Hepatic lipid accumulation in apolipoprotein C-I-deficient mice is potentiated by cholesteryl ester transfer protein[S]

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Abstract The impact of apolipoprotein C-I (apoC-I) deficiency on hepatic lipid metabolism was addressed in mice in the presence or the absence of cholesteryl ester transfer protein (CETP). In addition to the expected moderate reduction in plasma cholesterol levels, apoCIKO mice showed significant increases in the hepatic content of cholesteryl esters (+58%) and triglycerides (+118%) and in biliary cholesterol concentration (+35%) as compared with wild-type mice. In the presence of CETP, hepatic alterations resulting from apoC-I deficiency were enforced, with up to 58% and 302% increases in hepatic levels of cholesteryl esters and triglycerides in CETPTg/apoCIKO mice versus CETPTg mice, respectively. Biliary levels of cholesterol, phospholipids, and bile acids were increased by 88, 77, and 20%, respectively, whereas total cholesterol, HDL cholesterol, and triglyceride concentrations in plasma were further reduced in CETPTg/apoCIKO mice versus CETPTg mice. Finally, apoC-I deficiency was not associated with altered VLDL production rate. In line with the previously recognized inhibition of lipoprotein clearance by apoC-I, apoC-I deficiency led to decreased plasma lipid concentration, hepatic lipid accumulation, and increased biliary excretion of cholesterol. The effect was even greater when the alternate reverse cholesterol transport pathway via VLDL/LDL was boosted in the presence of CETP.—Gautier, T., U. J. F. Tietge, R. Boverhof, F. G. Perton, N. Le Guern, D. Masson, P. C. N. Rensen, L. M. Havekes, L. Lagrost, and F. Kuipers. **Hepatic lipid accumulation in apolipoprotein C-I-deficient mice is potentiated by cholesteryl ester transfer protein.** ***J. Lipid Res.*** **2007.** 48: **30–40.**

Supplementary key words liver • bile • cholesterol • triglycerides

The cholesteryl ester transfer protein (CETP) mediates the exchange of neutral lipids, i.e., cholesteryl esters and triglycerides, between circulating lipoproteins (1, 2). The action of CETP in vivo results in the net transfer of cholesterol from HDL to triglyceride-rich lipoproteins and in the reciprocal enrichment of HDL with triglycerides (1, 2). Although CETP has a potentially deleterious impact on the lipoprotein profile, its influence on the progression of atherosclerosis has been a matter of debate. Indeed, studies in transgenic mice as well as epidemiological investigations in human populations with CETP deficiency have led to conflicting results, and CETP has been described as either a pro- or an antiatherogenic protein, depending on the metabolic context (1). Several groups have suggested that CETP, by allowing some cholesterol to be returned to the liver via the apoB-containing lipoproteins, offers an alternative route for hepatic delivery of lipids to the classical HDL-mediated reverse cholesterol transport (3). In addition, previous studies in transgenic mice have shown that the presence of CETP increases hepatic cholesterol content (4, 5). However, this mechanism depends on the clearance capacities of the apoB-containing lipoproteins by the liver. Apolipoprotein C-I (apoC-I) is one of the proteins that might interfere with this clearance process because of its inhibitory effect on the uptake of triglyceride-rich lipoproteins.

ApoC-I is a small circulating apolipoprotein (6.6 kDa) that is mainly secreted by the liver (6). This highly exchangeable protein is associated predominantly with HDL (80%) and, to a lesser extent, with VLDL and chylomicrons (7). ApoC-I interferes with lipoprotein metab-

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Abbreviations: ABCB4, ATP binding cassette B4; ALAT, alanine transaminase; apoC-I, apolipoprotein C-I; ASAT, aspartate transaminase; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; NBD-CE, nitrobenzoxadiazole-labeled cholesteryl esters.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of a table.

olism in a complex fashion. Early studies indicated an inhibitory effect on the clearance of triglyceride-rich lipoproteins by the liver (8), and further in vitro work showed that apoC-I can inhibit the interaction between VLDL and LDL receptor-related protein (LRP) (9–11). More recent studies in transgenic mice overexpressing apoC-I revealed its hyperlipidemic effect and added to the complexity by showing that apoC-I is also able to impair the binding of VLDL to the VLDL receptor (VLDLR) (12) and to inhibit lipoprotein lipase (LPL) (13). Although apoC-I overexpression in transgenic animals leads to severe hyperlipidemia (14, 15), apoC-I-deficient mice show a very discrete phenotype on a chow diet, i.e., only a trend toward lower plasma lipids (16–18), which is enforced on an apoE-knock-out background (19). It was also shown that apoC-I, probably by reducing the uptake of fatty acids by peripheral tissues (20), can indirectly modify peripheral insulin sensitivity (20, 21). Finally, in vitro studies have demonstrated that apoC-I can activate LCAT activity, but to a much lesser extent than apoA-I (22), and this effect has not been documented in vivo. Strikingly, in addition to the direct effects of apoC-I on lipoprotein clearance, it has also been extensively shown that apoC-I is a potent inhibitor of CETP in vitro and in vivo (16, 23), suggesting that in the presence of CETP, apoC-I can interfere with the reverse cholesterol transport process by two distinct manners: *i*) by directly inhibiting CETP activity, and *ii*) by blocking the hepatic uptake of cholesterol-enriched apoB-containing lipoproteins.

In mice, the return of excess peripheral cholesterol to the liver for eventual excretion into bile is mainly mediated by HDL particles (24), whereas in humans, most of the cholesterol is transferred to apoB-containing lipoproteins prior to hepatic uptake (3). This difference might be due to the fact that the mouse is a CETP-deficient species (25), and the transgenic mouse expressing human CETP appears as a more relevant model for studying the processes involved in reverse cholesterol transport in a human-like situation. In parallel, apoC-I-deficient mice show only moderate alterations of the lipoprotein profile (18) and may thus constitute a relevant model for studying lipid fluxes without overt changes in plasma lipid levels. Consequently, to determine the influence of apoC-I on hepatic lipid metabolism, either directly or via its inhibitory effect on its natural target, CETP, we analyzed hepatic lipid levels, bile composition, and hepatic gene expression in apoC-I-deficient (apoCIKO) mice (18) compared with wild-type C57BL/6 mice. The same was done in apoCIKO mice that were crossbred with transgenic mice expressing human CETP, thus comparing CETPTg mice (26) to apoC-I-deficient mice expressing human CETP (CETPTg/apoCIKO mice).

MATERIALS AND METHODS

Animals

Four distinct mouse lines were used in the present study, all on a homogenous C57BL/6 genetic background: wild-type C57BL/6 mice, mice expressing human CETP under the control of natural flanking regions (CETPTg) (26), apoC-I knock-out mice (apoCIKO) (18), and CETPTg/apoCIKO mice obtained by crossbreeding (16). All mice were males of 3 months of age. ApoCIKO mice were all homozygous for the apoC-I-deficient trait. The mice had free access to food and water, and they were fed a standard chow diet (A03; Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France).

Plasma, bile, and liver sampling

Mice were weighed and then anesthetized by intraperitoneal injection of pentobarbital after an overnight fast. Blood samples were drawn by cardiac puncture and collected into heparin-containing tubes. Plasma was separated by centrifugation and stored at −80°C before analysis. Bile was collected by direct puncture of the gallbladder and stored at −80°C before analysis. Livers were excised, weighed, and separated into two parts: one part was immediately frozen and stored at −80°C for further biochemical analysis and for microscopic examination; the other part was directly homogenized in 500 μl Trizol (Invitrogen, Paisley, UK), and the homogenate was stored at −80°C before RNA isolation.

Plasma analyses

Commercially available kits were used for the determination of free cholesterol (Wako, Neuss, Germany), total cholesterol and triglycerides (Roche Molecular Biochemicals, Mannheim, Germany), free fatty acids (Roche), and phospholipids (Wako) in plasma. For cholesterol and triglyceride profiles, pooled plasma samples from all animals of one group were used for lipoprotein separation by fast-protein liquid chromatography on a Superose 6 HR 10/30 column (Amersham Biosciences) as described previously (27). Fractions 7–13, 14–21, and 22–30 contained VLDL, IDL/LDL, and HDL, respectively. Cholesterol content of HDLs from individual plasmas was determined after sequential ultracentrifugation. Briefly, HDLs were separated as the $1.063 < d < 1.21$ g/ml fraction with one 3 h, 100,000 rpm (356,000 *g*) spin in a TLA120 rotor in a TLX ultracentrifuge (Beckman). Densities were adjusted by the addition of solid potassium bromide. Plasma bilirubin concentration, aspartate transaminase (ASAT), and alanine transaminase (ALAT) activities, as well as glucose levels, were determined by routine clinical chemistry. Plasma insulin was determined using an enzyme-linked immunoassay kit (Ultrasensitive Mouse Insulin ELISA; Mercodia, Uppsala, Sweden). The homeostasis model assessment (HOMA) index was calculated as [glucose (mmol/l) × insulin (μU/ml)]/22.5 (28).

Hepatic and biliary lipid analysis

Hepatic and biliary lipids were extracted according to Bligh and Dyer (29). Phospholipids in bile and liver were determined as described by Böttcher, van Gent, and Pries (30). Cholesterol in bile was measured according to Gamble et al. (31). Hepatic cholesterol and triglyceride contents were analyzed by enzymatic methods as described above. Total bile salt levels were measured enzymatically (32). Bile salt profiles were analyzed according to Setchell et al. (33).

Neutral fat staining in liver sections

Liver histology was examined on frozen liver sections after Oil-Red-O staining for neutral lipid by standard procedures.

Measurement of CETP activity

Plasma CETP activity was measured in mouse plasma by using a fluorescent assay that was performed in microplates by using

donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (NBD-CEs) (Roar Biomedical, New York, NY) as described previously (16). In brief, incubation media contained 4 μl of donor liposomes and 10 μl of total plasma in a final volume of 200 μl TBS. Incubations were performed in triplicate for 3 h at 37°C in an FL600 Microplate Fluorescence Reader (Bio-Tek, Winooski, VT). The CETP-mediated transfer of NBD-CEs from self-quenched donors to acceptor endogenous plasma lipoproteins was monitored by the increase in fluorescence intensity (excitation, 465 nm; emission, 535 nm). The amounts of NBD-CE transferred (in pmol) were calculated by using a standard curve, which plotted fluorescence intensity and the concentration of NBD-CEs dispersed in propan-2-ol. Results were expressed as the initial transfer rate of NBD-CEs after deduction of blank values.

RNA isolation and PCR methods

Total RNA was isolated with Trizol (Invitrogen) and quantified using Ribogreen (Molecular Probes, Inc., Eugene, OR). cDNA synthesis was done according to Bloks et al. (34). Real-time quantitative PCR (35) was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. Primers were obtained from Invitrogen. Fluorogenic probes, labeled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine, were made by Eurogentec (Seraing, Belgium). Primer and probe sequences used in these studies have been described elsewhere [*Abcg5, Abcg8, Mdr2, Bsep, Hmgr, Cyp7a1, Cyp 27, Sr-b1, srebp1c, srebp2, Lxrα*, and *18s* (36); *Ldlr, Vldlr, Lpl, Hl, Fas, Acc1*, and *Mtp* (37); *Acc2* (38); *Cpt1a* (39), except *Lrp* (NM_008512; forward TCAGACGAGCCTCCAGACTGT, reverse ACAGATGAAGGCAGGGTTGGT, probe CCAGTTCCAGTGCTCCACCGGC), *ChREBP* (NM_021455; forward GATGGTGCGAACAGCTCTTCT, reverse CTGGGCTGTGTCATGGTGAA, probe CCAGGCTCCTCCTCGGAGCCC), *Aox* (NM_007585; forward CCC AAG TGG ATC AGC ATC ATG, reverse GCT CTT GTA CCT TTC GAA CAC TTT C, probe CTG AGC GCA GTG TGT GCC ACC TCC), *Ho1* (NM_010442; forward CACAGGGTGACAGAAGAGGCTAA, reverse CTGGTCTTTGTGTTCCTCTGTCAG, probe CAGCTCCTCAAACAGCTCAATGTTGAGC), *Il6* (NM_031168; forward CCGGAGAGGAGACTTCACAGA, reverse AGAATTGCCATTGCACAACTCTT, probe ACCACTTCACAAGTCGGAGGCTTAATTACA), *iNos* (AF049656; forward CTATCTCCATTCTACTACTACCAGATCGA, reverse CCTGGGCCTCAGCTTCTCAT, probe CCCTGGAAGACCCACATCTGGCAG), *Tnfα* (NM_013693; forward GTAGCCCACGTCGTAGCAAAC, reverse AGTTGGTTGTCTTTGAGATCCATG, probe CGCTGGCTCAGCCACTCCAGC)]. All expression data were subsequently standardized for 18S rRNA, which was analyzed in separate runs.

Western blot analysis

Frozen liver pieces were homogenized in PL3K lysis buffer supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and protein concentration in the homogenates was determined by using bicinchoninic acid reagent (Pierce, Rockford, IL). Liver homogenates (protein, 20 μg) were diluted (1:20, v/v) in TBS (100 mM, pH 6.8) containing SDS (25 g/l) and dithiothreitol (33 g/l) and then incubated at 80°C for 5 min. The treated samples were then applied on an SDS polyacrylamide 4-15% ready gradient gel (Bio-Rad) and transferred onto nitrocellulose membranes (Trans Blot; Bio-Rad) for ATP binding cassette B4 (ABCB4) immunodetection. The resulting blots were blocked for 2 h in 10% low-fat dried milk in PBS (100 mM, pH 7.4) containing 0.1% Tween and then washed with PBS/Tween. Mouse ABCB4 was revealed by successive incubations with rabbit anti-mouse ABCB4 antibodies (Abgent, San Diego, CA) and horseradish peroxidase-coupled goat anti-rabbit secondary antibodies (DakoCytomation, Glostrup, Denmark). Blots were finally developed by using the SuperSignal® Chemiluminescent kit (Pierce).

Triglyceride production rate in vivo

Fasted mice were injected intraperitoneally with Poloxamer 407 (P-407) (1,000 mg/kg body weight) as a 75 mg/ml solution in saline as previously described (40). Blood samples were drawn into heparin-containing tubes at 0, 30, 120, and 240 min after injection, and plasma was prepared by centrifugation. Plasma triglyceride levels were determined, and the triglyceride production rate was calculated from the slope of the curve and expressed as mg/kg body weight/h.

Statistical analyses

All data are reported as mean ± SD. Statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL). Differences between the groups were analyzed by the Mann-Whitney U-test. Differences with a P value smaller than 0.05 were considered statistically significant.

RESULTS

Effect of CETP expression

Impact of apoC-I deficiency on plasma lipid levels. In agreement with previous studies (16–18), plasma total cholesterol, cholesteryl esters, and phospholipids, which are mainly present in the HDL fraction of wild-type mice, were decreased in apoCIKO mice (supplemental Table 1; **Fig. 1A**, left panel). Extending our previous findings (16–18), plasma free fatty acid levels were also lower in apoCIKO mice than in C57BL/6 mice (−35.0%, $P < 0.05$). No significant differences were observed in plasma concentrations of free cholesterol and triglycerides, which are mainly confined to the VLDL fraction, in both wild-type and apoCIKO mice (supplemental Table 1; Fig. 1A, right panel).

ApoC-I deficiency in the presence of CETP induced a further reduction in plasma lipids. In good agreement with previous observations (16–18), total cholesterol, cholesteryl esters, triglycerides, and phospholipids were lowered by 47.0, 52.3, 53.2, and 37.2%, respectively in CETPTg/apoCIKO mice compared with CETPTg mice ($P < 0.05$ in all cases; supplemental Table 1). The decrease in plasma cholesterol was mainly due to a 53.6% reduction in the HDL fraction (Fig. 1B, left panel; supplemental Table 1), whereas the decrease in plasma triglyceride levels was due to a reduction in the VLDL fraction (Fig. 1B, right panel). As previously reported (16), lipoprotein-dependent CETP activity was significantly higher in the absence of its physiological inhibitor, apoC-I (+71.2% in CETPTg/apoCIKO mice vs. CETPTg mice; $P < 0.05$) (supplemental Table 1).

Impact of apoC-I deficiency on hepatic lipid levels. In contrast to what was observed for plasma lipid parameters, the hepatic lipid content was markedly increased in apoC-I-deficient mice as compared with C57BL/6 mice (**Table 1**). Indeed, hepatic cholesterol content was 31.4% higher in

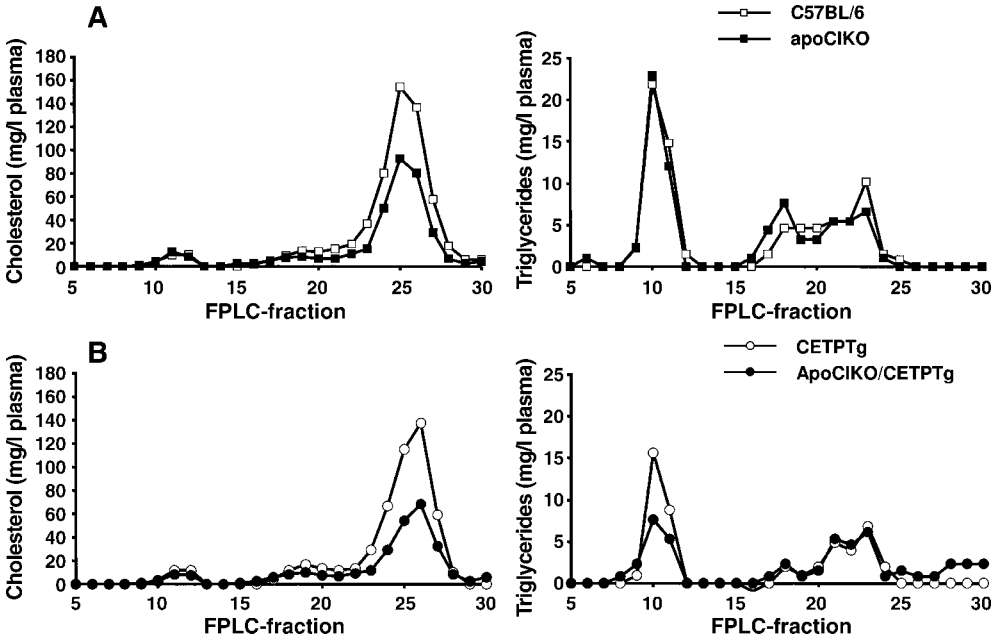

Fig. 1. Plasma distribution of cholesterol and triglycerides in C57BL/6, CETPTg, apoCIKO, and CETPTg/apoCIKO mice. Pooled plasmas from C57BL/6 (open squares; n = 11), apoCIKO (filled squares; n = 8), CETPTg (open circles; n = 6), and CETPTg/apoCIKO mice (filled circles; n = 6) were analyzed by fast-protein liquid chromatography on a Superose 6-HR column as described in Materials and Methods. Panel A shows cholesterol (left) and triglyceride (right) profiles in C57BL/6 mice (open squares) and apoCIKO mice (filled squares). Panel B shows cholesterol (left) and triglyceride (right) profiles in CETPTg mice (open circles) and CETPTg/apoCIKO mice (filled circles). Fractions 7–13, 14–21, and 22–30 contained VLDL, IDL/LDL, and HDL, respectively.

apoCIKO mice ($P < 0.05$, Table 1), due to a 57.8% increase in cholesteryl ester content compared with control mice ($P < 0.05$, Table 1), whereas hepatic free cholesterol levels were not significantly altered. The most dramatic effect of isolated apoC-I deficiency was a 118.4% increase in hepatic triglyceride levels in apoCIKO mice compared with C57BL/6 mice ($P < 0.05$, Table 1). Hepatic phospholipid levels were not different between C57BL/6 and apoCIKO mice (see Table 1), and liver weight (as percent body weight) was similar in both strains (not shown). The accumulation of neutral fat in livers of apoCIKO mice was confirmed by Oil-Red-O staining of frozen liver sections from representative C57BL/6 and apoCIKO mice (**Fig. 2**).

The impact of apoC-I deficiency on hepatic lipid content was potentiated when mice expressed CETP. Indeed, cholesteryl ester and triglyceride levels in the liver were increased by 58.2% and 302.4%, respectively, in CETPTg/apoCIKO mice compared with CETPTg mice ($P < 0.05$ in all cases, see Table 1). In accordance with observations in apoCIKO mice, the hepatic contents of free cholesterol and phospholipids did not differ between CETPTg/apoCIKO and CETPTg mice.

Impact of apoC-I deficiency on biliary lipid concentrations. Lipid concentrations were determined in gallbladder bile from the various groups. As shown in **Fig. 3A, B**, biliary cholesterol

TABLE 1. Hepatic lipid content in C57BL/6, apoCIKO, CETPTg, and CETPTg/apoCIKO mice

	C57BL/6	ApoCIKO	CETPTg	ApoCIKO/CETPTg
Total cholesterol	4.17 ± 0.52	5.48 ± 0.16[a]	4.50 ± 0.69[b]	6.44 ± 1.25[a,b,c]
Free cholesterol	2.30 ± 0.52	2.53 ± 0.16	2.11 ± 0.72	2.66 ± 0.15
Cholesteryl esters	1.87 ± 0.54	2.95 ± 0.14[a]	2.39 ± 0.52[b]	3.78 ± 1.24[a,c]
Triglycerides	12.75 ± 6.72	27.84 ± 9.05[a]	9.63 ± 4.66[b]	38.73 ± 17.30[a,c]
Phospholipids	38.29 ± 3.52	36.00 ± 3.10	36.97 ± 2.08	36.55 ± 3.71

Livers from fasting C57BL/6 (n = 11), apoCIKO (n = 8), CETPTg (n = 6), and CETPTg/apoCIKO mice (n = 7) were homogenized, and hepatic lipids were extracted and analyzed as described in Materials and Methods. All values are expressed in nmol/mg liver and represent means ± SD.

Mann-Whitney U-test.

[a] Significantly different from C57BL/6 mice, $P < 0.05$.

[b] Significantly different from apoCIKO mice, $P < 0.05$.

[c] Significantly different from CETPTg mice, $P < 0.05$.

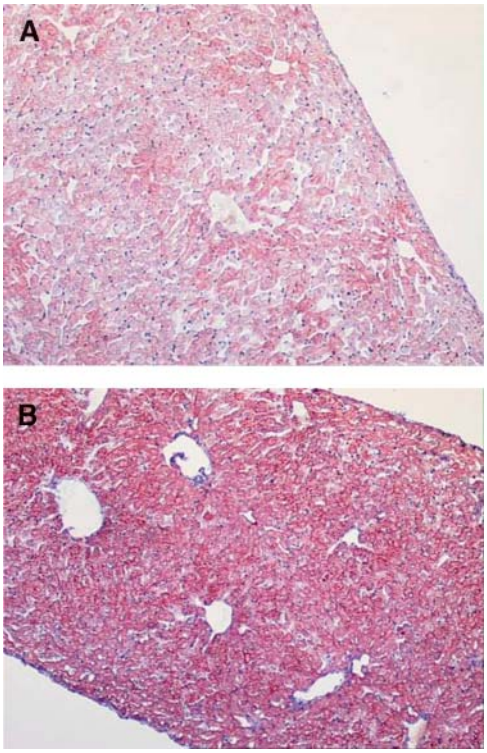

Fig. 2. Oil-Red-O staining of neutral lipids in livers from C57BL/6 and apoCIKO mice. Frozen liver sections from C57BL/6 mice (A) and apoCIKO mice (B) were examined for neutral lipid accumulation as described in Materials and Methods.

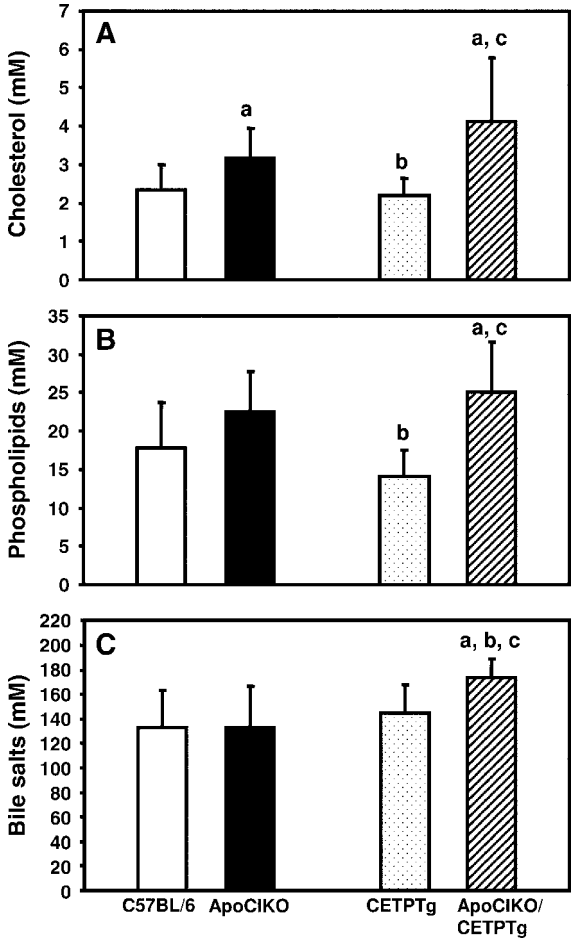

Fig. 3. Biliary lipid composition of gallbladder bile of C57BL/6, CETPTg, apoCIKO, and CETPTg/apoCIKO mice. Bile was collected from the gallbladder of overnight-fasted C57BL/6 (open bars; n = 11), apoCIKO (filled bars; n = 8), CETPTg (dotted bars; n = 6), or CETPTg/apoCIKO mice (hatched bars; n = 6). Biliary lipids were extracted and measured as described in Materials and Methods. Biliary concentrations of cholesterol, phospholipids, and bile acids are shown in panels A, B, and C, respectively. Values are means ± SD. [a], [b], and [c] indicate significant difference from C57BL/6, apoCIKO, and CETPTg mice, respectively ($P < 0.05$; Mann-Whitney U-test).

concentration was significantly higher in apoCIKO mice than in control C57BL/6 mice (+35.6%, $P < 0.05$), with a tendency toward a rise in phospholipid concentration ($P = 0.08$). Bile acid concentration was unchanged (Fig. 3C), and the resulting cholesterol-to-bile salt ratio was 37.8% higher in apoCIKO mice compared with C57BL/6 mice (0.024 vs. 0.018, respectively; $P < 0.05$).

Again, the presence of CETP enforced the effects of apoC-I deficiency, with a marked elevation of the concentrations of all the respective lipids in the gallbladder bile of CETPTg/apoCIKO mice compared with that of CETPTg mice (Fig. 3A, B, C). Biliary cholesterol, phospholipid, and bile acid levels were increased by 87.7, 76.9, and 19.6%, respectively ($P < 0.05$ in all cases). Furthermore, the cholesterol-to-bile salt ratio, as well as the phospholipid-to-bile salt ratio, was increased by 54.3% (0.023 vs. 0.015, $P < 0.05$) and 48.1% (0.143 vs. 0.097, $P < 0.05$), respectively, in CETPTg/apoCIKO mice compared with CETPTg mice. The biliary bile acid composition in CETPTg/apoCIKO mice was similar to that of CETPTg mice (not shown).

Hepatic expression of genes related to cholesterol and triglyceride metabolism is normal in mice lacking apoC-I

The mRNA levels of the LDL receptor (*Ldlr*; LDL uptake), the LDL receptor-related protein (*Lrp*; VLDL remnant uptake), the VLDL receptor (*Vldlr*; VLDL uptake), and the scavenger receptor B type I (*Sr-bI*; uptake of HDL-derived cholesterol) were unaltered in apoCIKO mice compared with C57BL/6 mice (**Fig. 4A**). Furthermore, no difference in the gene expression of HL and LPL was observed between the two mouse strains. The pattern was similar in CETP-expressing mice, except for the *Lrp* gene, which showed a discrete 20.8% increase in CETPTg/apoCIKO mice compared with CETPTg mice ($P < 0.05$, Fig. 4A).

Hepatic expression of the HMG-CoA reductase gene, catalyzing the rate-controlling step of cholesterol synthesis, as well as mRNA levels of its key regulator, *Srebp2*, were unchanged in apoCIKO and CETPTg/apoCIKO mice compared with C57BL/6 and CETPTg mice, respectively (Fig. 4B). Similarly, the expression of the *Cyp7a1* gene, which encodes the rate-limiting enzyme in hepatic bile acid synthesis, as well as the gene expression of *Cyp27* involved in the alternative pathway, did not differ between

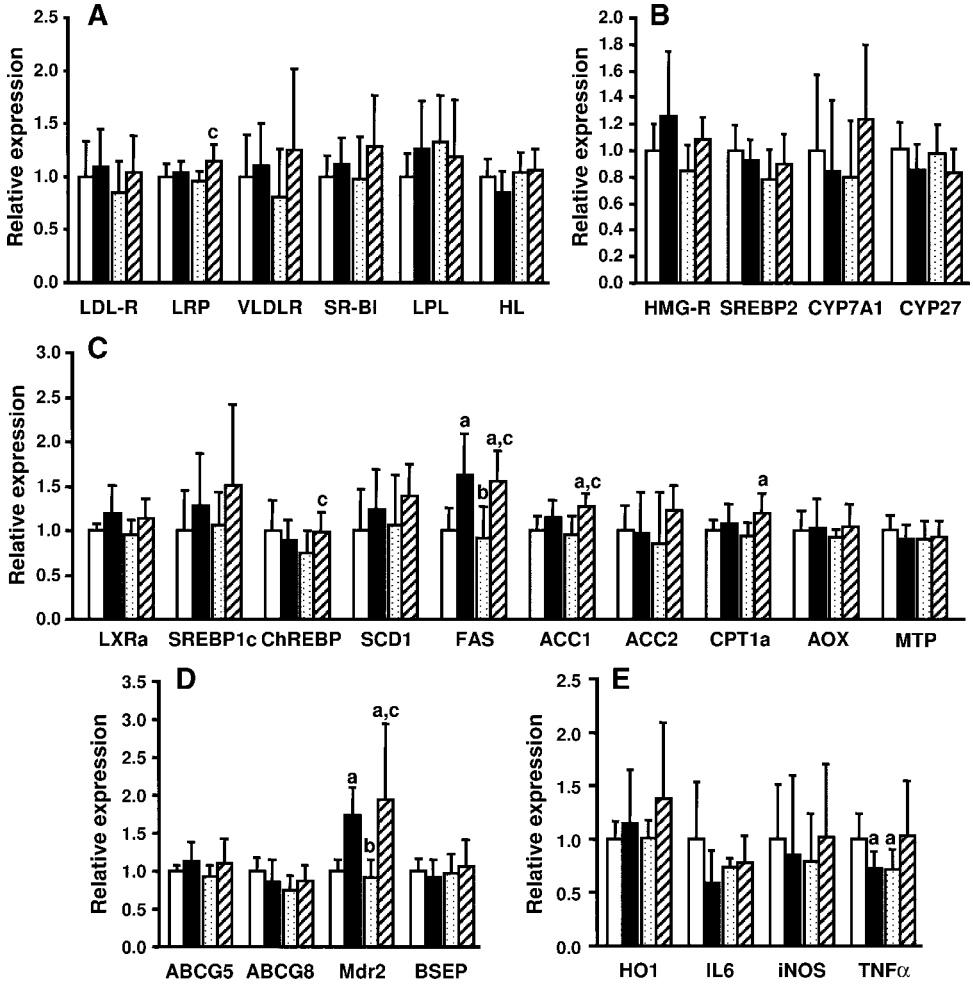

Fig. 4. Hepatic mRNA levels in C57BL/6, CETPTg, apoCIKO, and CETPTg/apoCIKO mice. Total RNA was extracted from the livers of C57BL/6 (open bars; n = 11), apoCIKO (filled bars; n = 8), CETPTg (dotted bars; n = 6), and CETPTg/apoCIKO mice (hatched bars; n = 6). Synthesis of cDNA and real-time quantitative PCR were performed as described in Materials and Methods. All data were standardized for 18S RNA expression. Expression levels in C57BL/6 mice were set to 1.00. A: Genes involved in lipoprotein metabolism. B: Genes involved in cholesterol synthesis and catabolism. C: Genes involved in fatty acid metabolism. D: Genes involved in bile formation. E: Genes involved in liver stress. Values are means ± SD. [a] Significantly different from C57BL/6 mice, $P < 0.05$; [b] significantly different from apoCIKO mice, $P < 0.05$; [c] significantly different from CETPTg mice, $P < 0.05$ (Mann-Whitney U-test).

C57BL/6 and apoCIKO mice or between CETPTg and CETPTg/apoCIKO mice (Fig. 4B).

With regard to hepatic triglyceride metabolism, the expression of the key regulators *Srebp1c*, *Lxrα*, and *Chrebp*, and their lipogenic target genes (*Acc1*, *Acc2*, and *Scd1*) were similar in both strains. Only the mRNA levels of *Fas* were increased in apoCIKO mice compared with C57BL/6 mice (+62.5%, $P < 0.05$; Fig. 4C). Finally, the gene expression of *Cpt1a* and *Aox*, both involved in β-oxidation, was unchanged in apoCIKO mice. In CETPTg/apoCIKO mice, hepatic mRNA levels of *Chrebp*, *Acc1*, and *Fas* were slightly increased (+32.2, +33.5, and +70.1%, respectively), compared with CETPTg mice ($P < 0.05$ in all cases, Fig. 4C) whereas the expression of *Scd1*, *Acc2*, *Srebp1c*, *Lxrα*, *Cpt1a*, and *Aox* was not significantly modified. Hepatic mRNA levels of *Mtp*, the key protein involved in VLDL assembly, were similar in the four groups of mice (Fig. 4C).

Finally, hepatic gene expression of the transporters involved in cholesterol secretion (*Abcg5* and *Abcg8*) and bile acid secretion (*Abcb11* or *Bsep*) into the bile did not differ between C57BL/6 and apoCIKO mice, whereas the expression of *Abcb4* (*Mdr2*), which drives phospholipid secretion into the bile, was 72.8% higher in apoCIKO mice compared with C57BL/6 mice ($P < 0.05$, Fig. 4D). Similarly, in CETP-expressing mice, *Abcg5/g8* and *Abcb11* expression was not altered by apoC-I deficiency, whereas the increase in *Abcb4* gene expression was more pronounced, with a 117.4% rise in CETPTg/apoCIKO mice compared with CETPTg mice ($P < 0.05$, Fig. 4B). This increase in *Abcb4* gene expression in the absence of apoC-I was also confirmed at the protein level, as shown by Western blot

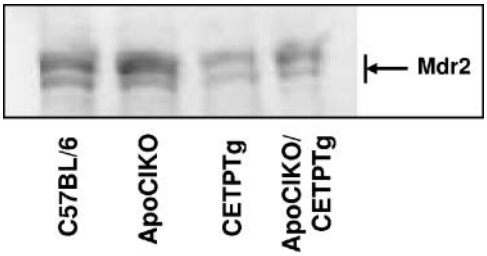

Fig. 5. Western blot analysis of Mdr2 protein levels in livers from C57BL/6, apoCIKO, CETPTg, and CETPTg/apoCIKO mice. Liver homogenates (protein, 20 μg) from C57BL/6, apoCIKO, CETPTg, and CETPTg/apoCIKO mice were subjected to gradient SDS-PAGE. Proteins were then blotted onto nitrocellulose membranes, and Mdr2 protein was subsequently detected, as described in the Materials and Methods section. Each lane represents six pooled liver samples. Mdr2 levels were assessed twice, each time on a different gel, with similar results.

on liver homogenates (**Fig. 5**), with approximate 37% and 45% increases in ABCB4 protein levels in apoCIKO and CETPTg/apoCIKO mice compared with C57BL/6 and CETPTg mice, respectively.

VLDL-triglyceride production by the liver and plasma markers of glucose and insulin metabolism are not modified in mice lacking apoC-I

To determine whether the dramatic accumulation of triglycerides in the liver was due to an impairment of the VLDL export system, VLDL-triglyceride production by the liver was determined in mice injected with P-407, a potent inhibitor of LPL (40). As shown in **Fig. 6**, VLDL-triglyceride production did not differ between groups.

Because insulin resistance is also a cause for lipid accumulation in the liver, plasma levels of glucose and insulin were assessed in the four groups of mice. As shown in **Table 2**, plasma glucose and insulin levels did not differ between the four groups, although a trend toward a decrease in glucose concentration could be observed in mice lacking apoC-I. Consequently, the calculated HOMA index, a marker of insulin resistance, was unchanged in mice lacking apoC-I (Table 2).

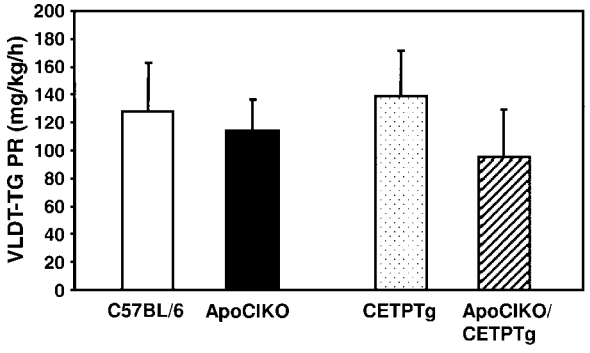

Fig. 6. VLDL triglyceride production rates in C57BL/6, CETPTg, apoCIKO, and CETPTg/apoCIKO mice. Poloxamer 407 (1,000 mg/kg body weight) was injected into fasted C57BL/6 (open bars), apoCIKO (filled bars), CETPTg (dotted bars), and CETPTg/apoCIKO mice (hatched bars) (six mice per group). Plasma triglyceride levels were determined during a 4-h period, and hepatic VLDL-TG production rates were calculated and subsequently corrected for body weight. Values are expressed as mg triglycerides/kg body weight/h and are means ± SD.

TABLE 2. Plasma glucose, insulin, ASAT, and ALAT levels in C57BL/6, apoCIKO, CETPTg, and CETPTg/apoCIKO mice

	C57BL/6	ApoCIKO	CETPTg	ApoCIKO/CETPTg
Glucose (mmol/l)	8.4 ± 0.6	6.8 ± 2.1	8.9 ± 0.9	7.5 ± 1.3
Insulin (μU/ml)	8.3 ± 1.0	8.5 ± 2.5	5.6 ± 3.7	6.8 ± 3.1
HOMA index	3.0 ± 0.4	2.5 ± 1.2	2.1 ± 1.4	2.2 ± 1.0
ASAT (U/l)	67 ± 16	82 ± 30	85 ± 7	102 ± 55
ALAT (U/l)	7 ± 6	5 ± 3	3 ± 2	7 ± 6
Bilirubin (μmol/l)	0.9 ± 0.7	1.0 ± 0.5	1.1 ± 0.7	0.9 ± 0.8

ALAT, alanine transaminase; ASAT, aspartate transaminase; HOMA, homeostasis model assessment. Blood samples were drawn from fasting C57BL/6 (n = 11), apoCIKO (n = 8), CETPTg (n = 6), and CETPTg/apoCIKO mice (n = 7) by cardiac puncture, and measurements were performed as described in Materials and Methods. HOMA index was calculated as [glucose (mmol/l) × insulin (μU/ml)]/22.5. All values represent means ± SD.

Markers of hepatic stress are not modified in mice lacking apoC-I

To determine whether apoC-I deficiency results in hepatotoxicity and appreciable liver damage, different markers of hepatic stress were measured. Plasma levels of bilirubin and of liver enzymes ASAT and ALAT were not different between groups (Table 2). Hepatic gene expression of heme oxygenase 1 (HO1; upregulated by oxidative stress), interleukin 6 (IL6; proinflammatory), as well as inducible nitric oxide synthase (iNOS; upregulated by inflammation) were identical between the four strains of mice. Expression of the tumor necrosis factor α (TNFα; proinflammatory) gene was even reduced in apoCIKO mice compared with C57BL/6 mice (Fig. 4E).

DISCUSSION

The present work reports for the first time a profound effect of apoC-I deficiency on hepatic lipid metabolism in mice. These effects were enforced in the presence of the human CETP transgene, emphasizing the close interaction between CETP and apoC-I in the processes involved in plasma-derived lipid uptake by the liver. When fed a chow diet, apoC-I-deficient mice display a significant increase in the cholesteryl ester content of the liver and a dramatic rise in its triglyceride content (Table 1, Fig. 2). This phenotype is in contrast with most other mouse models with altered expression of plasma apolipoproteins or hepatic receptors. Indeed, apoA-I-deficient mice (41) and mice overexpressing apoE (42, 43) or the LDL receptor (44, 45) show no difference in the hepatic cholesterol content compared with control mice. Conversely, mice overexpressing the hepatic SR-BI receptor (46) or deficient in apoE (47) show increased hepatic amounts of free cholesterol and severe changes in plasma lipid levels, whereas apoC-I-deficient mice showed an accumulation

of cholesteryl esters and displayed only a moderate decrease in HDL levels (Fig. 1, Table 1). Overall, these data show that apoC-I exerts its effect on hepatic lipid metabolism in a specific manner.

The expression of the HMG-CoA reductase and SREBP2 genes was not modified by apoC-I deficiency, whether CETP was expressed or not (Fig. 4). This indicates that the hepatic accumulation of cholesterol in the absence of apoC-I is in all likelihood not due to an increase in the endogenous synthesis by the liver. Cholesterol accumulation in the liver of apoC-I-deficient mice is not due to an impaired catabolism of cholesterol either, with no change in the expression of the *Cyp7a1* and *Cyp27* genes. Similar conclusions can be drawn for triglycerides, according to the gene expression pattern of enzymes involved in fatty acid metabolism: although the increase in *Fas* gene expression in apoCIKO and CETPTg/apoCIKO mice (Fig. 4) could contribute to the increase in hepatic triglycerides, it was not accompanied by a coordinated gene regulation of other enzymes involved in fatty acid synthesis, and the further accumulation of triglycerides in CETPTg/apoCIKO mice was not associated with a greater induction of *Fas* (Fig. 4B). Additionaly, the expression of the *Cpt1* and *Aox* genes coding for two key enzymes of β-oxidation, was not modified by the absence of apoC-I, indicating that the observed triglyceride accumulation is not due to an impaired catabolism of fatty acids. Overall, neutral lipid accumulation in apoC-I-deficient mice is due neither to increased lipid production nor to reduced lipid catabolism.

Biliary cholesterol concentration was increased in apoCIKO mice compared with control mice, and a similar trend could be observed for phospholipids. This excludes a reduced capacity for hepatobiliary cholesterol removal. The effect of apoC-I deficiency on biliary lipids was magnified in the presence of CETP, with further increases in cholesterol, phospholipid, and bile acid concentrations (Fig. 3). The increase in biliary cholesterol concentration was not related to higher gene expression of the Abcg5/g8 sterol transporters. The increase in bile acid concentration in CETPTg/apoCIKO mice could not be explained by modifications of the expression of Abcb11, encoding for the canalicular bile acid transporter, or Cyp7a1 (bile acid synthesis). However, mRNA levels of *Abcb4*, coding for the protein responsible for phospholipid secretion into the bile, were almost doubled in apoC-I-deficient mice (Fig. 4), and this increase could be confirmed at the protein level (Fig. 5). Because biliary secretion of cholesterol and that of phospholipids are interrelated (48), this increase in *Abcb4* gene expression can explain in part the rise in biliary cholesterol concentration in the case of apoC-I deficiency. The mechanism underlying the upregulation of *Abcb4* expression is not clear. On the one hand, it has been shown that Abcb4 expression can be induced by PPARα activation (49, 50), and the continuous turnover of the high triglyceride content in the livers from apoC-I-deficient mice might be the source of increased amounts of PPARα ligands (fatty acids). On the other hand, plasma free fatty acids, which are mainly taken up by the liver, were reduced in apoCIKO mice. Alternatively, the persistence of a high transhepatic bile acid flux may induce *Abcb4* expression via the activation of the nuclear receptor FXR (51–53). Overall, these results suggest that cholesterol in excess in the liver of apoC-I-deficient mice is at least in part eliminated via the bile.

Increased liver lipid levels can be due to impaired VLDL production, i.e., the export pathway of triglycerides from the liver to plasma and peripheral tissues. However, in our hands, apoC-I-deficient mice, whether expressing CETP or not, showed neither a reduction in VLDL-triglyceride production (Fig. 6) nor a modification of *Mtp* gene expression (Fig. 4C). These results are in line with previous studies in apoC-I-transgenic or apoC-I knock-out mice, which reported no impact of apoC-I expression on hepatic VLDL production (13, 15, 17). Accordingly, markers of insulin resistance and liver stress, two parameters that can affect VLDL production, were not modified between the four groups of mice (Table 2, Fig. 4E). Thus, the most likely explanation for the higher hepatic cholesterol and triglyceride content in apoC-I-deficient mice is an enhanced lipoprotein uptake from plasma by the liver. The gene expression of receptors involved in the uptake of HDL-, LDL-, VLDL- and remnant-derived cholesterol and triglycerides was not changed in apoC-I-deficient mice. This observation suggests that the cholesterol clearance capacity of the liver is maintained (Fig. 4A) and that the permissive effect on lipoprotein clearance does not occur via increased numbers of receptors, although a slight trend toward increased gene expression could be observed for all of them, but rather via enhanced interaction between lipoproteins and receptors. This view is strongly supported by studies conducted on perfused rat livers (8, 54), on isolated cells (10, 11, 55, 56), and in apoC-I-transgenic mice (14, 15), which have shown that apoC-I inhibits the clearance of triglyceride-rich lipoproteins and their remnants by impairing their interaction with LDLR, VLDLR, and LRP. In addition, apoC-I also displays antilipolytic properties by inhibiting LPL (13) and HL (57) in transgenic mice, thus reducing the processing of triglyceride-rich lipoproteins into remnants that are subject to hepatic uptake. Interestingly, the inhibitory effect of apoC-I on HL is specific to the apoC-I moiety that is located on the HDL fraction (57), and apoC-I inhibits the HL-mediated remodeling of HDL (58), a mechanism that facilitates the SR-BI-mediated cholesterol uptake from HDL (59, 60). Overall, all these lines of evidence indicate that apoC-I has a global inhibitory effect on cholesterol and triglyceride uptake by the liver via apoB-containing lipoproteins and, for cholesterol, probably also via HDL. Evidently, this suggestion requires further experimental proof. Remarkably, the proposed enhanced cholesterol uptake is not accompanied by reduced HMG-CoA reductase/LDLR expression, indicating partial compensation by increased biliary excretion and/or entry of plasma-derived cholesterol into specific, nonregulatory pools.

Importantly, the effects of apoC-I deficiency on hepatic lipid homeostasis were potentiated in the presence of CETP (Table 1, Fig. 3). The magnification of triglyceride

clearance from the plasma is reflected by the 300% increase in hepatic triglycerides (Table 1) as well as by the reduction of the abundance of triglycerides in the VLDL fraction in the plasma (Fig. 1). Furthermore, the significant increase in biliary phospholipid concentration might reflect the stimulation of an escape route for the overt excess of hepatic fatty acids in CETPTg/apoCIKO mice. In vivo, CETP promotes the net transfer of cholesterol from HDL to triglyceride-rich lipoproteins and the reciprocal enrichment of HDL with triglycerides (1, 2). Consequently, CETP, by allowing some cholesterol to be returned to the liver via the apoB-containing lipoproteins, offers an alternative pathway of hepatic clearance to the classical HDL-mediated reverse cholesterol transport (3).

The potentiation by CETP of the effects of apoC-I deficiency on the liver can be explained in terms of an enhanced return of lipids and lipoproteins to the liver by three complementary mechanisms. First, the CETP diverts some cholesterol from HDL to apoB-containing lipoproteins, providing an additional, more efficient way for clearance by the liver. Second, the reciprocal transfer of triglycerides from apoB-containing lipoproteins to HDL may participate, in combination with the enhanced actions of HL in the apoC-I-deficient state (57, 58), to a more efficient remodeling of these lipoproteins prior to HDL-cholesterol uptake via SR-BI (61). The increased HDL clearance in the presence of CETP is illustrated by the further reduction of the HDL fraction in CETPTg/apoCIKO mice versus CETPTg mice, compared with that of apoCIKO mice versus C57BL/6 mice (Fig. 1). Third, apoC-I itself is a potent inhibitor of CETP (16, 23, 62) and, in line with previous studies, the CETP activity is increased in CETPTg/apoCIKO mice compared with CETPTg mice (supplemental Table 1). Because the conditions of lipoprotein clearance are improved in the apoC-I-deficient context, the increased CETP activity itself may contribute to the enhancement of hepatic lipid uptake. Whether hepatocyte-derived CETP might directly mediate the selective uptake of HDL-derived cholesterol and thus facilitate the last step of centripetal cholesterol transport via HDL will deserve particular attention (63).

In conclusion, the present work shows for the first time that apoC-I deficiency leads to lipid accumulation in the murine liver as a result of an enhanced hepatic clearance of cholesterol and triglycerides. This phenotype was magnified by CETP expression, i.e., in the presence of a protein that facilitates some steps of the reverse cholesterol transport in this context. Although the potentially beneficial effect of apoC-I deficiency on liver lipid uptake might attenuate the marked reductions in HDL size and cholesterol content observed in CETPTg/apoCIKO mice (16), the hepatic accumulation of triglycerides might constitute an additional, yet unrecognized drawback of apoC-I deficiency.

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