertriglyceridemia. As suggested by in vitro studies (10, 23), changes in the relative amounts of CETP and LTIP will likely have marked effects on the flux of lipids between lipoproteins. Defining the consequences of altered LTIP/CETP ratios on lipoprotein composition and metabolism is important, especially as we anticipate the development of pharmacologic approaches to decrease levels of functional CETP.

This research was supported by Grant HL-60934 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

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