ApoF knockdown increases cholesteryl ester transfer to LDL and impairs cholesterol clearance in fat-fed hamsters

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Abstract  Cholesteryl ester transfer protein (CETP) regulates intravascular lipoprotein metabolism. In vitro studies indicate that ApoF alters CETP function by inhibiting its activity with LDL. To explore in vivo the complexities driving ApoF’s effects on CETP, we developed a siRNA-based hamster model of ApoF knockdown. In both male and female hamsters on chow- or fat-fed diets, we measured lipoprotein levels and composition, determined CETP-mediated transfer of cholesteryl esters (CEs) between lipoproteins, and quantified reverse cholesterol transport (RCT). We found that apof knockdown in chow-fed hamsters had no effect on lipoprotein levels or composition, but these ApoF-deficient lipoproteins supported 50–100% higher LDL CETP activity in vitro. ApoF knockdown in fat-fed male hamsters created a phenotype in which endogenous CETP-mediated CE transfer from HDL to LDL increased up to 2-fold, LDL cholesterol increased 40%, HDL declined 25%, LDL and HDL lipid compositions were altered, and hepatic LDLR gene expression was decreased. Diet-induced hypercholesterolemia obscured this phenotype on occasion. In fat-fed female hamsters, ApoF knockdown caused similar but smaller changes in plasma CETP activity and LDL cholesterol. Notably, ApoF knockdown increased cholesteryl ester transfer of cholesteryl esters (CEs) between lipoproteins, and quantified reverse cholesterol transport (RCT). We found that apoF knockdown in chow-fed hamsters had no effect on lipoprotein levels or composition, but these ApoF-deficient lipoproteins supported 50–100% higher LDL CETP activity in vitro. ApoF knockdown in fat-fed male hamsters created a phenotype in which endogenous CETP-mediated CE transfer from HDL to LDL increased up to 2-fold, LDL cholesterol increased 40%, HDL declined 25%, LDL and HDL lipid compositions were altered, and hepatic LDLR gene expression was decreased. Diet-induced hypercholesterolemia obscured this phenotype on occasion. In fat-fed female hamsters, ApoF knockdown caused similar but smaller changes in plasma CETP activity and LDL cholesterol. Notably, ApoF knockdown impaired HDL RCT in fat-fed hamsters but increased sterol excretion in chow-fed animals. These in vivo data validate in vitro findings that ApoF regulates lipid transfer to LDL. The consequences of ApoF knockdown on lipoproteins and sterol excretion depend on the underlying lipid status. By minimizing the transfer of HDL-derived CE to LDL, ApoF helps control LDL cholesterol levels when LDL clearance mechanisms are limiting. ApoF knockdown increases cholesteryl ester transfer to LDL and impairs cholesterol clearance in fat-fed hamsters. J. Lipid Res. 2019. 60: 1868–1879.

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ApoF is a minor apolipoprotein found predominately on HDL but also bound to LDL (1, 2). The human APOF gene encodes a 308 amino acid protein containing a 22 amino acid signal peptide and a 286 amino acid proprotein. Pro-ApoF is cleaved extracellularly by proprotein convertase PC7 (3) to produce ApoF, which is composed of 162 amino acids derived from the carboxy-terminal end of ProApoF (4).

Cholesteryl ester transfer protein (CETP) facilitates the movement of cholesteryl ester (CE) and triglyceride (TG) between lipoproteins. Through its capacity to exchange CE in one lipoprotein for TG in another (5), CETP modifies lipoprotein composition and controls lipoprotein metabolism (6–8). Its role in modulating lipoprotein metabolism and altering HDL levels in humans has been extensively studied (9). CETP activity is also critical to whole-body cholesterol clearance mechanisms in humans because 70% of tissue-derived cholesterol is first transferred (as CE) from HDL to VLDL and LDL by CETP prior to its removal by the liver for eventual excretion (10, 11).

Factors altering CETP activity have a high potential for modifying intravascular lipid metabolism. We previously identified a plasma protein that regulates CETP activity (12), and subsequently determined that this protein, originally called lipid transfer inhibitor protein, is identical to ApoF (4). Although we initially considered ApoF to be a general CETP inhibitor, in vitro studies over the ensuing years revealed a more complex function (1, 2, 13–15). Those in vitro studies demonstrated that ApoF preferentially suppresses CETP-mediated lipid transfers involving LDL. These findings led us to hypothesize that ApoF regulates lipoprotein metabolism by impeding the CETP-mediated transfer of HDL-derived CE to LDL and redirecting this lipid to VLDL.

Abbreviations:  CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; PL, phospholipid; qPCR, quantitative PCR; RCT, reverse cholesterol transport; S/C, surface to core; TC, total cholesterol; TG, triglyceride.

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While ApoF studies have been reported in genetically modified mice (16, 17), none of the noted effects are related to CETP regulation because mice do not express this protein. In order to properly investigate the hypothesis above, we selected an animal model where both of these proteins are naturally expressed. Hamsters express CETP and ApoF (18, 19), synthesize abundant LDL (an important ApoF target), and exhibit a lipoprotein profile similar to humans upon fat/cholesterol feeding (19, 20). We report here the development of a hamster model of siRNA-mediated ApoF knockdown. These in vivo studies confirm the hypothesized function of ApoF in regulating CE transfer into LDL by CETP, demonstrate its role in intravascular lipid metabolism, and show that ApoF depletion alters reverse cholesterol transport (RCT).

METHODS

ApoF knockdown in cells

Golden Syrian hamster (Mesocricetus auratus) APOF cDNA (XM_005079728.1), engineered to contain a C-terminal myc tag, was synthesized de novo by GenScript USA Inc. (Piscataway, NJ) and inserted into pcDNA3 using HindIII and BamHI restriction sites. A proprietary siRNA design algorithm was used to identify candidate sequences, which were then synthesized by Sigma-Aldrich (St. Louis, MO).

HEK293 cells (American Type Culture Collection, Manassas, VA) at 80% confluence were washed with warm Opti-MEM (Life Technologies Corp., Grand Island, NY) and then transfected with pcDNA3-hamster APOF-myc in Lipofectamine 2000 (Life Technologies Corp.) following the manufacturer’s instructions. After 5 h, medium containing 10% FBS was added and the cells were incubated overnight. The following day, the cells were washed with warm Opti-MEM and then transfected with either universal negative control duplex siRNA or with duplex hamster APOF siRNA (Sigma-Aldrich) using Mission siRNA transfection reagent (Sigma-Aldrich) following the manufacturer’s protocol.

For injection into hamsters, jugular vein catheters were flushed with sterile saline followed by injection of either control or APOF siRNA-Invitofectamine complexes, a second saline flush, and then reblocked with heparin/glycerol catheter lock solution (Braintree Scientific, Inc., Braintree, MA). Animals were either maintained on standard chow or switched to a fat/cholesterol diet consisting of chow diet supplemented with 20% hydrogenated coconut oil and 0.12% cholesterol (Envigo, Madison, WI). Chow and fat/cholesterol diets were provided ad libitum for the duration of the study. Animals were euthanized at the indicated time. Blood, collected in EDTA-containing tubes, was centrifuged to yield plasma.

Western blot for ApoF and ApoAI

ApoF in hamster plasma and isolated HDL were quantified by Western blot as previously described (19). Bands were visualized by Western Lightning Plus ECL reagent (PerkinElmer, Inc.). ApoAI was used as a loading control. For this, ApoF blots were stripped (Restore stripping buffer; Sigma-Aldrich) and reprobed with goat anti-human apoAI (Sigma-Aldrich) followed by rabbit anti-goat IgG HRP-conjugated secondary antibody, each used at 1/10,000 dilution, and bands detected with ECL reagent. Chemiluminescence was captured on a digital imager (GE Healthcare, Marlborough MA) and quantified by ImageJ (National Institutes of Health).

Isolation and radiolabeling of lipoproteins

Human and hamster plasma lipoproteins were isolated by sequential ultracentrifugation as described by Havel, Eder, and Braggdon (21). In some instances, human lipoproteins were doubly labeled with 3H-TG and 14C-CE by a dispersion method (12) before their isolation from plasma. Alternatively, purified LDL and HDL isolated from either human or hamster plasma were labeled with 3H-CE by CETP-mediated transfer of the radiolabel from phosphatidylcholine/cholesterol liposomes to the lipoprotein (14, 22), followed by re-isolation of the lipoprotein within its original density limits (21). CETP used for this labeling method was recombinant human CETP contained in the conditioned media from HEK293 cells transfected with pcDNA3-human CETP (23). This approach avoided contaminating isolated lipoproteins with ApoF, which is present at low levels in the human plasma-derived partially purified CETP typically used for this labeling procedure. 3H-CE ([1,2-3H(N)]cholesterol oleate), 14C-CE (cholesterol-[1-14C] oleate), and 3H-TG ([9,10-3H(N)]triolein) were purchased from PerkinElmer, Inc. (Waltham, MA).
Lipid transfer activity between isolated lipoproteins

Lipid transfer from \(^{3}H\)-CE-labeled hamster LDL to unlabeled hamster HDL was assayed as previously described (24, 25). CE transfer from LDL to HDL was mediated by recombinant human CETP (23). At the end of the assay, LDL was precipitated (25) and the percentage of \(^{3}H\)-CE transferred to HDL was calculated (24).

Endogenous plasma CETP activity

CETP transfer activity between endogenous lipoproteins in intact plasma was assayed by spiking native plasma with a small amount of radiolabeled lipoprotein. To measure the transfer of CE from LDL to HDL, hamster plasma was combined with human \(^{3}H\)-CE LDL (<2% of the endogenous LDLs) and incubated at 37°C for 0 (assay blank) or 4.5 h. At the indicated time, triplicate 20 µl aliquots were combined with 480 µl of 50 mM Tris-HCl, 150 mM NaCl (pH 7.4), and 200 µl 3.5% BSA followed by the addition of sodium phosphate and MnCl2, as previously described (25), to precipitate VLDL and LDL. HDL-associated \(^{3}H\)-CE in the supernatant was quantified by liquid scintillation counting. Assays of \(^{3}H\)-CE transfer from hamster HDL to the plasma LDL-VLDL fraction followed the same general design as described above. Because hamster HDL contains significant ApoF, for these assays HDL was isolated from control, APOF siRNA group 1, and APOF siRNA group 2 animals (as defined later), radiolabeled, and added to hamster plasma of the same type. After incubation, LDL and LDL were precipitated, the pellet containing these lipoproteins was washed once as previously described (26), and \(^{3}H\)-CE in the subsequent pellet was measured. For both assays, CE transfer was expressed as the micrograms of CE transferred per milliliter of plasma. This was calculated for each plasma sample by multiplying the fraction of radiolabeled CE transferred to the acceptor (after subtracting zero time blank values) by the concentration of CE in the donor lipoprotein fraction of that plasma.

CETP quantification

Hamster plasma CETP levels were determined by assaying plasma samples in the presence of excess exogenous lipoproteins. Under these conditions, CETP transfer activity correlates well with CETP mass (19, 27). Briefly, hamster plasma (10 µl) was combined with 100 µg \(^{3}H\)-TG, \(^{14}C\)-CE labeled human LDL, 100 µg unlabeled human HDL, and Tris-buffered 1% BSA in 0.7 ml final volume as previously described (19). After 5 h at 37°C, lipid transfer was stopped by the addition of 200 µl of 0.45 M sodium phosphate (pH 7.4) and 300 µl of 0.1 M MnCl2. After centrifugation to pellet the VLDL/LDL precipitate, radiolabel in the HDL-containing supernatant was determined by scintillation counting. Transfer was calculated as previously described (24).

Plasma lipoprotein separation by FPLC

Plasma lipoproteins were fractionated by tandem Superose 6 columns as previously described (19, 28). The column eluate was continuously combined with Infinity cholesterol detection reagent (Thermo Fisher Scientific) and the reaction product was monitored at 505 nm. VLDL, LDL, and HDL peaks were identified based on the elution profile of hamster lipoproteins isolated by ultracentrifugation. The area under each lipoprotein absorption peak was measured and reported as the fraction of cholesterol recovered in each lipoprotein fraction. These values were multiplied by the total cholesterol (TC) concentration of the plasma sample to determine the plasma concentration of each lipoprotein fraction.

HDL-CE RCT

Human HDL was labeled with \(^{3}H\)-CE as described above. HDL was extensively dialyzed versus endotoxin-free PBS and then sterile filtered (0.22 µm). Seventy-two hours after injection of siRNA to initiate an experiment, hamster jugular vein catheters were flushed with saline, injected with \(^{3}H\)-CE HDL (300 µl containing ~125 µg protein and 2 µCi \(^{3}H\)) followed by a second saline flush and reblooding the catheter with lock solution. Animals were maintained on their existing diet, and transferred to cages with wire-bottom platforms to facilitate complete feces collection. Animals were euthanized 4 h after \(^{3}H\) injection. A segment of fresh liver was minced and homogenized (100 mg of liver per milliliter of water) with a Tissue-Tearor (BioSpec Products, Bartlesville, OK). The remaining portion of liver was immediately snap-frozen in liquid N2 and stored at −80°C for future mRNA analysis. Feces were dried overnight at 55°C, weighed, suspended in 50% ethanol (100 mg of feces per milliliter), and homogenized as above. The \(^{3}H\) content of liver and fecal homogenates was determined by liquid scintillation counting of triplicate aliquots. Samples were counted a second time after the addition of a \(^{3}H\) internal standard to quantitate quenching caused by sample color. Original sample \(^{3}H\) values were then corrected for differences in counting efficiency. Plasma \(^{3}H\) was determined by direct scintillation counting of whole plasma. Total plasma \(^{3}H\) calculations assumed a plasma volume of 3.5% of body weight.

mRNA qPCR

Liver tissues were homogenized by a Tissuelyser II (Qiagen, Germantown, MD). Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized using random primers and reverse transcriptase (Promega, Madison, WI). Quantitative (q)PCR was performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) and a StepOnePlus RT PCR system (Life Technologies Corp.). The qPCR primers are listed in Table 1. mRNA values were normalized to ACTB. Gene expression was calculated using the 2^-ΔΔCT method (29) and reported relative to control cells.

Analytical methods

Protein was measured by a modification of the Lowry et al. method (30) with BSA as standard. TC, free cholesterol (FC), and TG were quantified by enzyme-based kits from Thermo Fisher Scientific (TC, TG) or Wako Diagnostics Inc., Mountain View, CA (FC). CE was calculated as TC minus FC times 1.69 to adjust for the fatty acid contained in this molecule. Phospholipid (PL) phosphorus was determined chemically by the method of Bartlett (31). For plasma protein analysis, samples were depleted of albumin and IgG by a PureProteome Albumin-IgG Depletion kit (Sigma-Aldrich). The remaining proteins were separated by SDS-PAGE (32) and stained with colloidal Brilliant Blue G (Sigma-Aldrich). HDL particle size distribution was determined by native gradient gel electrophoresis (33, 34). Statistical analysis was performed by unpaired t-test (Instat 3; GraphPad Software, San Diego, CA). P values <0.05 were considered statistically significant. Group sizes are indicated in the tables and figures.

RESULTS

ApoF knockdown

An algorithm-based strategy was used to identify candidate siRNA sequences for APOF. Three candidate siRNA constructs were synthesized (Table 2), and their ability to inhibit the synthesis of hamster ApoF was evaluated in HEK293 cells transiently transfected with a myc-tagged hamster APOF plasmid as described in the Methods. All
three siRNAs reduced ApoF synthesis but the S1 siRNA was the most effective, reducing ApoF protein secreted into the media by more than 75% under the conditions tested (4 μg APOF plasmid, 24 pmoles siRNA). Subsequent studies evaluated the in vivo knockdown potential of S1 siRNA. The S1 construct is hereafter referred to as APOF siRNA.

For initial in vivo studies, the impact of APOF siRNA on hamster plasma ApoF levels was evaluated 5 days after siRNA injection because this time frame was desirable for future studies. The liposome-based siRNA delivery strategy used primarily targets the liver, which is the principal site of ApoF gene expression in hamsters (19). An initial dosage of 1 mg siRNA per kilogram was used. Under these conditions, APOF siRNA reduced plasma and HDL ApoF protein levels by 90% compared with control siRNA (Fig. 1A). In a dose response study, 0.5 mg APOF siRNA per kilogram was found to be equally effective as 1 mg/kg in reducing ApoF protein levels (Fig. 1B). At the 0.5 mg/kg dosage, ApoF levels were consistently reduced ~90% for up to 5 days after injection; however, ApoF knockdown was markedly less effective and more variable thereafter (Fig. 1C). APOF siRNA treatment did not have any apparent effect on the level of other plasma proteins, which are primarily of hepatic origin (Fig. 1D). Also, a specific assay for plasma CETP revealed no change in the level in this ApoF target following siRNA treatment (Fig. 1E). ApoF knockdown in these chow-fed animals had no apparent effect on total plasma cholesterol or its distribution among lipoproteins (Table 3) or on the lipid composition of LDL and HDL (data not shown).

Impact of ApoF depletion on CETP activity in vitro

Our previous studies with isolated lipoproteins showed that CETP activity, especially lipid transfer involving LDL, is reduced by the addition of purified ApoF (1, 2, 14). To determine whether reducing lipoprotein ApoF content had the opposite effect, LDL and HDL were isolated from control and APOF siRNA-treated male hamsters and assayed in vitro with recombinant CETP. CETP activity was measured in assays where LDL was the CE donor and HDL the acceptor. CETP-dependent CE transfer was 2-fold higher when the assay donor and acceptor lipoproteins were depleted of ApoF (Fig. 2A). In other experiments, compared with assays that contained LDL and HDL from the same treatment group, CETP-mediated lipid transfer was intermediate when one of the lipoproteins in the transfer assay contained native levels of ApoF (i.e., isolated from control animals) and the other was ApoF-depleted (Fig. 2B). Because active ApoF is bound to LDL (1), these data suggest that LDL can acquire ApoF from HDL when ApoF-deficient LDL is incubated with native HDL. Relative to control lipoproteins, the increase in CETP activity caused by ApoF depletion varied between lipoprotein preparations. This may arise from variations in the ability of control LDL preparations to bind ApoF. Overall, these data show that ApoF-deficient LDL and HDL support greater CETP activity than their ApoF-replete counterparts.

Effect of ApoF depletion on lipoproteins in fat/cholesterol-fed male hamsters

CETP activity is strongly influenced by plasma TG levels and is typically not rate limiting when TG-rich lipoprotein levels are low (35, 36). Because ApoF depletion did not alter lipoproteins in chow-fed animals, we performed similar studies in animals fed a diet enriched in fat and cholesterol. Although this diet increases hamster plasma ApoF protein levels (19), the siRNA knockdown strategy remained effective at reducing plasma ApoF (Fig. 3A).

Based on changes in LDL and HDL cholesterol, two distinct phenotypes were observed in APOF siRNA-treated animals. Representative lipoprotein profiles of control and both ApoF knockdown groups are shown in Fig. 3B–D. In APOF siRNA group 1 animals, LDL cholesterol levels were modestly reduced compared with control animals, whereas the levels of other lipoprotein classes were unchanged (Table 4). In contrast, in ApoF-deficient animals with a group 2 phenotype, LDL cholesterol levels were 40% higher and HDL cholesterol levels decreased by 25% (Table 4). Compared with control and group 1 ApoF knockdown animals, the ratio of LDL cholesterol to HDL cholesterol was 1.8-fold higher in ApoF knockdown animals with the group 2 phenotype (Fig. 4A). Overall, group 2 animals showed a shift in cholesterol distribution from HDL into ApoB-containing lipoproteins, whereas group 1 animals did not. These two ApoF siRNA phenotypes did not arise from differences in residual plasma ApoF levels, which were 14.5 ± 5.0% and 9.3 ± 2.7% of control values in groups 1 and 2, respectively.

### Table 1. qPCR primers for the indicated golden Syrian hamster (Mesocricetus auratus) genes

| Gene | Forward Primer 5′ to 3′ | Reverse Primer 5′ to 3′ |
|------|-------------------------|-------------------------|
| ACTB | GGTGGATCACCAAGGGGAGGTT | CTGAGGAAAGCGCCTAGAC |
| CYP2A1 | TACTAGCTAGCTAAGGAGGCTC | CCATGCCAGTCGGGGTCTG |
| HMGR | GCTAGGGTTCTAGGGAGGCT | CCAACATCTTGGGCAAG |
| LDLR | AGAGCAGGCTACAGGCTCGAGG | TCTCGTCATGCTGGGGGTT |
| MTP | ATGCCTGCTGCTGTTACACAA | AGCATCAGGAACATCCGATT |
| SCARB1 | ATGCTCCTGCTGCTGCTC | CTCAGCACTTGTGGCTTC |

### Table 2. siRNA duplexes

| APOF51 | CAGUGCGUGCUCUUAAGGCC[dT][dT] | UGGCUUAAAGCGACAGU[dT][dT] |
|--------|--------------------------------|--------------------------|
| APOF52 | CCGGCUAGUCGGUCUA[dT][dT] | UCAUAAACCGACUAGG[dT][dT] |
| APOF53 | CUGCCUUUGGGUUGAGA[dT][dT] | UCUCUGAGGAGCAAG[dT][dT] |

Shown are the 5′ to 3′ ribonucleotide sequence of APOF siRNA duplexes with two overhanging deoxythymidine residues on each 3′ end.
When lipoprotein levels were quantified by their protein content instead of their cholesterol content, *ApoF* siRNA group 1 animals continued to show reduced LDL, whereas group 2 LDL protein levels tended to be higher, but this increase did not reach statistical significance compared with controls (Fig. 4B). The protein content of LDL is largely due to ApoB. Because LDL contains a single ApoB molecule per particle, these data indicate that the number of LDL particles is decreased in group 1 animals. In contrast, in both ApoF siRNA group 1 and 2 animals, HDL protein levels were reduced by 25–30% (Fig. 4C).

Analysis of the lipid components of LDL also shows differences in the chemical composition of LDL from *ApoF* siRNA groups 1 and 2 animals (Fig. 5A–F). LDL from *ApoF* group 1 animals contained more FC and PL but less TG than control LDL. As a result, the ratio of surface to core (S/C) components in these LDLs increased compared with the control. An increase in this ratio indicates that group 1 LDLs are smaller than control LDLs. By comparison, group 2 LDLs have elevated CE content compared with group 1 LDLs and reduced TG content, resulting in a 2.7-fold increase in the CE/TG ratio of these LDLs but no change in the sum of these core lipids or in the calculated S/C ratio relative to the control. The changes in group 2 LDLs are consistent with increased transfer of CE into these particles.

Changes in HDL composition, in comparison to LDL, were small (Table 5). HDLs from both ApoF knockdown groups have elevated PL content, and group 1 HDLs have higher TC content, reflecting a modest increase in CE content. These changes in HDL lipid composition and loss of ApoF protein had no detectable effect on the distribution of HDL among its five recognized size subfractions (Table 5) or the functional properties of HDL as assessed in cholesterol efflux assays (not shown). Because HDL size was not affected, the reduction in *ApoF* group 1 and 2 HDL protein levels noted above indicates that HDL particle number is reduced in these animals.

**Effect of ApoF depletion on CE transfer in plasma**

Because ApoF knockdown in fat/cholesterol-fed male hamsters produced two distinct lipoprotein phenotypes, we questioned whether these phenotypes might arise from differences in plasma CETP activity. Differential CETP activity would explain the observed changes in CE content and CE/TG ratio in group 1 versus 2 LDLs because these lipids are substrates for CETP. These phenotypes were not due to the quantity of CETP present in plasma (Fig. 6A). However, CETP activity is influenced by the chemical properties of its lipoprotein targets (37–42). To measure plasma CETP activity under physiologic conditions, native plasmas were spiked with 3H-CE-labeled hamster HDL and the transfer of CE from HDL to the VLDL + LDL fraction was quantified. CE transfer from HDL was increased 20% in *ApoF* siRNA group 2 plasmas compared with the control, but was decreased in *ApoF* siRNA group 1 animals (Fig. 6B). While this assay measures the overall extent to which CETP promotes the removal of CE from HDL, it likely underestimates the impact of removing ApoF because ApoF has little impact on HDL to VLDL transfers (13). To quantify CE transfer between HDL and LDL specifically, this transfer event was measured by adding a small quantity of 3H-CE-labeled LDL to plasma and measuring the transfer of radiolabeled CE to HDL. CE transfer between LDL and HDL was increased 2-fold in *ApoF* siRNA group 2 plasmas compared with the control; however, this transfer was modestly reduced in *ApoF* siRNA group 1 (Fig. 6C).
It is noteworthy that the two distinct plasma CETP activity phenotypes observed here in APOF siRNA animals were not as evident in animals fed the fat/cholesterol diet for a shorter time. Instead of the near equal distribution of animals between groups 1 and 2 observed above, in these ApoF-depleted animals, four of five had increased plasma CETP activity like group 2 animals. This suggests that the divergent CETP activity phenotypes appear as hyperlipidemia develops.

The higher activity of CETP in LDL-HDL transfer assays in APOF siRNA group 2 but not group 1 animals is consistent with the observation that group 2 animals have larger alterations in LDL and HDL levels. When the relationship between endogenous CETP activity and plasma lipoprotein levels was assessed for the three experimental groups, there was a direct relationship between steady-state plasma LDL cholesterol and HDL cholesterol concentrations and endogenous CETP transfer activity. For both assays of CETP activity described above, the transfer of donor lipoprotein CE was positively associated with plasma LDL cholesterol concentrations and negatively associated with plasma HDL cholesterol levels (Fig. 6D, E).

APOF siRNA group 1 plasmas have the lowest endogenous CETP activity with correspondingly low LDL cholesterol and high HDL cholesterol levels, whereas group 2 plasmas have the highest CETP activity, which is associated with the lowest HDL cholesterol levels and the highest LDL cholesterol levels. Control plasmas were intermediate in this continuum.

**ApoF knockdown in female hamsters**

The existence of two distinct phenotypes in APOF knockdown fat/cholesterol-fed male hamsters, but not in control animals, suggests an interaction between diet and ApoF deficiency. To examine the role of diet-induced changes on the divergent ApoF deficiency phenotypes, similar studies were performed in female hamsters. Previous studies have shown that female hamsters are relatively resistant to diet-induced changes in plasma lipids (43). Like male hamsters, APOF siRNA reduced plasma ApoF levels to \( \leq 10\% \) of control (Fig. 7A). Plasma CETP levels were unchanged (Fig. 7B). Unlike male hamsters, however, ApoF knockdown in female hamsters created a single lipoprotein phenotype. Depletion of ApoF increased plasma cholesterol by 20%, which was due to an increase in LDL cholesterol (Fig. 7B). These changes are similar, but lower in magnitude, to those observed in male APOF siRNA group 2 animals. Also, like male group 2 animals, ApoF knockdown in female hamsters affected LDL lipoprotein composition. LDLs from ApoF-deficient female hamsters were enriched in PL content and reduced in TG content. Although the increase in CE content did not reach statistical significance, their activity with correspondingly low LDL cholesterol and high HDL cholesterol levels, whereas group 2 plasmas have the highest CETP activity, which is associated with the lowest HDL cholesterol levels and the highest LDL cholesterol levels. Control plasmas were intermediate in this continuum.

**TABLE 3. Plasma lipoprotein levels in chow-fed male hamsters**

| siRNA     | Plasma Cholesterol | VLDL | LDL | HDL |
|-----------|-------------------|------|-----|-----|
|            | mg/dl             | mg cholesterol/dl |
| Control    | 96.6 ± 6.2        | 5.2 ± 10.7 | 42.1 ± 3.9 | 49.3 ± 3.8 |
| APOF       | 94.1 ± 3.0        | 3.1 ± 0.5 | 38.2 ± 2.5 | 52.8 ± 2.3 |

The distribution of cholesterol among lipoprotein classes was determined by FPLC chromatography of plasmas from hamsters 5 days after injection of 0.5 mg/kg control or APOF siRNA. Results are the mean ± SEM, \( n = 4 \) per group.

Fig. 2. Effect of ApoF deletion on CETP activity between LDL and HDL. CE transfer activity from isolated \(^3\)H-CE-labeled hamster LDL to unlabeled hamster HDL (equal amounts based on cholesterol content) mediated by recombinant human CETP was measured as described in the Methods. Lipoproteins were isolated from hamster plasmas collected 5 days after a 0.5 mg/kg siRNA injection. A: CE transfer activity between LDL and HDL isolated from animals receiving the same siRNA treatment. B: Donor LDLs and acceptor HDLs isolated from the indicated treatment groups were combined and assayed for CE transfer activity. + ApoF LDL and HDL were from control siRNA-treated animals. −ApoF LDL and HDL were isolated from APOF siRNA-treated animals. Data are expressed as mean ± SD. \( *P < 0.05, **P < 0.01 \).

**Fig. 3.** Plasma cholesterol distribution among lipoproteins in fat/cholesterol-fed animals after ApoF knockdown. Male hamsters consuming a fat/cholesterol diet were injected with 0.5 mg/kg siRNA and then euthanized at day 5. A: Western blot of ApoF in representative plasmas from control and APOF siRNA-injected animals. B: FPLC gel filtration profile of plasma cholesterol from an animal receiving control siRNA. V, L, and H designations indicate elution positions of VLDL, LDL, and HDL, respectively. C: FPLC cholesterol elution profile of a representative animal from APOF siRNA group 1. D: FPLC cholesterol elution profile of a representative animal from APOF siRNA group 2.
mRNA levels were unchanged. Markers of hepatic HMGCR compared with group 1 animals or controls (Fig. 8B). Data are expressed as mean ± SEM. *P < 0.05 versus control.

In contrast to that seen in fat/cholesterol-fed male hamsters, endogenous plasma CE transfer activity was consistently increased in ApoF-deficient fat/cholesterol-fed female hamsters (Fig. 7D). This increase in plasma CETP transfer activity in ApoF knockdown female hamsters is consistent with the single lipoprotein phenotype observed in these animals. Together, these data suggest that the form of the group 1 phenotype in male ApoF siRNA animals is due to a unique interaction between ApoF deficiency and significant diet-induced changes in lipoproteins, which does not occur in diet-resistant female hamsters.

Hepatic gene expression in ApoF knockdown animals

Fat/cholesterol feeding led to the expected downregulation of hepatic LDLR and HMGCR mRNA levels (Fig. 8A). In contrast, this diet increased SCARB1 mRNA 2-fold, whereas two genes involving in hepatic processing of cellular cholesterol and fatty acids, CYP7a1 and MTTP, were unchanged. We questioned whether expression of these genes might be different in fat/cholesterol-fed male hamsters where enhanced CE transfer from HDL increased plasma LDL cholesterol levels (i.e., group 2 animals). Interestingly, in group 2 animals, hepatic mRNA levels for SCARB1, LDLR, CYP7a1, and MTTP were reduced ~50% compared with group 1 animals or controls (Fig. 8B). HMGCR mRNA levels were unchanged. Markers of hepatic inflammatory status, TNFα and F4/80 (ADGRE1), were not different in the two ApoF siRNA phenotype groups compared with the control (not shown). Overall, these findings suggest that the altered plasma lipoprotein content of group 2 animals significantly alters how liver gene expression is modified by circulating lipids.

Impact of ApoF depletion on RCT

Most plasma CE is derived from FC that is esterified on the HDL surface by lecithin:cholesterol acyltransferase. The above data indicate that ApoF siRNA group 2 animals have enhanced capacity to transfer this CE to other lipoproteins. The impact of this difference in HDL-CE metabolism on sterol excretion was assessed by a RCT assay. 3H-CE-labeled HDL was injected into fat/cholesterol-fed male hamsters and the recovery of 3H in plasma, liver, and feces was determined after 48 h. Plasma 3H was higher in ApoF siRNA group 2 male hamsters compared with either ApoF siRNA group 1 or controls (Fig. 9A). However, the percentage of HDL-derived CE excreted into feces was decreased by ~40% in ApoF siRNA group 2 animals compared with other groups. Expression of these fecal 3H values as milligrams of HDL-CE excreted into feces, instead of as the percent of dose, yielded a larger reduction (55%) in the amount of HDL-CE delivered to feces in ApoF siRNA group 2 animals (Fig. 9B). Such calculations assume that the injected 3H-CE remains associated with HDL, which is not the case in animals expressing CETP. Calculations of fecal CE that make the opposite assumption, i.e., that the injected 3H-CE instantaneously equilibrates with the CE in all plasma lipoproteins, still show that the recovery of 3H in feces in group 2 animals is reduced 33% compared with control or group 1 animals (Fig. 9B). In reality, the in vivo transfer of 3H-CE from HDL to other lipoproteins during

### Table 4. Plasma lipoprotein levels in fat/cholesterol-fed male hamsters

| siRNA Group | Cholesterol mg/dl | VLDL LDL mg cholesterol/dl | HDL mg cholesterol/dl |
|-------------|-------------------|-----------------------------|-----------------------|
| Control     | 281.6 ± 13.5       | 34.1 ± 4.1                  | 142.8 ± 6.4           |
| ApoF group 1| 237.6 ± 11.8       | 30.1 ± 2.5                  | 115.9 ± 5.8           |
| ApoF group 2| 324.6 ± 21.0       | 47.1 ± 7.3                  | 199.5 ± 17.8          |

The distribution of cholesterol among lipoprotein classes was determined by FPLC chromatography on plasmas isolated from hamsters 5 days after injection of 0.5 mg/kg control or ApoF siRNA. The results are the mean ± SEM of values from n = 6, 7, and 5 control, ApoF group 1, and ApoF group 2 animals, respectively.

**Fig. 4.** Altered plasma LDL and HDL levels in ApoF knockdown in male animals. Lipoproteins were isolated from fat/cholesterol-fed hamsters 5 days after receiving 0.5 mg/kg control or ApoF siRNA. A: Ratio of LDL cholesterol (LDLc) to HDL cholesterol (HDLc) in plasma. B: Plasma LDL protein levels. C: Plasma HDL protein levels. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01.

**Fig. 5.** Effect of ApoF knockdown on LDL composition. Analysis of LDL isolated from fat/cholesterol-fed male hamsters 5 days after receiving 0.5 mg/kg control or ApoF siRNA. A–E: LDL content of the indicated lipid component relative to LDL protein. F: LDL S/C ratio, which was calculated as the sum of surface components (protein, FC, PL) in a given LDL divided by the sum of its core components (CE, TG). *P < 0.05, **P < 0.01. The results are the mean ± SEM.
the time course of the RCT assay lies between these two extremes. These calculations demonstrate that the delivery of HDL-derived CE to feces is markedly impaired in fat/cholesterol-fed ApoF-deficient animals where CETP-mediated transfer of CE to LDL is enhanced.

In other studies, we measured RCT in ApoF-deficient animals consuming the fat/cholesterol diet for a shorter time where hypercholesterolemia was less developed (≤200 mg cholesterol per deciliter vs. ≤100 mg/dl in chow control) compared with that in group 2 animals (Table 4). In these animals, ApoF was reduced by ~90% and plasma CE transfer from HDL to the LDL/VLDL fraction was increased (96 ± 8 μg CE per milliliter vs. 120 ± 12 μg CE per milliliter, P < 0.05), but neither the increased plasma LDL cholesterol nor the decreased hepatic LDLR mRNA characteristic of group 2 animals had developed. In these animals, fecal RCT was not different from control (7.7 ± 1.2% vs. 6.8 ± 1.2%, n = 5). To investigate this relationship further, HDL RCT assays were performed in chow-fed male hamsters where all lipoprotein levels are lower. In marked contrast to that observed in fat/cholesterol-fed animals, ApoF knockdown in chow-fed animals stimulated the delivery of HDL-derived CE into feces more than 24-fold (Fig. 9C).

DISCUSSION

The existence of factors in human plasma that regulate the expression of CETP activity has been well-documented. Various in vitro studies have noted that rather high levels of some apolipoproteins inhibit CETP activity (44–47); however, most of these have proven ineffective in vivo (48–51). An exception is ApoCI. Lagrost and colleagues provided strong evidence that CETP activity in CETP transgenic mice is increased when the APOC1 gene is disrupted (52, 53). In humans, plasma CETP activity correlates negatively with
ApoCI levels (54) and immunoadsorption of ApoCI from plasma stimulates CETP activity by $\sim 40\%$ (52). ApoCI disrupts the binding of CETP to HDL by altering the surface electrostatic charge of HDL (55). This effectively prevents the lecithin:cholesterol acyltransferase-derived CE in HDL from being transferred to VLDL or LDL.

In contrast to ApoCI, in vitro studies show that ApoF preferentially suppresses lipid transfers involving LDL (13, 56), while having the least effect on CETP-mediated transfers between VLDL and HDL. These studies show that this selectivity likely occurs because ApoF is active primarily when bound to LDL (1). We have hypothesized that ApoF provides a mechanism for fine-tuning where CE goes once removed from HDL by CETP.

The studies here provide in vivo evidence for the importance of ApoF in regulating CETP activity in an animal that naturally expresses CETP. Within the short time frame of these studies, ApoF knockdown did not result in measurable changes in lipoprotein levels in Chow-fed animals. However, studies of LDL and HDL isolated from these animals showed for the first time that ApoF-deficient lipoproteins support greater CETP activity than control lipoproteins. And, in fat/cholesterol-fed animals, ApoF knockdown increased CETP activity among endogenous plasma lipoproteins up to 2-fold in male group 2 animals and to a lesser extent in female hamsters. CE transfer between HDL and LDL was increased much more than that between HDL and the combined VLDL/LDL pool, consistent with the removal of ApoF primarily enhancing LDL-HDL transfers rather than VLDL-HDL transfers.

In both group 2 male hamsters and female hamsters fed the fat/cholesterol diet, ApoF knockdown increased LDL cholesterol levels and altered LDL lipid composition, including an increased ratio of CE/TG, the two substrates of CETP. Such changes did not occur in male group 1 animals where CETP activity was not enhanced, showing a direct correlation between enhanced CETP activity and lipoprotein alterations. In fat/cholesterol-fed male hamsters where the impact of ApoF removal was larger, LDL cholesterol levels were decreased. These data are consistent with an overall shift in plasma cholesterol distribution from HDL into LDL in ApoF-deficient animals.

The origin of the two phenotypes observed in fat-fed male hamsters remains to be determined but it may reflect unique conditions created by the concurrent knockdown of ApoF and initiation of the fat/cholesterol diet combined with differences in the response of individual animals to the diet. We speculate that compositional changes occurring in group 1 lipoproteins prevent an increase in CETP activity even though ApoF levels are low. This may arise from the unique alterations in particle size and surface lipid composition that were observed for group 1 LDL (Fig. 5). CETP’s lipid substrates reside in the PL surface of lipoproteins and changes in the composition and molecular organization of this lipid layer have documented regulatory effects on CETP activity (37–42). The role of response to diet in the genesis of these two phenotypes is further supported by the observation that the group 2 phenotype was the major phenotype present earlier in the development of diet-induced hyperlipidemia, and the only phenotype observed in fat/cholesterol-fed female hamsters. Female hamsters are relatively resistant to diet-induced changes in lipoproteins. Understanding the mechanisms at play in group 1 male hamsters that counter the effects of ApoF removal will shed additional light on factors that control CETP activity.

Enhanced LDL compositional remodeling and altered plasma cholesterol distribution by ApoF knockdown had downstream consequences on lipid metabolism. In **APOF**

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**TABLE 6. Composition of LDL from fat/cholesterol-fed female hamsters**

| siRNA  | TC       | FC       | CE       | TG       | PL       | CE/TG     |
|--------|----------|----------|----------|----------|----------|-----------|
| Control| 1.15 ± 0.06 | 0.32 ± 0.01 | 1.40 ± 0.08 | 0.57 ± 0.05 | 0.99 ± 0.03 | 2.63 ± 0.46 |
| APOF   | 1.28 ± 0.05 | 0.36 ± 0.03 | 1.56 ± 0.04 | 0.38 ± 0.01* | 1.23 ± 0.10* | 4.07 ± 0.19* |

LDLs were isolated from animals 5 days after injection with 0.5 mg/kg control or APOF-siRNA. Values are the mean ± SEM, n = 5.

*P < 0.01 versus control.

*P < 0.05 versus control.
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mRNA levels of several hepatic genes involved in lipid uptake and metabolism (SCARB1, LDLR, Cyp7a1, and MTTP) were downregulated compared with control or group 1 animals. In general, these genes are regulated by lipid ligands that control gene expression through sterol regulatory element-binding protein or the liver X receptor. Because ApoF deficiency in these animals changes the distribution of circulating lipids between VLDL, LDL, and HDL, this likely impacts how lipids are delivered to the liver and how they subsequently influence gene expression.

The impact of ApoF knockdown on the excretion of HDL-derived CE in feces was model dependent. In fat/cholesterol-fed animals where ApoF depletion was associated with increased LDL levels (group 2), the excretion of radiolabeled cholesterol to feces was decreased. In mildly hypercholesterolemic hamsters where ApoF deficiency did not increase LDL levels above those induced by the fat diet alone, RCT was not altered. And in chow-fed hamsters where LDL levels are low, fecal RCT was stimulated by ApoF depletion. These data suggest that the effect of ApoF knockdown on RCT depends on underlying plasma LDL levels. For example, in ApoF-deficient group 2 animals, the increased transfer of CE to LDL combined with downregulation of hepatic LDL receptors by dietary lipids (57, 58) leads to an accumulation of cholesterol in the LDL fraction and to reduced delivery of CE to the liver. The lower HDL cholesterol levels in these animals, combined with decreased SCARBI expression, may also contribute to the lower RCT. Conversely, the increased sterol excretion in chow-fed ApoF-deficient animals likely occurs because even though more HDL-CE is transferred to LDL, hepatic LDL receptor expression is high and plasma LDL levels remain low, allowing the CE diverted to LDL to be efficiently cleared by the liver. Because HDL cholesterol levels are not altered in these chow-fed animals, combined with continued delivery of HDL-CE by direct mechanisms provide the additional cholesterol substrate for excretion.

We observed here that ApoF knockdown in both group 1 and group 2 animals reduced plasma HDL particle number. ApoF resides primarily on HDL, but this pool of ApoF appears to have no impact on CETP activity (1). HDL ApoF may serve as a reservoir for LDL ApoF, which varies dependent on the physiochemical properties of LDL (1, 2). It is also possible that ApoF directly influences HDL metabolism,

Fig. 8. Liver mRNA levels. mRNA levels of the indicated genes were measured by qPCR in livers obtained from male hamsters euthanized 5 days after receiving 0.5 mg/kg siRNA. A: Hepatic mRNA levels in animals fed chow or fat/cholesterol diets receiving control siRNA. B: Liver mRNA levels in fat/cholesterol-fed animals injected with control or APOF siRNA. APOF groups 1 and 2 (Gr1, Gr2) are defined in the text. Data are expressed as mean ± SEM. *P < 0.05 and **P < 0.01.

Fig. 9. RCT assay. Hamsters received 3H-CE-labeled HDL 3 days after a 0.5 mg/kg siRNA injection and were euthanized after 48 h. A: Percent recovery of 3H in plasma, liver, and feces from fat/cholesterol-fed male hamsters as described in the Methods. B: Panel A fecal percent 3H values were converted to CE mass values based either on the plasma content of HDL-CE or the total CE content of plasma. C: Percent recovery of 3H in plasma, liver, and feces from chow-fed male hamsters treated with control siRNA (n = 5) or APOF siRNA (n = 4). Values are mean ± SEM. *P < 0.05 and **P < 0.01.
leading to the altered HDL levels seen here. Consistent with this, overexpression of ApoF in mice, which naturally lack CETP, alters HDL levels (16). However, ApoF knockdown mice have no change in HDL (17), but interpretation of data from this model is complicated by the simultaneous hypomorph expression of the nearby major inflammatory gene, Stat2 (39).

In summary, these studies show that ApoF knockdown enhances the transfer of CE from HDL to LDL, resulting in increased LDL cholesterol levels in hypercholesterolemic animals. Increased plasma LDL cholesterol is associated with altered hepatic gene expression and impairment of fecal cholesterol excretion. These studies validate previous in vitro studies and establish in vivo that ApoF controls the flow of HDL-derived CE to LDL. In this context, ApoF may optimize RCT by channeling the CE synthesized on HDL into VLDL instead of LDL. VLDL has a very short plasma residence time (t₁/₂ = 45 min vs. ~10 h for LDL) (60) and its hepatic clearance mechanisms are largely independent of the LDL receptor that is downregulated by elevated plasma cholesterol (61–63). By selectively inhibiting the transfer of CE from HDL into LDL, ApoF reduces LDL cholesterol levels and promotes hepatic sterol clearance through VLDL when LDL receptors are downregulated (59).

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