Molecular cloning of hamster lipid transfer inhibitor protein (apolipoprotein F) and regulation of its expression by hyperlipidemia

Lahoucine Izem and Richard E. Morton[1]

Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

Abstract **Lipid transfer inhibitor protein (LTIP) is a regulator of cholesteryl ester transfer protein (CETP) function. Factors affecting plasma LTIP levels are poorly understood. In humans, plasma LTIP is elevated in hypercholesterolemia. To define possible mechanisms by which hyperlipidemia modifies LTIP, we investigated the effects of hypercholesterolemic diets on plasma LTIP and mRNA levels in experimental animals. The hamster, which naturally expresses CETP, was shown to express LTIP. Hamster LTIP mRNA, exclusively detected in the liver, defined a predicted LTIP protein that is 69% homologous to human, with an isoelectric point of 4.15 and Mr = ~16.4 kDa. Hyperlipidemia induced by feeding hydrogenated coconut oil, cholesterol, or both lipids increased plasma LTIP mass up to 2.5-fold, with LTIP mass correlating strongly with plasma cholesterol levels. CETP mass was similarly affected by these diets. In contrast, these diets reduced LTIP hepatic mRNA levels by >50%, whereas CETP mRNA was increased. Similar results for both CETP and LTIP were also observed in cholesterol-fed rabbits. In conclusion, we report in hamster and rabbit that dietary lipids regulate LTIP. Diet-induced hypercholesterolemia markedly increased plasma LTIP mass while concomitantly depressing LTIP gene expression. CETP and LTIP have distinct responses to dietary lipids.—**Izem, L., and R. E. Morton. **Molecular cloning of hamster lipid transfer inhibitor protein (apolipoprotein F) and regulation of its expression by hyperlipidemia. *J. Lipid Res.* 2009.** 50: **676–684.**

Supplementary key words rabbit • cholesterol • hydrogenated coconut oil

Cholesteryl ester transfer protein (CETP) is an important regulator of lipoprotein composition, and its activity affects plasma lipoprotein levels (1–3). Lipid transfer inhibitor protein (LTIP), also known as apolipoprotein F (apoF), impacts CETP activity in a unique way compared with other factors that have been proposed to regulate CETP activity. Unlike general inhibitors of CETP activity, LTIP tailors CETP-mediated transfer events, inhibiting some and promoting others (4, 5). We have proposed that the balance of CETP and LTIP activities is important in defining the flux of CETP substrates, cholesteryl ester, and triglyceride, between lipoproteins (5–7).

Factors affecting plasma levels of LTIP are poorly understood. In several human studies, LTIP levels were shown to correlate negatively with triglyceride levels (6, 8), although this correlation appears to occur only in males (6). However, LTIP levels are elevated in hypercholesterolemia (8). In comparison, CETP levels are increased in hypercholesterolemic subjects and typically unchanged in hypertriglyceridemic subjects (9–11). In cholesterol-fed animals, increased plasma CETP levels are accompanied by marked increases in hepatic and extrahepatic CETP mRNA (12–15), but it is not known how cholesterol feeding affects plasma levels of LTIP or LTIP gene expression.

To understand the changes in plasma LTIP that are observed in various hyperlipidemic patient populations, we have investigated the response of LTIP in cultured cells treated with nuclear hormone agonist and in animals subjected to diet-induced hyperlipidemia. Selection of a suitable experimental model was initially complicated because the two common laboratory animals in which CETP expression has been studied, hamster and rabbit (12, 13), were not known to express LTIP. However, we report here that hamsters synthesize an LTIP (apoF) homolog, and the recently reported rabbit genome sequence data substantiates the presence of the LTIP gene in this species as well. Most studies were performed in hamsters, where animals were fed diets enriched in saturated fat and/or cholesterol to assess acute (3 day) and chronic (30 day) effects of these lipids on plasma lipoproteins, LTIP levels, and LTIP gene expression. We report that LTIP and CETP respond uniquely to these diet challenges.

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Abbreviations: apoF, apolipoprotein F; CETP, cholesteryl ester transfer protein; HCO, hydrogenated coconut oil; LTIP, lipid transfer inhibitor protein; LXR, liver X receptor.

[1] To whom correspondence should be addressed.
e-mail: mortonr@ccf.org

MATERIALS AND METHODS

Cell culture

C3A cells, a human hepatocellular carcinoma cell line (American Type Culture Collection, Manassas, VA), were grown to confluence in MEM containing 10% FBS. For experiments, cells were washed with serum-free media and incubated for 24 h in media containing 5% human lipoprotein-deficient serum, 10 μM retinoic acid, and the indicated agonist. mRNA levels were determined by real-time PCR using commercially available primer sets (SuperArray Bioscience Corp., Frederick, MD) for LTIP (apoF, PPH02612A) and GAPDH (PPH00150A). To determine mRNA turnover, 1 μg/ml actinomycin D was added to cells following agonist treatment, and the decline in LTIP mRNA over time was determined. LTIP mass in conditioned cell media was determined by Western blot as previously described (6). Media was concentrated ~20-fold by cold acetone precipitation.

Rabbit diet study

Plasma and liver samples were obtained from other investigators in our institution who were conducting approved atherosclerosis studies in rabbits. New Zealand White rabbits were fed a control diet (NIH-09) or the control diet containing 1% cholesterol for 8 weeks. Plasma and liver tissues were frozen at −80°C until analysis. Owing to very high lipid levels in cholesterol-fed rabbit plasma, it was necessary to remove endogenous lipoproteins before performing CETP and LTIP activity assays. Rabbit lipoprotein-deficient plasma was prepared by dextran sulfate-manganese chloride precipitation (16) and extensively dialyzed against 50 mM Tris-HCl, 150 mM NaCl, 0.02% EDTA, and 0.2% NaN_3, pH 7.4. LTIP was determined from the ability of dialyzed samples (10 μl) to inhibit exogenous human CETP in a lipid transfer assay containing 40 μg cholesterol of ^{3}H[CE]LDL and 40 μg unlabeled HDL acceptor (8). CETP activity was determined under the same assay conditions but in the absence of exogenous CETP. Hepatic CETP and LTIP mRNA levels were measured as described below.

Hamster diet study

Male Syrian Golden hamsters (~100 g) were purchased from Charles River (Wilmington, MA) and placed on Purina rodent laboratory chow for 2 weeks. Animals were divided randomly into 3 day and 30 day study groups. Within each study group, animals were further randomly divided into four diet groups of four to five animals each. On day 0, animals received either standard chow, or chow containing 20% hydrogenated coconut oil (HCO), 0.12% cholesterol (Chol), or both 20% HCO and 0.12% cholesterol (HCO+Chol). After 3 or 30 days, animals were fasted overnight, anesthetized with pentobarbital, and exsanguinated, and tissues were collected. Plasma was isolated by centrifugation and held at 4°C for immediate analysis. Aliquots of plasma were stored at −80°C for LTIP and CETP analyses. All tissues were frozen in liquid nitrogen immediately after removal and stored at −80°C until analysis. All animal studies conformed to the Public Health Service policy on the humane care and use of laboratory animals, and were approve by the Cleveland Clinic's Institutional Animal Care and Use Committee.

Lipid and lipoprotein analysis

Fresh plasma samples were assayed for total cholesterol and triglyceride by colorimetric, enzymatic kits (Infinity, Sigma). Lipoprotein cholesterol profiles were obtained by fast-protein liquid chromatography. Samples (10 μl diluted to 100 μl in column buffer) were applied to tandem Superose 6 HR columns (GE Healthcare, Piscataway, NJ) equilibrated in 0.9% NaCl, 0.01% EDTA, and 0.02% NaN_3, pH 7.4, and eluted at 0.3 ml/min. The cholesterol content of the eluant was measured by an on-line method as previously described (17), except that Infinity Cholesterol (Sigma) reagent was used without modification. The post-column eluant was continuously combined with this cholesterol reagent (2 vol eluant to 1 vol reagent) and incubated for 10 min at 37°C prior to absorbance detection at 505 nm. Absorbance profiles were integrated to determine the distribution of cholesterol among lipoprotein fractions. Lipoproteins were identified by their coelution with human VLDL (60–70 min), LDL (78–98 min), and HDL (110–130 min) isolated from normolipidemic plasma by sequential ultracentrifugation (18).

Cloning of hamster LTIP (apoF)

Following reverse transcription, hamster LTIP cDNA was cloned using primers based on the published (19) human LTIP precursor sequence (apoF precursor, Accession NM_001638). Primers were 5′-GTGGAAGCCCTGGCCTCTGCTCTGCAGCT-3′ (forward) and 5′-TAATACTGGATCACTGCTGAACCCCAG-3′ (reverse), and were selected from regions of high homology with mouse LTIP precursor (Accession AF_411831). Primers were ≥93% homologous between these species. The resulting 357 nucleotide product was TA cloned into pCR2.1-Topo (Invitrogen Corp., Carlsbad, CA) and sequenced. A second cloning step was accomplished using 3′ RACE (Invitrogen Corp.) with the help of a gene-specific primer obtained in the first cloning step (5′-CCAGGTCCAAAGAGGGTC-3′) and an oligo dT reverse primer. The 714 nucleotide product was ligated into pCR2.1-Topo, amplified, and sequenced.

LTIP mass determination

LTIP mass in frozen plasma was determined by Western blot. Hamster plasma was diluted 10-fold, combined with 5× SDS-glycerol buffer (without β mercaptoethanol), and heated as previously described (6), then electrophoresed on 4–20% gradient polyacrylamide gels (Lonza, Rockland, ME)). Separated proteins were electrotransferred to polyvinylidene fluoride, and the membrane was blocked with PBS containing 5% dry milk plus 1% calf serum for 1 h at room temperature. Blots were reacted with rabbit anti-hamster LTIP or preimmune rabbit sera, washed three times with PBS, then reacted with peroxidase-conjugated secondary antibody. LTIP-antibody complexes were visualized by ECL reagent (Perkin Elmer). Images were captured by a Fotodyne Luminary system (Hartland, WI), and bands were quantified by National Institutes of Health Image J software. Rabbit anti-hamster LTIP antibody was prepared by injecting rabbits with the synthetic hamster LTIP peptide DEKEASTPQPEITKDGSDFE, conjugated to keyhole limpet hemocyanin, following a 70 day immunization protocol (Open Biosystems, Huntsville, AL).

CETP mass determination

Hamster CETP mass was determined from CETP activity measured in the presence of excess exogenous lipoproteins. Under these assay conditions, CETP activity directly reflects CETP mass because the high exogenous lipoproteins added to the transfer assays nullify the effects of endogenous lipoprotein levels on CETP activity and override the inhibitory effects of LTIP (20). CETP assays were performed essentially as previously described (21, 22), except that assays contained 100 μg cholesterol of ^{3}H[CE]LDL and unlabeled HDL acceptor, and incubations were at 42°C for 6 h. Values, reported as percent transfer, are for CETP activity in 5 μl of hamster plasma. By comparison, pooled human plasma (5 μl) mediated 3.1% transfer in this assay.

RNA isolation from tissues

Total RNA was extracted from flash-frozen (liquid N_2) hamster tissues using Trizol solution following the manufacturer's instructions (Invitrogen Corp.). Hamster and rabbit tissue samples (0.3 g) were homogenized on ice in Trizol solution by a Tissue Tearor (Biospec Products Inc., Bartlesville, OK) set at medium speed. The integrity of the isolated total RNA was assessed on agarose gel stained with ethidium bromide.

Northern blot analysis

For hamster Northern blot analysis, 20 μg of total RNA was separated on 1.4% denaturing agarose gel, then transferred to a nylon membrane and cross-linked using Stratalinker 1800 (Stratagene, La Jolla, CA). Blots were prehybridized overnight in ExpressHyb solution (Clontech, Mountain View, CA) at 42°C. The hybridization was carried out overnight at 63°C in the same solution containing ^{32}P-labled DNA probe (Random Primers Labeling, Invitrogen Corp.). The 471 bp DNA hamster LTIP probe corresponded to nucleotides 19–489 (**Fig. 1**). After washing three times for 40 min at room temperature with solution containing 0.1× SSC and 0.1% SDS, the blots were washed two times for 40 min at 55°C with a 0.2× SSC and 0.1× SDS solution. Blots were exposed to a phosphor screen (8 to 48 h), and the images were collected using the STORM840 PhosphorImager (GE Healthcare). The same conditions were used for Northern blot analysis of human LTIP, starting with a commercially available human multiple tissue blot (Clontech). The radiolabeled human LTIP DNA probe was 482 bp in length and corresponded to nucleotides 545–1,027 of the published sequence (19).

mRNA quantification by real-time PCR

Total RNA was isolated using Trizol as described above then treated with DNase (Invitrogen Corp.) before analysis by real-time PCR to quantify the mRNA levels of target genes. The sequences of primers used in these experiments are shown in **Table 1**. The reverse transcriptase reaction was carried out using 5 μg total RNA, oligo dT, and reverse transcriptase at 50°C for 60 min. Five microliters of this reaction was used in the real-time PCR reaction employing SYBR Green according to the manufacturer's instructions (SuperArray Bioscience Corp.).

RESULTS

LTIP gene regulation in C3A cells

In hypercholesterolemic humans, plasma LTIP levels are elevated. We hypothesized that hypercholesterolemia may increase plasma LTIP levels by inducing LTIP gene expression. To investigate this possibility, C3A human hepatocytes (an HepG2 subclone) were treated with 10 μM retinoic acid plus agonists for nuclear receptors known to regulate lipid metabolism. Cholate (15 μM, farnesoid X receptor agonist) had no effect on LTIP mRNA levels; however, both 22-hydroxycholesterol [25 μM, liver X receptor (LXR) agonist] and clofibrate (300 μM, peroxisome proliferator-activated receptor α agonist) reduced LTIP message levels to 43 ± 6% (n = 5) and 48 ± 23% (n = 3), respectively, of control values. These treatments tended to reduce LTIP mRNA turnover ($t_{1/2}$ = 1.0–1.5 h versus 0.8 h for control cells), indicating that the lower mRNA levels reflect reduced transcription, not decreased mRNA stability. As determined by Western blot, 22-hydroxycholesterol and clofibrate treatment also reduced LTIP secretion into the media to 34–38% of control cells. The reduction in LTIP mRNA and LTIP protein secretion by sterol was surprising because hypercholesterolemia is associated with increased plasma LTIP. To determine whether these cell culture findings accurately reflect the physiological setting, we investigated LTIP expression in vivo.

Cloning of hamster LTIP

Hamsters express CETP naturally, but it is unknown whether they express LTIP (apoF). To determine this, we sought to clone hamster LTIP. Human LTIP is the C-terminal 162 amino acid fragment of a 308 amino acid precursor protein (19), referred to here as LTIP precursor. To clone putative hamster LTIP, regions of human LTIP precursor cDNA that are highly conserved among human, mouse, and rat were selected for primer set design. Following this approach, primers were identified that permitted the cloning of a 357 nucleotide fragment from hamster liver cDNA. Using an internal primer derived from this sequence, 3′ rapid amplification of complementary DNA ends cloning yielded the sequence shown in Fig. 1. This fragment of hamster LTIP cDNA was 67% identical to human LTIP at the nucleotide level, with a 163 codon open reading frame. Hamster LTIP retained the proteolytic site where human LTIP is cleaved from LTIP precursor (hamster AA 13–14). Assuming that hamster LTIP precursor is cleaved at the same proteolytic site, the C-terminal fragment (i.e., LTIP) is 150 amino acids, slightly smaller than human LTIP, with a calculated molecular weight of 16,372. Like human LTIP, hamster LTIP appears to be markedly glycosylated, as evidenced by its anomalously high molecular weight on SDS-PAGE (6). The theoretical isoelectric point of 4.15 is identical to that calculated for human LTIP. The deduced hamster LTIP amino acid sequence contained 69% identical or conserved amino acid substitutions compared with the human protein. This homology was not uniformly distributed. The N-terminal half of hamster LTIP contained amino acids that were 83% identical or conserved substitutions compared with human, whereas the C-terminal half of the protein was only 45% homologous.

By Northern blot analysis, a 1.7 kb hamster transcript was observed only in liver (**Fig. 2A**). Similarly, the 1.7 kb human LTIP transcript was observed predominately in the liver (Fig. 2B), although when this blot was overexposed, LTIP message was also readily detected in spleen and placenta (not shown).

Having established that hamsters have the LTIP gene, we investigated the effects of hyperlipidemia on LTIP gene expression and plasma protein levels.

Effect of cholesterol and saturated fat on hamster plasma lipids

Hamsters fed diets containing cholesterol and/or HCO developed hyerlipidemia within 3 days, with further manifestations of these abnormalities observed after 30 days on these diets (**Table 2**). As would be expected, cholesterol feeding increased VLDL and LDL levels, and this effect

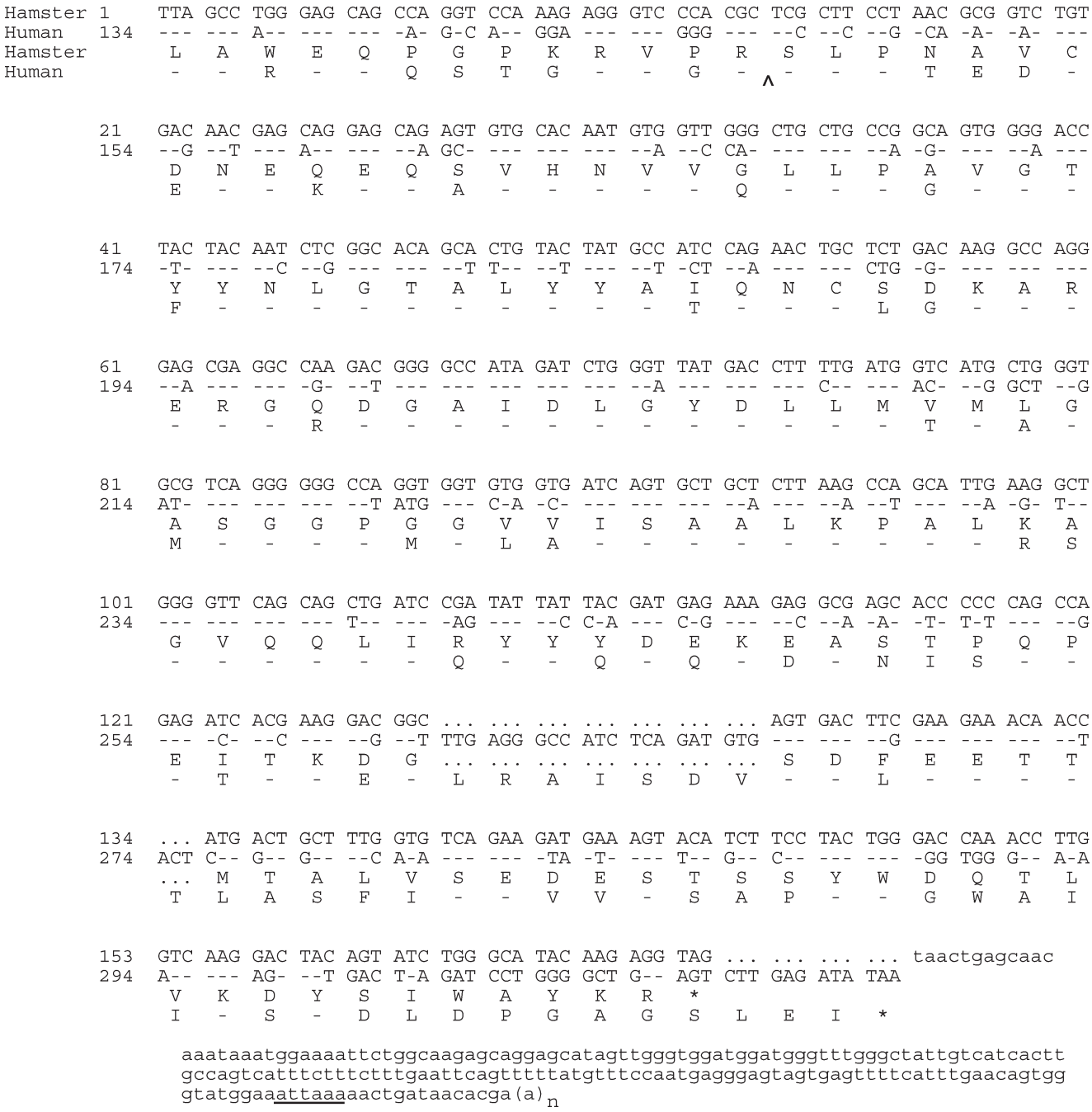

Fig. 1. Comparison of hamster and human lipid transfer inhibitor protein (LTIP) nucleotide and amino acid sequence. Hamster LTIP cDNA was cloned as described in Materials and Methods. The top two lines of each row are cDNA sequences. The bottom two rows are the predicted protein sequences. Codon numbers are shown on the left. Hamster codon 1 was signed to the most 5′ codon of the sequence obtained. Human codon numbering is based on its published sequence (19). Dashes (–) represent identical residues. Dots (...) represent missing residues. The carat (^) denotes the proteolytic cleavage site for the production of LTIP from LTIP precursor. The polyadenylation signal of the hamster cDNA is underlined. Sequences were aligned by BLAST analysis.

was magnified when diets were also enriched in saturated fat. Representative lipoprotein elution profiles are shown in **Fig. 3**. Notably, unlike that seen in other fat-fed animal models, hyperlipidemic hamster plasma did not contain significant intermediate density lipoprotein regardless of the diet treatment. Based on their earlier elution time, LDL and HDL in hamsters fed a cholesterol-enriched diet for 3 days were larger than control lipoproteins [LDL: control = 89.2 ± 0.2 min, cholesterol-fed = 87.2 ± 0.7 min ($P < 0.001$); HDL: control = 120.2 ± 0.4 min, cholesterol-fed = 119.3 ± 0.4 min ($P < 0.01$)]. HCO alone had no statistically significant effect on lipoprotein size, but when HCO was added to the cholesterol-containing diet, HDL size was significantly larger [control = 120.2 ± 0.4 min vs. 119.5 ± 0.4 min ($P < 0.02$), respectively]. After 30 days on these diets, the lipoprotein size increases noted at 3 days were

TABLE 1. Primers for quantitative real-time PCR

Species	Gene	Primers
Hamster	LTIP (apoF)	F: 5′-CAACCATGACTGCTTTGGTG-3′
		R: 5′-TCCTGCTCTTGCCAGAATTT-3′
	CETP	F: 5′-AAGGGTGTCGTGGTCAGTTCT-3′
		R: 5′-ACTGATGATCTCGGGGTTGAT-3′
	Actin	F: 5′-AGGCATTGCTGACCGGATG-3′
		R: 5′-CTAGAAGCATTTGCGGTGGACGAT-3′
Rabbit	LTIP (apoF)	F: 5′-TGTGTACAGCATTGTCCGGCTCTT-3′
		R: 5′-TGGCCATCAGAAGGTCATAACCCA-3′
	CETP	F: 5′-GGTTGGGCATCAATCAGTCT-3′
		R: 5′-CAGCCATGATGTTGGAGATG-3′
	GAPDH	F: 5′-TCACCATCTTCCAGGAGCGA-3′
		R: 5′-TGATGGCGTGCACCGTGGTC-3′

apoF, apolipoprotein F; F, forward primer; R, reverse primer; LTIP, lipid transfer inhibitor protein; CETP, cholesteryl ester transfer protein.

magnified, as evidenced by the larger absolute difference between their elution times compared with controls. For example, LDL in cholesterol-fed animals eluted almost 3 min earlier than control LDL [control = 89.8 ± 0.3 min versus cholesterol-fed = 87.0 ± 0.7 min ($P < 0.001$)]. And in animals fed cholesterol or HCO plus cholesterol, HDL particles eluted up to 1.5 min before control HDL [control = 119.9 ± 0.5 min vs. cholesterol-fed = 118.5 ± 0.5 min ($P < 0.002$), and HCO plus cholesterol-fed animals = 118.4 ± 0.6 min ($P < 0.003$)]. Overall, these earlier elution times indicate that these diets increased LDL and HDL size up to 25% and 11%, respectively.

Influence of diet-induced hyperlipidemia on LTIP and CETP

The three dietary regimens had unique effects on plasma LTIP mass levels (**Fig. 4A**). Whereas HCO alone had no effect on plasma LTIP levels after 3 days, cholesterol feeding modestly decreased LTIP mass. In contrast, HCO plus cholesterol diets caused a large, 2.7-fold increase in plasma LTIP levels. After 30 days of diet supplementation, all lipid-supplemented diet groups had elevated plasma LTIP mass levels compared with chow-fed controls. At this latter time point, cholesterol and HCO feeding increased plasma LTIP levels, and these effects were additive when both lipids were present in the diet. In the 30 day diet groups, plasma LTIP levels correlated positively with plasma cholesterol (Fig. 4B) and plasma LDL (Fig. 4C) concentrations.

In contrast to LTIP, all diets, regardless of feeding duration, either had no effect on CETP levels or were stimulatory (**Fig. 5A**). After 30 days of the combined HCO plus cholesterol diet, CETP was significantly elevated, but in animals consuming diets containing only one of these lipids, CETP was modestly but not statistically increased. However, when the results for all 30 day diet animals were considered individually, CETP levels were positively correlated with plasma cholesterol levels ($r = 0.989$, $P = 0.012$) (Fig. 5B).

To understand the basis for the changes in plasma CETP and LTIP concentrations, mRNA abundance was determined. As has been previously reported, dietary cholesterol and saturated fat caused marked increases in CETP mRNA (**Fig. 6A**). HCO alone was most effective in inducing CETP gene expression, whereas cholesterol alone or together with HCO caused similar stimulatory responses. In contrast to CETP, LTIP mRNA levels were decreased by feeding HCO or cholesterol. After 3 days, consumption of HCO, cholesterol, or both lipids reduced LTIP gene expression up to 50% (Fig. 6B). LTIP mRNA levels remained depressed following 30 days of fat or cholesterol consump-

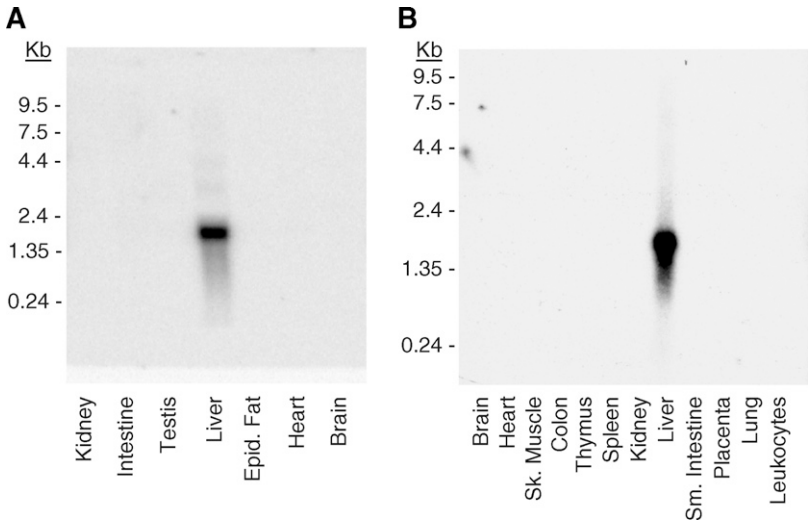

Fig. 2. Tissue distribution of LTIP mRNA. A: Total RNA was extracted from the indicated tissues, and 20 μg was applied to a 1.4% agarose formaldehyde gel. Following transfer, blots were hybridized with a 471 bp ^{32}P-labeled hamster DNA probe. ^{32}P was detected by PhosphorImager analysis; a 16 h exposure is shown. See Materials and Methods for details. B: A commercially available multiple-tissue Northern blot was hybridized with a 482 bp ^{32}P-labeled human DNA probe. A 6 h exposure is shown. RNA size markers are shown on the left of each blot. Abbreviations: Epid. Fat, epididymal fat; Sk. Muscle, skeletal muscle; Sm. Intestine, small intestine.

TABLE 2. Diet-induced changes in plasma lipid and lipoprotein levels

Diet	Duration	*n*	Cholesterol	Triglyceride	VLDL	LDL	HDL
	days				*mg/dl*		
Chow	3	4	91 ± 12	126 ± 45	8 ± 4	17 ± 9	66 ± 9
HCO	3	5	131 ± 20[b]	157 ± 39	12 ± 12	19 ± 4	100 ± 10[b]
Chol	3	5	164 ± 18[b]	211 ± 79	34 ± 12[b]	60 ± 6[c]	70 ± 7
HCO + Chol	3	5	156 ± 26[b]	242 ± 110	35 ± 14[b]	36 ± 13[a]	84 ± 9[a]
Chow	30	5	104 ± 7	125 ± 42	9 ± 4	20 ± 4	75 ± 3
HCO	30	4	141 ± 20[b]	127 ± 35	11 ± 3	32 ± 13	97 ± 16[a]
Chol	30	4	213 ± 31[c]	178 ± 72	60 ± 21[b]	61 ± 6[c]	92 ± 15[a]
HCO + Chol	30	4	304 ± 53[c]	474 ± 52[c]	99 ± 15[c]	114 ± 41[b]	91 ± 14[a]

HCO, hydrogenated coconut oil; Chol, cholesterol. Hamsters were fed the indicated diet for 3 or 30 days. Diets consisted of chow alone, chow + 20% wt/wt HCO, chow + 0.12% wt/wt Chol, and chow containing both coconut oil and cholesterol supplements. Following an overnight fast, animals were euthanized and blood was collected. The distribution of plasma cholesterol among lipoprotein classes was determined by fast-protein liquid chromatography analysis as described in Materials and Methods. Values are the mean ± SD.

[a] $P < 0.05$ versus chow-fed control value in each group.

[b] $P < 0.01$ versus chow-fed control value in each group.

[c] $P < 0.001$ versus chow-fed control value in each group.

tion. Overall, these data show that dietary HCO and cholesterol have distinctly different effects on LTIP protein and mRNA levels.

Influence of a hypercholesterolemic diet on rabbit CETP and LTIP

We sought to determine whether the response of LTIP to cholesterol feeding was observable in another CETP-expressing species. Cholesterol feeding is widely recognized to induce massive hypercholesterolemia in rabbits. In the absence of antibodies to rabbit CETP and LTIP, we measured the plasma activities of these proteins. When rabbits were fed 1% cholesterol, the 25-fold increase in plasma cholesterol [42.3 ± 10.0 (n = 7) vs. 1,034 ± 333 mg/dl (n = 4)] was associated with a 50% increase in CETP activity and LTIP activity (**Fig. 7A, B**). At the mRNA level, CETP and LTIP had markedly different responses to dietary cholesterol (Fig. 7C, D). Whereas dietary 1% cholesterol increased hepatic CETP mRNA levels 2.5-fold, LTIP message levels were reduced by more than 50%. Overall, these results, which are consistent with those seen in hamsters, substantiate the marked and distinctly different effects of dietary cholesterol on CETP and LTIP.

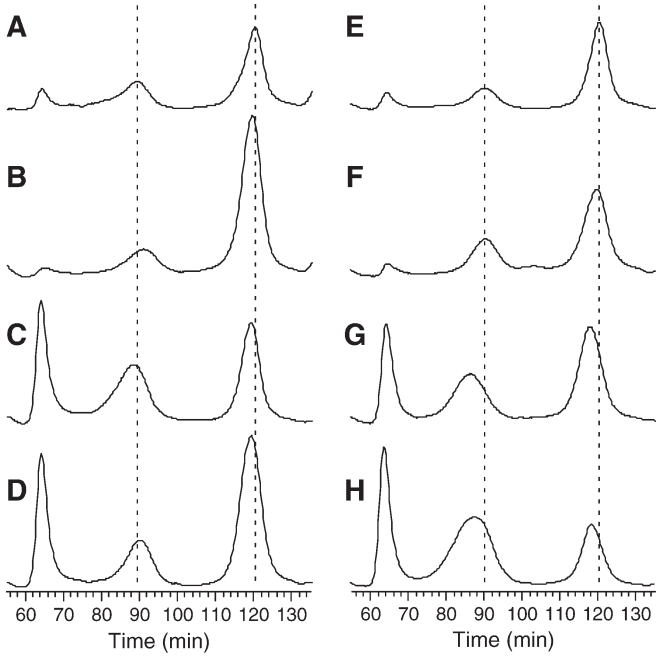

Fig. 3. Fast-protein liquid chromatography elution profiles. Representative cholesterol elution profiles are shown for hamster plasma (10 μl) applied to tandem Superose 6 columns. See Materials and Methods for details. The *y* axis (relative cholesterol content) scale is the same for all plots. Animals were fed chow (A, E), hydrogenated coconut oil (HCO) (B, F), cholesterol (C, G), or HCO plus cholesterol (D, H) for 3 days (A–D) or 30 days (E–H). Vertical dashed lines mark the elution time for LDL and HDL in the chow-fed animals. The three peaks in each profile are (left to right) VLDL, LDL, and HDL based on their coelution with isolated human lipoproteins.

DISCUSSION

We report here that hamsters, in addition to expressing CETP, also synthesize LTIP (apoF). The partial amino acid sequence deduced for hamster LTIP contains a proteolytic cleavage site homologous to the site utilized in humans to convert precursor LTIP to LTIP (19). Cleavage at this site would produce a hamster protein slightly smaller than human LTIP. However, because human and hamster LTIPs behave anomalously on SDS electrophoresis, presumably owing to their high carbohydrate content, the location of the cleavage site remains to be established. Human and hamster LTIPs show considerable homology at the nucleotide and amino acid levels, especially in the N-terminal half of LTIP, where amino acid homology exceeds 80%. LTIP mRNA has a very limited tissue distribution, observed only in the liver of the hamster, which is similar to that found for humans. This differs markedly from the widespread expression of CETP in these species (12, 23), suggesting that some functions of CETP, such as its role in intracellular lipid transfer (24), may not be regulated by LTIP.

Previous human studies indicated that plasma LTIP levels are altered in hyperlipidemic subjects, with LTIP increased

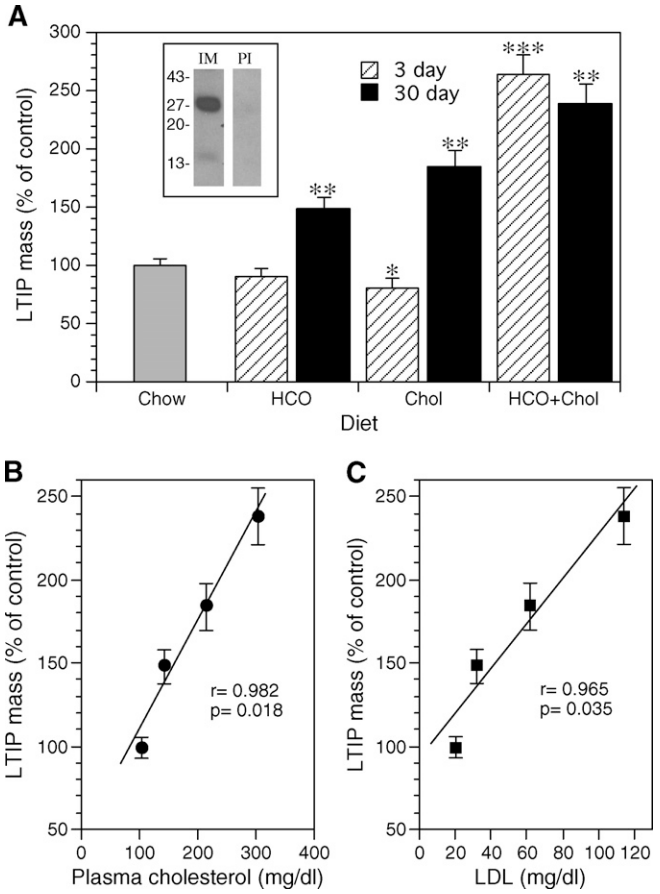

Fig. 4. Diet-induced changes in hamster plasma LTIP. A: LTIP mass. Relative LTIP mass was determined by Western blot as described in Materials and Methods. Shown are the mean ± SD (n = 3) of values determined on plasmas pooled from animals consuming the same diet, and are representative of two similar analyses. Inset: Western blot detection of LTIP in control hamster plasma by immune (IM) and preimmune (PI) antisera. Molecular mass markers, in kDa units, are shown on the left. HCO, hydrogenated coconut oil; Chol, cholesterol. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with chow-fed animals. B, C: Plasma LTIP mass versus plasma lipid levels. Values shown are mean ± SD (n=3).

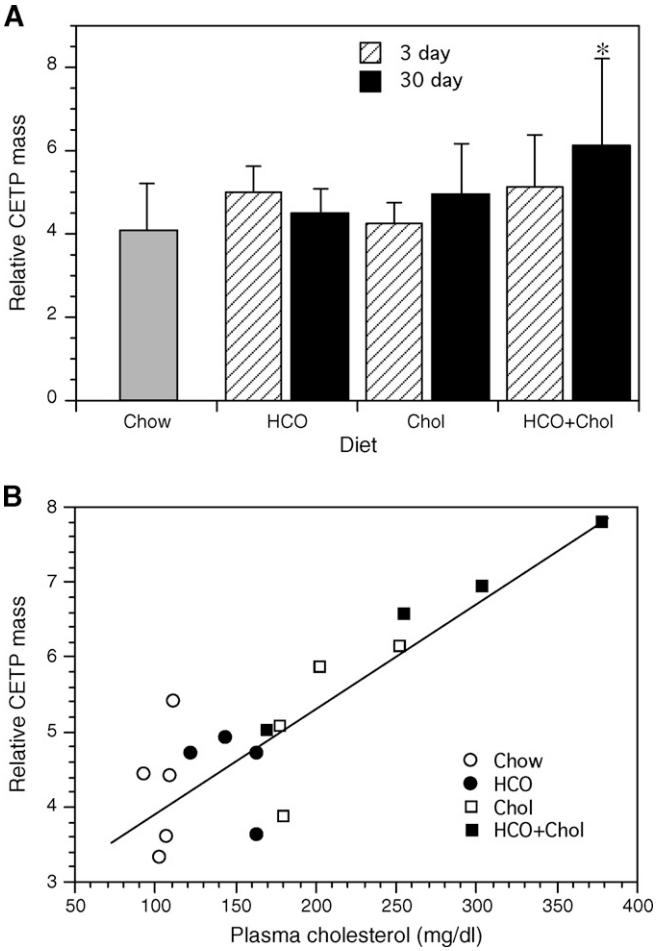

Fig. 5. Response of hamster cholesteryl ester transfer protein (CETP) to dietary lipid modifications. A: CETP mass. Hamster CETP mass was determined by an activity assay under conditions in which CETP activity is proportional to CETP mass (see Materials and Methods for details). Error bars indicate the mean ± SD of the values for animals fed the same diet. Individual plasma samples (5 μl) were assayed in duplicate. * $P < 0.05$ compared with chow animals. B: CETP mass versus plasma cholesterol. Relative CETP mass levels, determined in A, are plotted versus plasma cholesterol levels. Symbols show values for individual animals. See the legend to Fig. 4 for abbreviations.

in hypercholesterolemic subjects but reduced in some hypertriglyceridemic individuals (6, 8). As an initial inquiry into a possible basis for these changes in LTIP levels, we investigated the response of hamster LTIP to diet-induced hyperlipidemia. We show for the first time that the LTIP gene is subject to dietary regulation. Feeding saturated fat, which induces endogenous hypercholesterolemia in hamsters, reduced hepatic LTIP mRNA levels by half. Likewise, cholesterol feeding or cholesterol plus saturated fat feeding, both of which induced hypercholesterolemia and hypertriglyceridemia, also reduced LTIP mRNA to a similar extent. These reductions in LTIP gene expression were also mirrored in cholesterol-fed rabbits and in human hepatic cells treated with 22-OH cholesterol. Collectively, these results show that LTIP mRNA abundance is depressed by cholesterol or a cholesterol metabolite. Suppression of LTIP gene expression by hypercholesterolemia is contrary to anticipation on the basis of hyperlipidemic human studies.

Despite reduced LTIP mRNA expression, hyperlipidemic hamsters showed marked increases in plasma LTIP. Like that seen in hypercholesterolemic humans, hamster LTIP mass was increased up to 3-fold, and among all diet groups, the increase in LTIP mass was positively correlated with both plasma cholesterol and LDL. In humans, hypertriglyceridemia mitigates the rise in plasma LTIP in subjects with concomitant hypercholesterolemia (8). For the present diet studies, the group size was not adequate to determine whether such a relationships exists in hamsters.

The mechanism underlying the multi-fold increase in plasma LTIP mass in the face of a 50% reduction in hepatic LTIP mRNA remains to be determined. Certainly, reduced mRNA levels do not necessitate reduced LTIP production, although these often do correlate. This association was observed in C3A cells, where agonist-mediated suppression of LTIP mRNA was accompanied by a similar

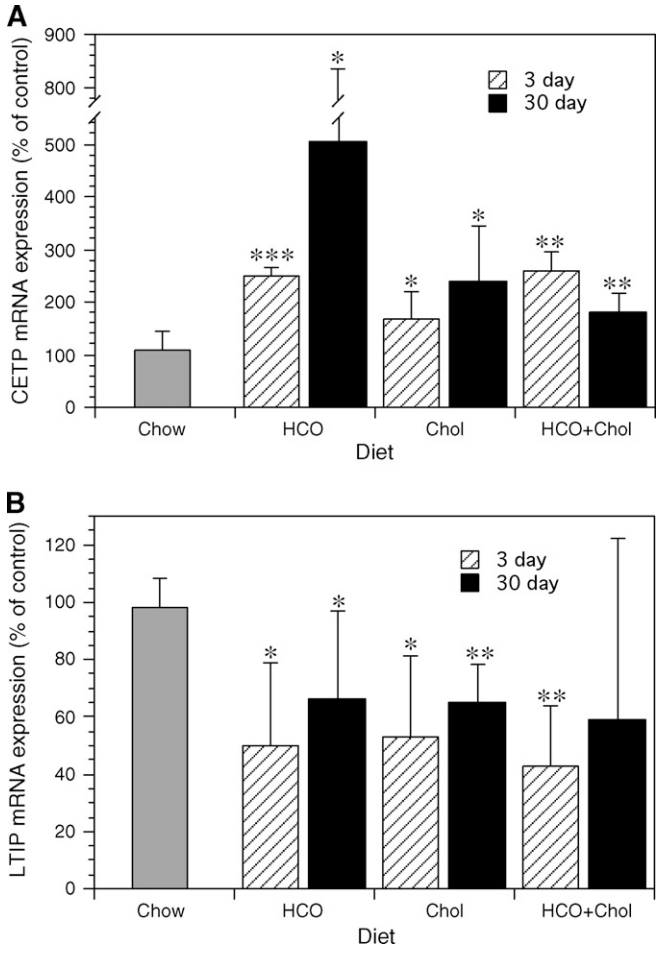

Fig. 6. Influence of dietary lipid modifications on hamster CETP and LTIP hepatic mRNA. mRNA was quantified by real-time PCR as described in Materials and Methods. A: CETP mRNA. B: LTIP mRNA. Individual RNA samples were assayed in duplicate. Values are normalized to actin mRNA levels, and are reported relative to the values found in chow-fed animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with chow-fed animals. See the legend to Fig. 4 for abbreviations.

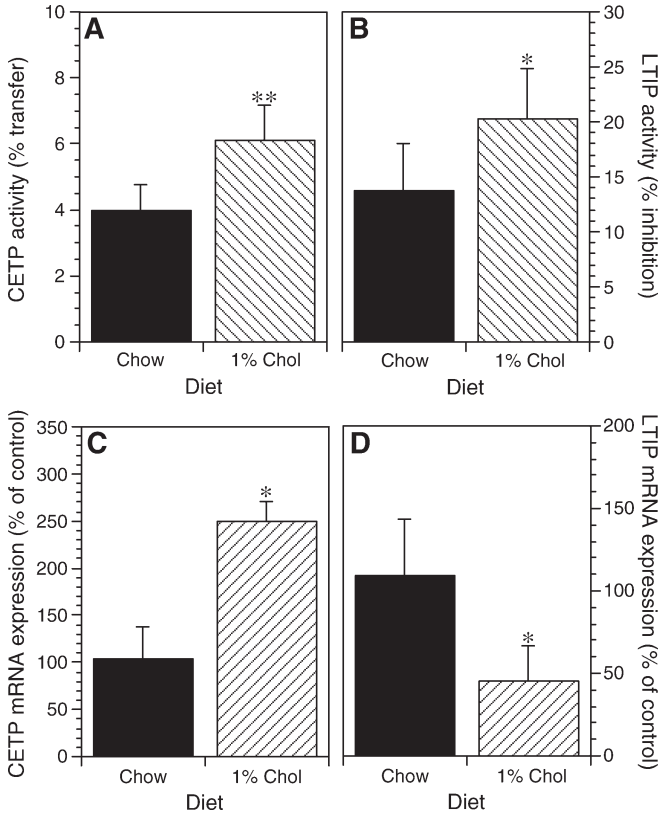

Fig. 7. Influence of dietary lipids on rabbit CETP and LTIP activity and mRNA. Rabbits were fed chow ± 1% cholesterol (Chol) diets for 8 weeks. Shown in A (CETP) and B (LTIP), activities of these two proteins were determined in lipoprotein-deficient samples as described in Materials and Methods. Values are the mean ± SD of n = 7 (chow) and n = 4 (1% Chol) animals. Individual animal samples were assayed in triplicate. In C (CETP) and D (LTIP), mRNA levels were determined by real-time PCR as described in Materials and Methods. Values are the mean ± SD of n = 4 (chow) and n = 4 (1% Chol) animals. Individual RNA samples were assayed in duplicate. Values are normalized to GAPDH mRNA levels and are reported relative to the values found in control (chow-fed) animals. * $P < 0.05$, ** $P < 0.01$, compared with chow-fed animals.

reduction in LTIP secretion. Additional studies will be required to define the processes that lead to increased plasma LTIP in vivo. One possible mechanism that warrants consideration is that hypercholesterolemia reduces the turnover rate for plasma LTIP. In humans, LTIP is bound to an inactive 470 kDa complex and to LDL, and hypercholesterolemia increases the association of LTIP with LDL (25). Although technical limitations of the hamster anti-LTIP antibody prevented us from verifying this distribution in hamsters, we have detected the presence of the more abundant 470 kDa LTIP component in hamster plasma. We propose that the association of LTIP with LDL may facilitate LTIP clearance from plasma when the lipoprotein is endocytosed. In the hypercholesterolemic state, LDL turnover is reduced, which may decrease LTIP turnover and potentially elevate steady-state levels of LTIP in plasma. For example, in the hypercholesterolemic hamster, the fractional catabolic rate for LDL is reduced 2-fold and 5-fold, respectively, by feeding the cholesterol or the cholesterol plus HCO diets used in this study (26). The viability of this hypothesis remains to be determined.

Diet-induced changes in CETP observed here confirm previous reports. For example, the increases we observed in the hepatic CETP mRNA of cholesterol- and coconut oil-fed hamsters are similar to the CETP mRNA changes noted by Jiang et al. (12) in other hamster tissues. Although hamster CETP expression is highest in adipose tissue (12), and CETP mRNA levels in this tissue correlate well with plasma CETP levels (14), our results show that hepatic CETP mRNA levels respond to dietary stimuli in a similar fashion. The marked diet-induced rise in CETP mRNA was accompanied by a rather modest increase in plasma CETP. This is consistent with the observations of others (14, 27). As has been commonly reported in various animal models and in humans, diet-induced hamster CETP levels correlated well with plasma cholesterol levels. This relationship is the same as we observed for hamster LTIP, although the mechanism underlying this correlation is

probably different, because hypercholesterolemia stimulates CETP gene expression but inhibits LTIP gene expression.

In conclusion, we report that in hamster and rabbit, diet-induced hypercholesterolemia increases circulating levels of LTIP even though LTIP gene expression is markedly suppressed. This effect on mRNA levels was mirrored in cultured human hepatocytes treated with oxysterol. This stands in marked contrast to CETP, whose gene expression is upregulated by these conditions. Sterol activation of CETP gene expression is mediated by the activity of LXR/retinoid X receptor on a direct repeat 4 element in the CETP promoter (28). The mechanism by which hypercholesterolemia downregulates LTIP is unknown, but probably does not involve LXR, because analysis of the human LTIP promoter did not reveal binding sites for this nuclear receptor. The LTIP promoter also lacks predicted binding sites for the farnesoid X receptor or sterol response element binding proteins, but does contain two putative peroxisome proliferator-activated receptor response elements. The latter may mediate the observed suppression of LTIP mRNA by clofibrate in cultured cells. Despite reduced LTIP gene expression, hypercholesterolemia is associated with increased plasma LTIP mass. In humans, much of the increased LTIP is associated with LDL (25). As we have discussed previously (25), higher LTIP levels should reduce lipid transfer to LDL and permit more cholesterol, as cholesteryl ester, to be transported from HDL to VLDL by CETP. Because of the brief plasma lifetime of VLDL, this process enhances the transport of HDL-derived cholesterol to the liver while maintaining the ability of HDL to mediate cholesterol removal from tissues. Thus, increased plasma LTIP may be an adaptive response whose goal is to reduce the participation of LDL in lipid transfer processes and, consequently, to optimize clearance pathways when cholesterol is in abundance.

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