LDL and HDL enriched in triglyceride promote abnormal cholesterol transport

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Abstract **Hypertriglyceridemia induces multiple changes in lipoprotein composition. Here we investigate how one of these modifications, triglyceride (TG) enrichment, affects HDL and LDL function when this alteration occurs under conditions in which more polar components can naturally re-equilibrate. TG-enriched lipoproteins were produced by co-incubating VLDL, LDL, and HDL with cholesteryl ester (CE) transfer protein. The resulting 2.5-fold increase in TG/CE ratio did not measurably alter the apoprotein composition of LDL or HDL, or modify LDL size. HDL mean diameter increased slightly from 9.1 to 9.4 nm. Modified LDL was internalized by fibroblasts normally, but its protein was degraded much less efficiently. This likely reflects an aberrant apolipoprotein B (apoB) conformation, as suggested by its resistance to V8 protease digestion and altered LDL electrophoretic mobility. TG-enriched LDL ineffectively down-regulated cholesterol biosynthesis compared with control LDL at the same protein concentration, but was equivalent in sterol regulation when compared on a cholesterol basis. TG-enriched HDL promoted greater net cholesterol efflux from cholesterol-loaded J774 cells. However, cholesterol associated with TG-enriched HDL was inefficiently esterified by lecithin:cholesterol acyltransferase, and TG-enriched HDLs were poor donors of CE to HepG2 hepatocytes by selective uptake. We conclude that TG-enrichment, in the absence of other significant alterations in lipoprotein composition, is sufficient to alter both cholesterol delivery and removal mechanisms. Some of these abnormalities may contribute to increased coronary disease in hypertriglyceridemia.**—Skeggs, J. W., and R. E. Morton. **LDL and HDL enriched in triglyceride promote abnormal cholesterol transport.** ***J. Lipid Res.*** **2002.** 43: **1264–1274.**

Supplementary key words cholesteryl ester transfer protein • lipoprotein composition • low density lipoprotein degradation • cholesterol efflux • lecithin:cholesterol acyltransferase • selective uptake

Hypertriglyceridemia is a recognized risk factor for coronary heart disease (1). High triglyceride (TG) is a trait common in many conditions, including insulin resistance, hypertension, and centrally mediated obesity (2), and in lipase deficiency (3, 4). Increased risk is thought to be manifested largely through a reduction in HDL cholesterol (1). However, since elevated triglycerides result in significantly altered composition of all plasma lipoproteins, the contribution of other pathways to this increased pathology is possible.

Delineating the atherogenic mechanisms of hypertriglyceridemia is complicated by the fact that multiple processes can lead to an elevated plasma TG phenotype. Each of these processes can result in unique alterations in lipoprotein properties. For example, LDL and HDL may be smaller when hypertriglyceridemia results from increased TG production (5, 6), whereas these particles are often larger when inefficient lipolysis is the cause of elevated TG (3, 4). Regardless of the underlying cause of hypertriglyceridemia, a common feature of hypertriglyceridemic LDL and HDL is a marked increase in their TG/cholesteryl ester (CE) ratio (1, 6, 7). For the most part, these altered ratios are the consequence of remodeling mediated by CE transfer protein (CETP). CETP mediates the equilibration of lipoprotein core lipids (TG and CE) by promoting the heteroexchange of these lipids down their concentration gradients (8). CETP increases the TG/CE ratio of CE-rich lipoproteins, i.e., LDL and HDL, and lowers this ratio in VLDL. In most individuals, plasma TG levels, not CETP concentrations, are rate limiting to this remodeling process (9). In hypertriglyceridemic individuals, this process is accelerated, resulting in TG enrichment of LDL and HDL.

LDL and HDL isolated from hypertriglyceridemic individuals are aberrant in their interactions with cell surface receptors (10–12), in their plasma clearance kinetics (13, 14), and in facilitating sterol balance (15). However, because the metabolic events that contribute to the properties of lipoproteins in vivo are complex, it is unclear to

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Abbreviations: apoB, apolipoprotein B; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; LPDS, lipoprotein-deficient serum; TG, triglyceride.

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what extent these altered functional properties arise from the increase in TG/CE alone. Several studies have investigated the consequences of altering the TG content of LDL (11, 16–18) and HDL (19) in vitro. However, these modifications were typically performed under conditions in which the natural re-equilibration of surface components among plasma lipoproteins was not possible. Further, in none of these studies were the functional properties of the resultant LDL and HDL examined concomitantly.

To identify the unique contribution of core lipid modification to altered lipoprotein function, we have modified the TG/CE ratio of lipoproteins by incubating VLDL, LDL and HDL, combined at ratios similar to those in mild hypertriglyceridemia, with CETP. This approach resulted in the TG enrichment of LDL and HDL to an extent commonly observed when steady-state TG levels are increased, permitted the secondary re-equilibration of surface-active components among lipoproteins, and avoided confounding influences by other plasma factors (lipases, LCAT, phospholipid transfer protein). We report that TG-enriched LDL and HDL are functionally aberrant; these changes may contribute to the atherogenicity of hypertriglyceridemia.

EXPERIMENTAL PROCEDURES

Preparation, modification, and radiolabeling of lipoproteins

Lipoproteins were isolated from fresh plasma by differential ultracentrifugation (20). All glassware used for lipoprotein isolations was rinsed three times with ultra-high-purity water and baked overnight at 120°C to remove residual detergents and endotoxin contamination, respectively. VLDL ($d < 1.006$ g/ml), LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml), isolated in NaBr solutions containing 0.02% EDTA, were extensively dialyzed against 0.9% NaCl, 0.02% EDTA (pH 7.4) (NaCl/EDTA buffer), and stored in the dark at 4°C. Lipoprotein oxidation was assessed by thiobarbituric acid reactivity (21), apolipoprotein lysine content (22), and electrophoretic mobility in agarose gels (23).

To prepare [^{3}H]cholesterol-labeled HDL, 4–7 μCi of [1,2-^{3}H(N)] cholesterol (NEN Life Sciences, Boston, MA) was dried onto filter paper under nitrogen gas. HDL (0.5–2 mg protein), labeled by incubation with the filter paper for 18 h at 37°C under N_2, was sterile filtered before use. Residual LCAT activity in isolated HDL was blocked by prior incubation of HDL with 1 mM paraoxon. Following extensive dialysis, HDL-specific activities ranged from 25 to 70 cpm/ng total cholesterol. [^{3}H]cholesteryl ether and ^{125}I-tyramine-cellobiose-labeled HDL were prepared as previously described (24). LDL was iodinated with carrier-free ^{125}I in 0.1 NaOH (NEN Life Sciences) following the iodine monochloride method (25, 26). The final specific activity of LDL averaged 50 cpm/ng protein. Acetylated LDL was produced by repetitive addition of acetic anhydride (27).

Preparation and analysis of CETP-modified lipoproteins

Partially purified CETP was isolated from lipoprotein-deficient plasma (28) as previously described (29). These preparations, which are free of phospholipid transfer protein and LCAT activities, are functionally identical to homogenous CETP (30, 31). LDL, VLDL, and HDL were mixed in the ratio of 2:1:1 based on cholesterol. Lipoproteins (4 mg) and partially purified CETP (80–240 μg protein), were combined with NaCl/EDTA buffer, 3.5% BSA, and 100 mM paraoxon (0.1% and 1 mM final concentration, respectively) in a final volume of ~4 ml. The mixture was incubated for 18–24 h at 37°C to yield CETP-modified lipoproteins, or at 4°C, where CETP is inactive, for control (unmodified) lipoproteins. VLDL, LDL, and HDL were re-isolated within their original density limits as described above. In selected instances, LDL and HDL were modified under more physiological conditions by incubating plasma (containing 1 mM paraoxon) at 37°C for 0 or 24 h followed by lipoprotein isolation as described above.

The protein content of the lipoproteins was determined using a modified Lowry protein assay (32). TG and free cholesterol content were assayed using enzymatic colorimetric kits (Sigma Chemical Co., St. Louis, MO). Total cholesterol was determined by the addition of cholesterol esterase (0.0625 U/ml) from *Candida cylindracea* (Boehringer Mannheim, Indianapolis, IN) to the free cholesterol color reagent and subsequent incubation at 37° for 45 min. Alternatively, total cholesterol was determined by a purchased kit (Sigma). Phospholipid content was quantitated by phosphorus analysis (33). The electrophoretic mobility of LDL and HDL was determined by 1% agarose gel electrophoresis (34).

In some instances, control and TG-enriched LDLs were partially digested with *Staphylococcus aureus* V8 protease (Pierce Chemical Co.) as described in the text (35). Hydrolyzed LDLs were then denatured under reducing conditions and analyzed using SDS polyacrylamide gel electrophoresis on 2–15% Mini-Plus Sepragels (Owl Separation Systems, Woburn, MA) as previously described (36). Proteins were visualized by Coomassie blue staining.

The particle size of control and TG-enriched HDL was determined by polyacrylamide gradient gel electrophoresis (4–30%) (Isolab, Akron, OH) or 4–20% (Owl Separation Systems)) under nondenaturing conditions (37). Proteins were stained with colloidal Coomassie blue stain (Owl Separation Systems). Scanned gels were analyzed by NIH Image software. Gel filtration was performed on tandem Superose 6 columns followed by on-line cholesterol detection (38).

^{125}I-LDL uptake and degradation

Measurements of LDL uptake and degradation were performed essentially as described by Goldstein and Brown (39). Human skin fibroblasts (ATCC# GM5757) were plated in 24-well tissue culture plates and grown to confluence (~48 h) in DMEM/F12 media (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (Biowhitaker, Inc., Walkersville, MD) containing penicillin, streptomycin, and amphotericin B (Life Technologies, Rockville, MD). This media was removed, and DMEM/F12 with 5% human lipoprotein-deficient serum (LPDS) was added for 18 h to up-regulate LDL receptor expression. Cells were washed with PBS, and then ^{125}I-LDL in DMEM/F12 media containing 3 mM Ca^{++} and 1% LPDS was added. After 5 h at 37°C, the media was removed and LDL degradation was quantitated from the trichloroacetic acid-soluble, non-iodide radioactivity (39). The cell monolayer was washed five times with cold PBS, dissolved in 0.1 N NaOH, and counted to determine cell-associated ^{125}I radioactivity.

Regulation of cholesterol synthesis

Cholesterol synthesis from [^{14}C]acetate was determined essentially as described previously (16). Confluent fibroblasts were cultured in DMEM/F12 + 5% LPDS for 18 h. LDL in 5% LPDS and 2 mM Ca^{++} was then added for 22 h. This was subsequently re-

moved, and cells were incubated in DMEM/F12 media containing 8 μCi/ml [1-^{14}C]acetic acid (sodium salt, NEN Life Sciences). After 4 h at 37°C, the media was removed and the cells washed four times with PBS. Washed cells were scraped from the plate and lipids extracted (40). Lipids were fractionated by thin layer chromatography on KS silica gel plates (250 μm; Whatman, Clifton, NJ) in a developing solution of hexane-ethyl ether-acetic acid (70:30:1, v/v/v). [^{14}C]acetate incorporated into cholesterol, identified by its co-migration with authentic exogenous cholesterol, was determined by scintillation counting.

Cholesterol efflux and influx

The bi-directional flux of free cholesterol from lipid-loaded macrophages was determined as described by Rothblat et al. (41). J774A.1 macrophages (ATCC# TIB 67) were enriched in cholesterol by overnight incubation with acetylated LDL (42). After 24 h, this media was removed and DMEM/F12 containing 3 μg/ml Sandoz compound 58-035 (a generous gift from John Heider, Sandoz, Inc.) was added for 18 h to inhibit acylCoA:cholesterol acyltransferase activity (43). The free cholesterol content of J774 cells was determined by a fluorescent assay (44).

For efflux experiments, the cells treated as above were simultaneously labeled with [^{3}H]cholesterol (0.2–0.3 μCi/ml) during the cholesterol-loading incubation with acetyl LDL. Final free cholesterol-specific activity averaged 4×10^3 cpm/μg free cholesterol. To measure the HDL-stimulated efflux of cholesterol, these cells were then treated with media containing HDL and 58-035 (3 μg/ml). At set time points after the addition of the HDL, aliquots of media were counted to determine the extent of cholesterol efflux.

To measure cholesterol influx from HDL, unlabeled, cholesterol-loaded cells were treated with the indicated amount of [^{3}H]cholesterol-labeled HDL (specific activity ~2.5×10^5 cpm/μg free cholesterol) in DMEM/F12 containing 3 μg/ml of compound 58-035. At set time points, the media was removed and the cell monolayers were washed rigorously with PBS, harvested, sonicated, and aliquots taken for scintillation counting to determine the influx of cholesterol from HDL. Cholesterol mass efflux and cholesterol mass influx values were calculated from the cholesterol radioactivity transferred to the acceptor compartment and the initial specific activity of cholesterol in the donor. Net cholesterol movement, which was always a net efflux, was calculated from the difference between these two flux values.

LCAT activity

The ability of HDL fractions to support LCAT activity was determined by the conversion of [^{3}H]cholesterol to labeled CE. [^{3}H]cholesterol-labeled HDL, typically containing ~1.5×10^5 cpm/μg free cholesterol, was incubated at the indicated concentration with 125 μl lipoprotein-deficient human plasma, as a source of LCAT, and saline/EDTA buffer containing 0.02% NaN_3 to yield 250 μl final volume. After incubation, lipids were extracted (40) and fractionated by thin layer chromatography as above. The % conversion of labeled cholesterol to CE was determined, and the mass of cholesterol esterified was calculated based this % conversion and the initial free cholesterol mass in HDL. LCAT activity was linear during 30–90 min.

CE selective uptake

Scavenger receptor class B type I (SR-BI)-mediated selective uptake of CE from doubly-labeled HDL ([^{3}H]cholesteryl ether, ^{125}I-tyramine-cellobiose) by HepG2 cells (ATCC# HB8065) was determined exactly as previously described (24). Selective uptake was calculated as the difference between total CE uptake (^{3}H content) and the amount of CE incorporation that could be accounted for by whole HDL uptake as determined by the accumulated cellular ^{125}I.

RESULTS

Characterization of TG-enriched lipoproteins

Native lipoproteins were promptly isolated from freshly drawn normolipidemic plasma by sequential ultracentrifugation. Isolated LDL and HDL were unoxidized as assessed by thiobarbituric acid reactivity (0.18 ± 0.10 and 0.05 ± 0.05 nmoles malondialdehyde/mg protein, respectively ($n \geq 20$) and electrophoretic mobility determinations ($Rf = 0.75 \pm 0.08$ and 2.66 ± 0.34 compared with transferrin, respectively). The oxidation status of LDL was further evaluated by quantitation of its lysine content. Lysine amine residues are rapidly derivatized during the initial stages of oxidation (45). LDL retained all of its lysly residues as determined by trinitrobenzenesulfonic acid reactivity (366 ± 34 mol lysines/mole apolipoprotein B (apoB), compared with a theoretical value of 357 based on published sequence (GenBank, accession LPHUB).

Co-incubation of VLDL, LDL, and HDL with CETP at 37°C to permit lipid transfer activity resulted in net CE transfer to VLDL and TG enrichment of LDL and HDL compared with untreated lipoproteins (not shown) or lipoproteins incubated with CETP at 4°C, where its lipid transfer activity is minimal (46) (**Table 1**). This resulted in a 2.5-fold increase in the TG/CE ratio of LDL and HDL, with a concomitant decline in this ratio for VLDL. These changes in lipoprotein cholesterol content were also readily observed when lipoproteins in the incubation mixture were separated by gel filtration chromatography instead of ultracentrifugation (**Fig. 1A**).

TABLE 1. TG enrichment of LDL and HDL

Incubation Temperature	Lipoprotein	Phospholipid	Free Cholesterol	Cholesteryl Ester	Triglyceride	Triglyceride/Cholesteryl Ester
			μg/μg protein			
4°C	VLDL	1.85	0.86	0.41	5.09	12.4
	LDL	0.83	0.54	1.02	0.20	0.20
	HDL	0.40	0.07	0.24	0.05	0.21
37°C	VLDL	1.98	1.14	0.93	4.89	5.26
	LDL	0.68	0.41	0.84	0.39	0.46
	HDL	0.44	0.08	0.18	0.10	0.56

VLDL, LDL, and HDL (1:2:1 cholesterol ratio) were incubated at the indicated temperature for 24 h in the presence of cholesterol ester transfer protein (CETP) as described in Experimental Procedures. The lipid composition of re-isolated lipoproteins, which are typical of multiple preparations, is shown relative to their protein content.

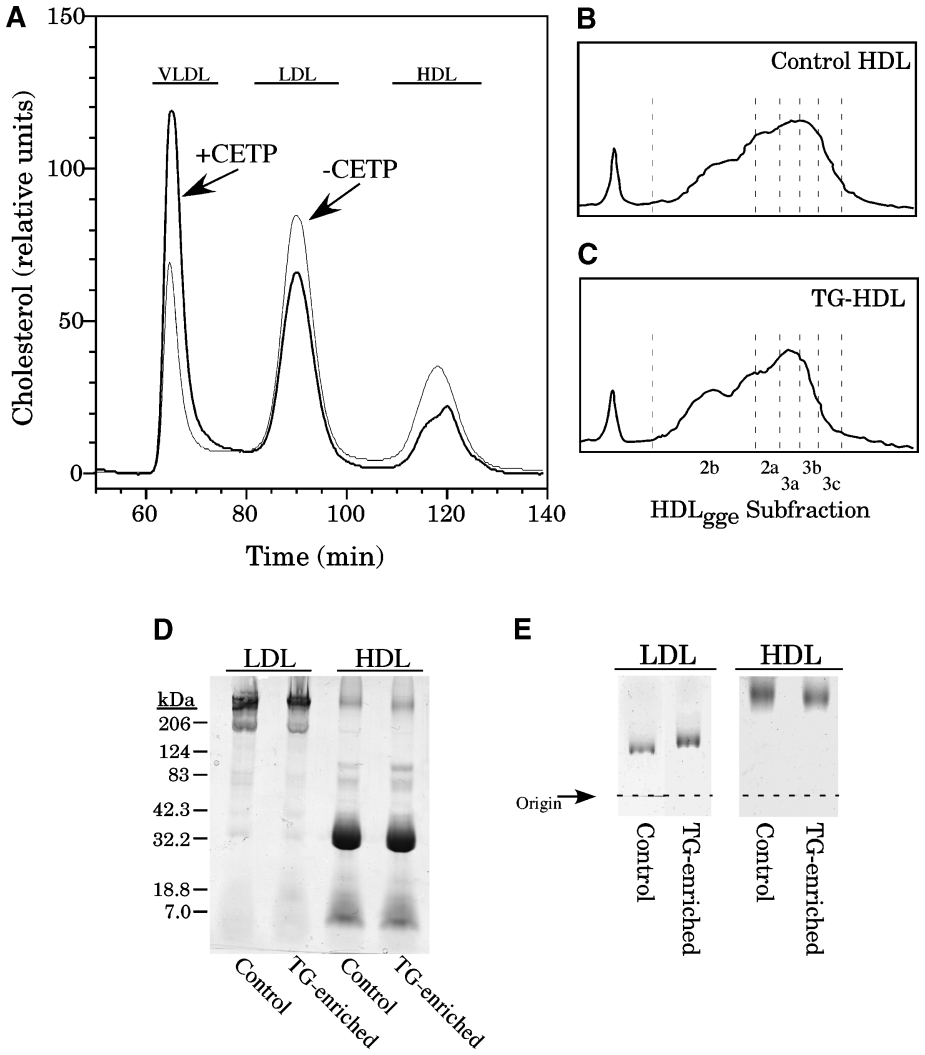

Fig. 1. Characterization of triglyceride (TG)-enriched lipoproteins. VLDL, LDL, and HDL (1:2:1 cholesterol ratio) were incubated with cholesteryl ester transfer protein (CETP) (300 μl) for 20 h at 4°C or 37°C to produce control and TG-enriched lipoproteins, respectively. Aliquots (100 μg cholesterol) of the incubation mixture were fractionated on tandem Superose 6 columns followed by on-line detection of cholesterol essentially as previously described (38) (A). Alternatively, lipoproteins were re-isolated by ultracentrifugation within their original density limits. Isolated lipoproteins were subjected to non-denaturing electrophoresis (B and C, HDL only), SDS-PAGE analysis on 4–20% gradient gels (D), and agarose gel electrophoresis (E). Proteins were detected by staining with Coomassie blue. See Experimental Procedures for additional details.

Based on its cholesterol profile (Fig. 1A) or protein elution (not shown), TG enrichment of LDL did not cause a measurable change in LDL particle size. This was not unexpected however, because, based on the differential in TG and CE partial specific volumes (7), TG enrichment would have resulted in only a 0.9% increase in particle diameter (~0.2 nm). There was, however, an alteration in the cholesterol distribution of TG-enriched HDL (Fig. 1A), suggesting that individual HDL subfractions are not equally active in CETP-mediated CE-TG heteroexchange. This was evident on non-denaturing PAGE analysis, where the HDL profile shifted to the left after TG enrichment (Fig. 1B, C). There was a significant reduction in HDL3c content (11.8% to 4.6%) and a small increase in the levels of the HDL2 (53.6% to 62.7%). Overall, HDL mean particle diameter increased modestly from 9.1 to 9.4 nm. TG enrichment of LDL and HDL did not result in detectable apoprotein changes for either lipoprotein (Fig. 1D).

Modified LDL had a slightly faster migration on agarose gel electrophoresis, whereas TG-enriched HDL migrated slightly slower (Fig. 1E). Among three preparations, the relative electrophoretic mobilities (relative to control lipoprotein) for TG-enriched LDL and HDL were 1.09 ± 0.17 and 0.91 ± 0.05, respectively (n = 3). The increased mobility of TG-enriched LDL was not due to lipoprotein oxidation, as the conjugated diene content (absorbance 234 nm) and the level of apoB amino group derivatization as evidenced by autofluorescence (360 nm ex/430 nm em) in modified LDL was not different from control LDL. Finally, consistent with the identical apoprotein patterns shown in panel D, the data in panel E illustrate that ultracentrifugally isolated control and TG-enriched LDL and

HDL are free of cross-contamination by other lipoprotein fractions.

Cellular processing of TG-enriched LDL

The catabolism of control and TG-modified LDL by fibroblasts was studied to assess the impact of TG enrichment on cellular interactions with LDL. A small but reproducible (in 6 of 8 experiments) difference was noted in the 4°C binding parameters of TG-enriched LDL. However, the higher maximum binding (V_{max} = 116 ± 17% of control LDL) and lower binding affinity observed for TG-enriched LDL (K_m = 9.7 ± 2.9 vs. 8.5 ± 3.0 μg protein, TG-modified vs. control LDL, respectively) failed to reach statistical significance.

The uptake of TG-modified LDL by fibroblasts over a 5 h incubation at 37°C was indistinguishable from control

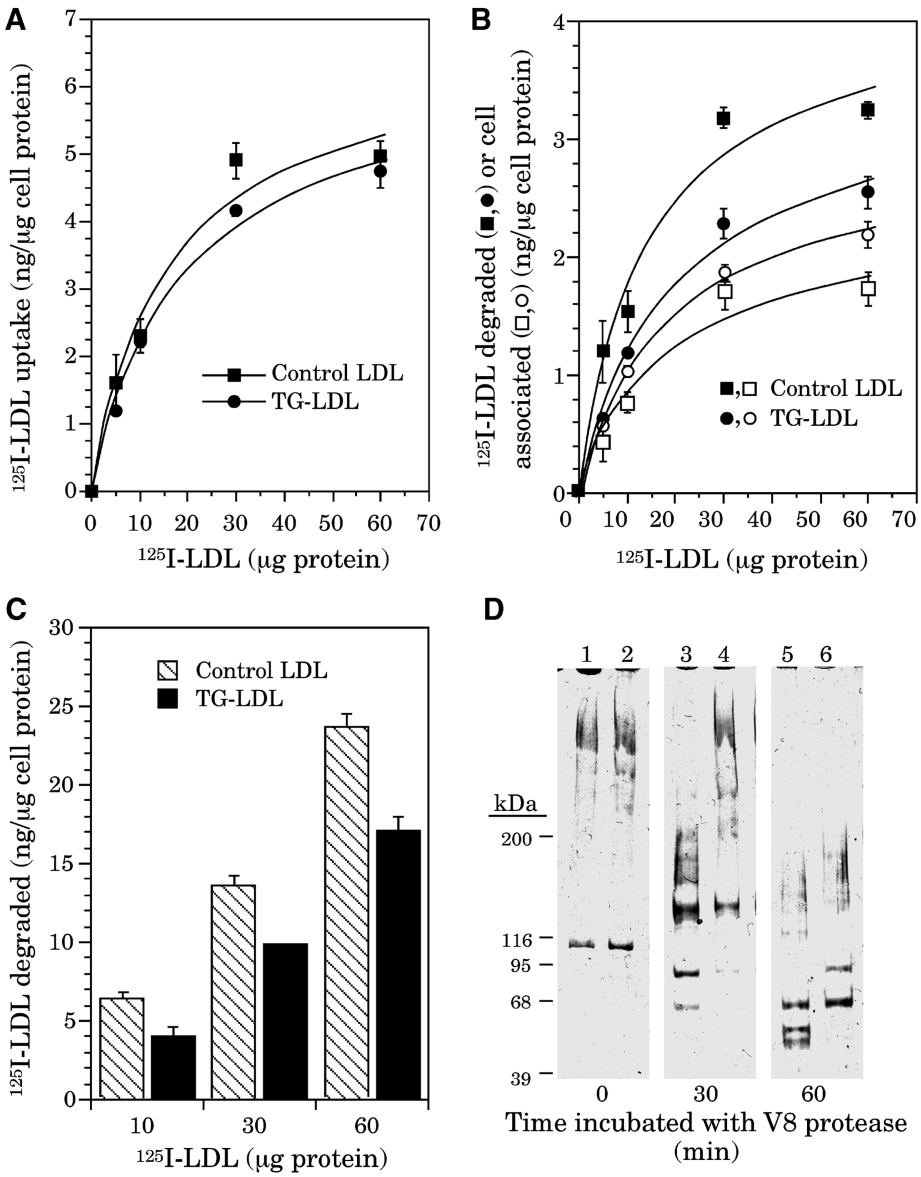

Fig. 2. Catabolism of control and TG-enriched LDL. Human skin fibroblasts were incubated with the indicated amount of ^{125}I-labeled control or TG-enriched LDL for 5 h. Cell media was removed and its content of non-iodide, trichloroacetic acid precipitable radioactivity determined to quantify lipoprotein degradation. Cell-associated radioactivity was measured after extensive washing. Total LDL uptake (degraded + cell-associated) is shown in (A). The extent of LDL degradation (closed symbols) and cell-associated ^{125}I (open symbols) are plotted in (B). Data points, the mean ± SD of duplicate wells, are representative of 4 experiments. To evaluate the properties of LDL modified under more physiological conditions, LDL was isolated from LCAT-inhibited plasma incubated (37°C) for t = 0 (control) or t = 24 h (TG-LDL). The degradation of these particles is shown in (C). To assess the sensitivity of LDL protein to proteolytic attack, 120 μg LDL protein was incubated with 0.6 units *Staphylococcus aureus* V8 protease. Aliquots were removed at the indicated times, denatured, and analyzed by SDS-PAGE gel electrophoresis on 2–15% gradient gels under reducing conditions. Results for control LDL (lanes 1, 3, and 5) and TG-enriched LDL (lanes 2, 4, and 6) are shown in (D). Each lane contains 7 μg LDL protein.

LDL (**Fig. 2A**). However, the cellular processing of this internalized lipoprotein was significantly altered. For control LDL, only 26–29% of the internalized lipoprotein remained in the cell, with the remainder having been degraded and released into the medium during the 5 h incubation (Fig. 2B). In contrast, the degradation of labeled protein associated with TG-enriched LDL was much lower. At all concentrations, almost half of the modified lipoprotein remained cell associated (Fig. 2B). Both cell-associated and degraded protein radioactivities could be reduced >80% by 10-fold excess unlabeled homologous lipoprotein. Also, for both lipoproteins, ≤10% of the cell-associated radioactivity could be released from the cell by dextran sulfate treatment (16) at the end of the experiment. This indicates that cell-associated lipoprotein protein is almost exclusively intracellular, representing a pool of internalized but incompletely degraded lipoprotein. LDL enriched in TG (TG/CE = 0.121 vs. 0.068 for control) under more physiological conditions, i.e., by incubation of LCAT-inhibited plasma, showed similar reduction in degradation (Fig. 2C). Thus, these data show that TG modification of LDL results in lipoproteins that are less efficiently processed by fibroblasts. Similar results were observed in HepG2 hepatocytes (not shown).

It is well-recognized that the conformation of apoB is significantly influenced by the lipid composition of LDL (10, 11). The slower degradation of apoB on TG-enriched LDL suggests that its conformation has been altered, rendering it less sensitive to proteolytic degradation. This was examined in vitro by assessing the degradation of apoB by bacterial protease as previously reported (35). ApoB in control LDL was rapidly degraded by V8 protease (Fig. 2D). After 30 min (lane 3), no intact apoB remained; products of 130–200 kDa dominated and were further reduced to 45–60 kDa fragments after 60 min (lane 5). In contrast, although yielding similar degradation products over time, apoB in TG-enriched LDL remained largely undegraded after 30 min (lane 4) and remained less-well-degraded even after 60 min (lane 6). These results are consistent with the cellular degradation studies above and further support the notion that TG-enrichment of LDL modifies the conformation of apoB, rendering it less susceptible to proteolytic cleavage.

It is possible that the reduced degradation of apoB in TG-enriched LDL reflects a more general defect in the degradation of the LDL particle. To investigate the cellular processing of lipoprotein lipid components, the capacity of LDL-derived cholesterol to down-regulate cellular cholesterol biosynthesis was examined. Fibroblasts were incubated in lipoprotein-free media to up-regulate cholesterol biosynthesis, followed by incubation with the indicated LDL. After overnight incubation, cholesterol biosynthesis was measured by [^{14}C]acetate incorporation. Control LDL was much more effective than TG-enriched LDL in down-regulating cholesterol synthesis (**Fig. 3A**). K_i values of 1.0 and 2.5 μg protein were found for control and TG-enriched LDL, respectively. Given the reduced CE content of modified LDL, this difference was not unexpected. When LDLs were compared on a cholesterol basis, TG-enriched and control LDLs were similarly effective in down-regulating cholesterol synthesis (K_i = 2.1 vs. 1.7 μg lipoprotein cholesterol, respectively). These data suggest that the cholesterol (free and esterified) contained in TG-enriched LDL is hydrolyzed and released from the lysosome to an extent equivalent to that for control LDL. Thus, unlike apoB, there does not appear to be a deficiency in cholesterol processing due to TG enrichment.

Cholesterol flux, esterification, and transport mediated by TG-enriched HDL

HDL plays an important role in the clearance of tissue cholesterol through the process of reverse cholesterol transport. Three key HDL-dependent aspects of this process are cholesterol efflux from cells, the conversion of HDL cholesterol to CE by LCAT, and hepatic clearance of HDL CE. We have examined the impact of TG enrichment on these processes.

Cholesterol efflux from and influx to J774 macrophages was measured after enriching cells with cholesterol by treatment with acetyl LDL followed by chemical inhibition of acylCoA:cholesterol acyltransferase activity. Cholesterol efflux (t = 2 h) from macrophages was 60% higher in the presence of TG-enriched HDL than with control HDL (**Fig. 4A**) at HDL concentrations ≤30 μg/ml. Cholesterol movement in the opposite direction, i.e., from HDL to cells (t = 2 h), also tended to be higher with TG-enriched HDL (Fig. 4B). Nevertheless, for both HDL sources, efflux exceeded influx by more than 5-fold, resulting in a net efflux of cholesterol from cells. TG-enriched HDL promoted >55% more net efflux of cholesterol (Fig. 4C), although this difference was overcome at higher concentrations (60 μg/ml). This greater efflux ca-

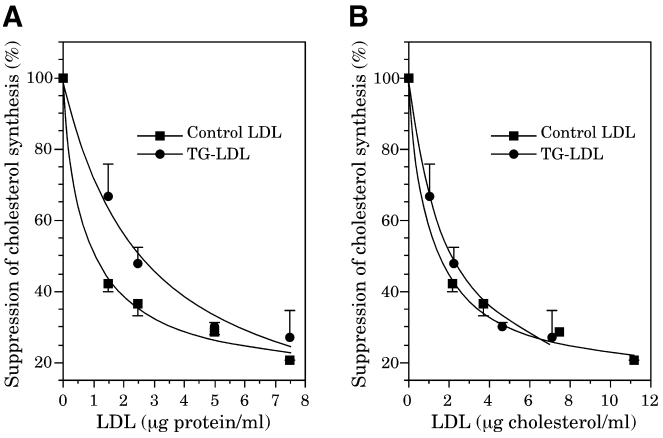

Fig. 3. Inhibition of cholesterol synthesis in fibroblasts by LDL. Cells, preincubated overnight in 5% lipoprotein-deficient serum (LPDS) to up-regulate cholesterol synthesis, were incubated (22 h) in media containing the indicated amount of LDL. The rate of cholesterol biosynthesis in these cells was subsequently determined from the incorporation of [^{14}C]acetate into cholesterol, as described in Experimental Procedures. A: The suppression of cholesterol synthesis based on the mass of input LDL protein. B: The suppression of cholesterol synthesis based on the mass of input LDL cholesterol. Data are the mean ± SD of duplicate wells and are representative of five experiments.

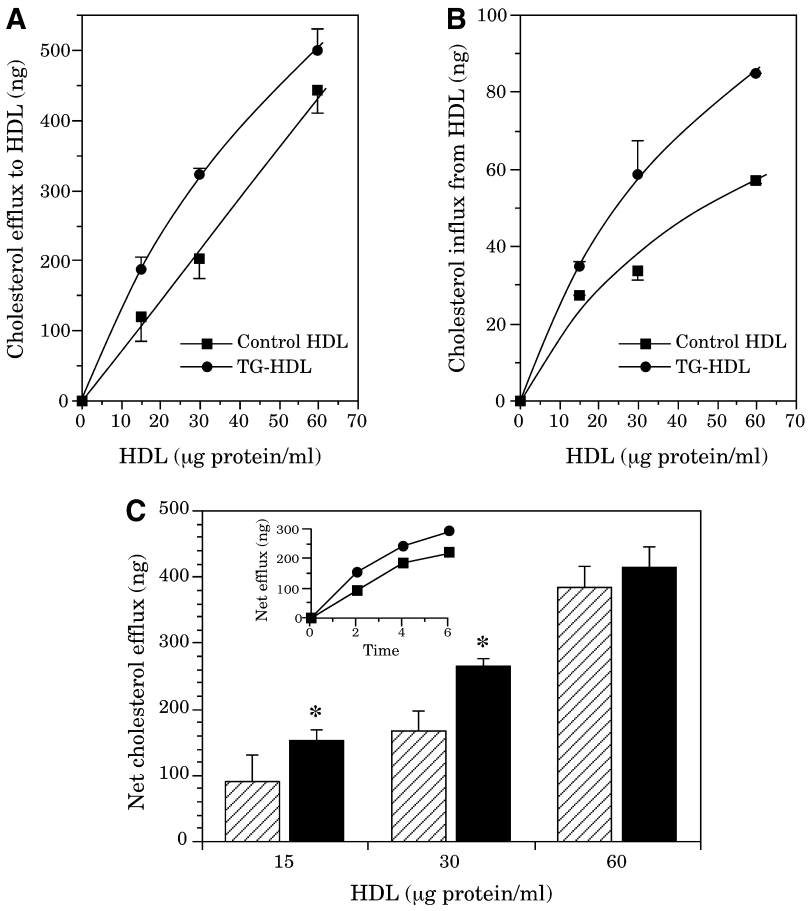

Fig. 4. HDL-mediated bidirectional flux of free cholesterol from J774 cells. The cholesterol pool in HDL or lipid-loaded J774 macrophages was radiolabeled, and the influx and efflux of free cholesterol mediated by control and TG-enriched HDL were measured as outlined in the Experimental Procedures section. A: Cholesterol transport from cells to HDL (efflux, $t = 2$ h). B: Cholesterol transport from HDL into cells (influx, $t = 2$ h). C: Net cholesterol efflux calculated from the difference in efflux and influx values shown in (A) and (B). * Significantly different from control HDL ($P < 0.04$). Inset: Net efflux as a function of incubation time in the presence of 15 μg/ml control HDL (squares) or TG-enriched HDL (circles). Data are the mean ± SD of duplicate wells and are representative of four experiments measuring both influx and efflux.

pacity for TG-enriched HDL persisted over a 6 h study window (inset). Unlike that shown in Fig. 4B, for some preparations of control and modified-HDL cholesterol, influx rates were similar. In this instance, the net efflux promoted by TG-enriched HDL, compared with control HDL, was greater than that shown in this figure. We have not determined whether TG enrichment alters the levels of lipid-poor cholesterol acceptors, such as pre β-HDL, that may not co-isolate with the HDL fraction ($1.063 < d < 1.21$ g/ml).

To assess the capacity of HDL to support LCAT activity, the free cholesterol pools of control and TG-enriched HDLs were radiolabeled, then incubated without or with a source of LCAT. In the absence of added enzyme, both HDLs supported minimal CE synthesis (Fig. 5A, open symbols), indicating that each lipoprotein preparation contained negligible LCAT contamination. In the presence of added LCAT, cholesterol esterification increased with HDL concentration and was up to 50% lower for TG-enriched HDL than for control HDL (Fig. 5A). This lower esterification activity was not due to an altered concentration of free cholesterol substrate in the modified lipoprotein (inset). However, in a subset of in vitro-generated, TG-enriched HDL, there was a reduction in the cholesterol/protein ratio similar to that seen in vivo (7, 47). In this instance, the defect in CE synthesis was even more profound (data not shown). LCAT activity was similarly lower when HDL, enriched in TG by incubation of paraoxon-treated plasma (TG/CE = 0.122 vs. 0.061 for control) instead of CETP plus isolated lipoproteins, was used as substrate (**Fig. 5B**).

SR-BI mediates the selective removal of HDL CE (48). Clearance of HDL CE by these receptors on the liver would facilitate reverse cholesterol transport. CE selective uptake by HepG2 hepatocytes was measured using the nondegradable ether analog of CE. Over a 3-fold concentration range, TG-enriched HDLs were inferior donors of CE to liver cells compared with control HDL (Fig. 5C). Control HDL_2 and HDL_3 were similar in their support of CE selective uptake when compared on an equal protein

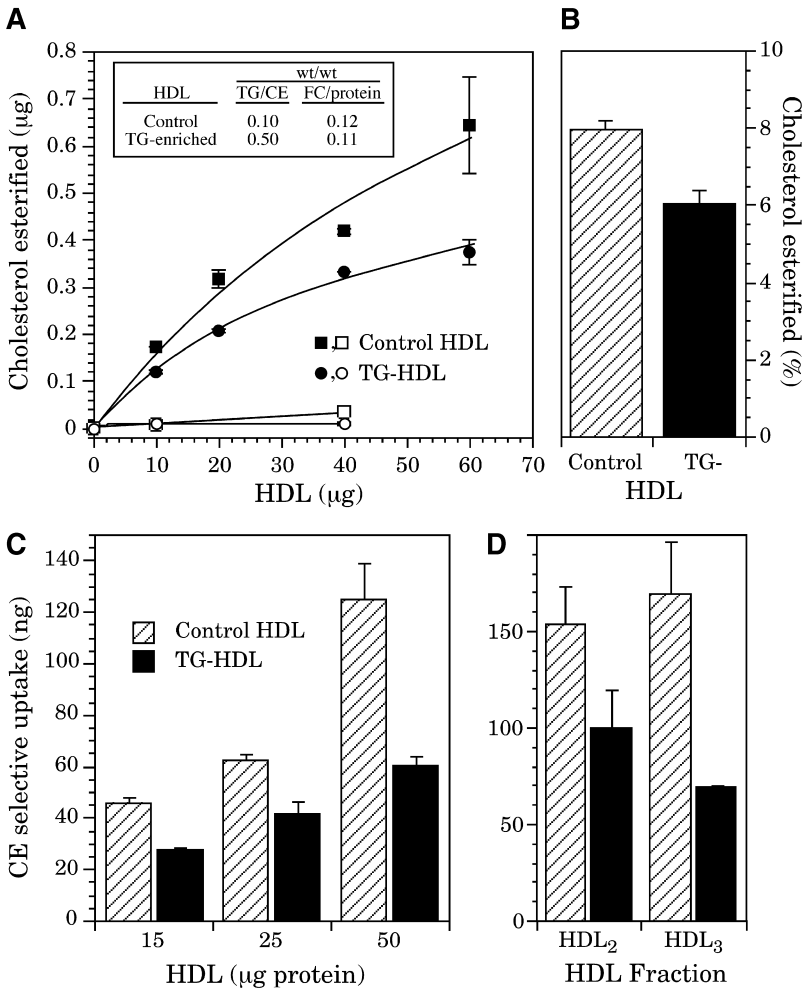

Fig. 5. Cholesteryl ester (CE) synthesis and removal in control and TG-enriched HDL. A: HDL were labeled with ^{3}H-free cholesterol, then incubated with lipoprotein-deficient human plasma as a source of LCAT. After 1 h, samples were extracted and the extent of [^{3}H]CE synthesis determined. Open symbols show esterification in the absence of added LCAT (i.e., without lipoprotein-deficient plasma). Data are the mean ± SD of duplicate wells and are representative of 4 experiments. B: LCAT activity, determined as in (A), supported by control and TG-enriched HDL (60 μg protein) that was isolated from plasma incubated as described in Experimental Procedures. C: [^{3}H]CE and ^{125}I-protein-labeled HDLs were incubated with HepG2 cells, as described in Experimental Procedures. CE selective uptake was calculated as total CE uptake minus CE that was derived from holo-HDL catabolism as determined from protein radioactivity. D: Same as (C), except that CE selective uptake was determined from isolated HDL subfractions (40 μg protein). Values in (C) and (D) are mean ± SD of triplicate wells.

basis (Fig. 5D). The lower CE selective uptake from TG-enriched HDL was also observed for both HDL_2 and HDL_3 subclasses (Fig. 5D). In all instances, cellular CE incorporation by whole HDL clearance was less than 10% of the selective uptake value and was unaffected by TG enrichment.

DISCUSSION

Hypertriglyceridemia causes marked changes in plasma lipoprotein composition and catabolism. A characteristic aberration is the marked rise in TG/CE content of LDL and HDL. This compositional change is facilitated, in large part, by CETP. To better understand the contribution of CETP-mediated remodeling to the compositional and functional abnormalities of TG-enriched lipoproteins, several studies have examined the altered lipoproteins that result when LDL or HDL are incubated with VLDL and CETP (16–19). However, there has been very limited investigation of the changes induced by CETP when all major plasma lipoproteins are co-incubated (11, 49). Here we describe some of the compositional and functional alterations that result when LDL and HDL are concomitantly enriched in TG by CETP.

After CETP-mediated remodeling, the TG/CE ratio in LDL and HDL increased 2- to 3-fold, resulting in final weight ratio of ~0.5. Similar core compositions are observed in LDL and HDL isolated from mildly hypertriglyceridemic subjects of varying etiologies (7, 10, 50). TG en-

richment of LDL had no measurable effect on its size or its apolipoprotein content. Apolipoprotein changes have been reported when LDL is modified by VLDL and CETP alone (16, 18), but not when LDL TG enrichment is achieved under more physiological conditions (11). By agarose gel electrophoresis, TG-rich LDLs were more electronegative; this alteration has been previously reported for LDLs from hypertriglyceridemic subjects (11) and in vitro modified LDL (11, 51). Increased electrophoretic mobility was not due to oxidation, indicating that TG enrichment of LDL alters the conformation of apoB such that its electronegative surface potential is increased (52). Changes in apoB conformation following TG enrichment have been reported by others, based on immunologic and proteolytic methods (11, 49), and are readily observed in patient-derived hypertriglyceridemic LDL (10).

The cellular uptake of TG-rich LDL was the same as control LDL. However, TG-enriched LDL protein was ineffectively process by cells, as illustrated by reduced degradation and elevated intracellular LDL protein. LDLs enriched in TG under more physiological conditions were also degraded less well. This novel defect in processing was also observed in vitro with exogenous protease, strongly suggesting that TG enrichment results in alterations in apoB conformation, rendering it more resistant to proteolytic attack. As suggested by the near-identical uptake and similar binding affinities of control and TG-enriched LDL, these conformational changes appear to be remote from the receptor binding region of apoB. Immunological studies have confirmed that the structure of apoB in TG-enriched LDL is unaltered in this region, as assesses by the MB47 antibody (11, 51).

TG-enriched LDL ineffectively down-regulated cholesterol biosynthesis compared with control LDL at the same protein concentration. When compared on an equal cholesterol basis, control and TG-enriched LDL were equivalent in their capacity for down-regulation of cholesterol biosynthesis. Thus, despite ineffectual hydrolysis of LDL protein, the cholesterol contained in TG-rich LDL is efficiently processed and exported from the lysosome. In TG-rich LDL, the lysosomal hydrolysis of CE may be facilitated by the reduced phase transition temperature of the core lipids that accompanies TG enrichment (11). Nonetheless, to achieve equivalent sterol synthesis control, greater numbers of TG-enriched LDL particles must be internalized to deliver the same cholesterol load. This reduced cholesterol content may lead to the need for higher steady-state levels of LDL receptors in vivo to achieve sterol regulation.

TG-enriched HDL were modestly larger but were apparently unaltered in apolipoprotein content. This differs from that observed when the TG-CE ratio of modified HDL reaches 1.3 (19), suggesting that apolipoprotein content may be altered under extreme remodeling conditions. TG-rich HDL displayed a slightly lower electrophoretic mobility than control HDL. This likely results from alterations in apolipoprotein A-I conformation, which has been shown to be very sensitive to core lipid composition (53).

In reverse cholesterol transport, HDL accepts tissue cholesterol, LCAT converts the cholesterol to CE on the HDL surface, and then CE is either transferred to VLDL by CETP for hepatic clearance or removed directly from HDL by the hepatic SR-BI (54). The high incidence of coronary heart disease in hypertriglyceridemia (1), which significantly exceeds that attributable to reduced HDL cholesterol levels alone (2), suggests that hypertriglyceridemic HDLs are functionally aberrant. We observed that TG-rich HDLs are abnormal in their support of cholesterol efflux, esterification, and transport. TG-enriched HDL stimulated greater net cholesterol efflux from cholesterol-loaded macrophages than control HDL. This resulted largely from a 50% greater efflux rate. However, cholesterol associated with TG-rich HDL produced by the action of CETP on purified lipoproteins or isolated from incubated plasma was esterified by LCAT at a markedly slower rate. Less than half of this reduction appears related to changes in HDL subclass levels (55, 56). We hypothesize that these two metabolic abnormalities are related to a redistribution of cholesterol from the HDL surface to the core, where up to 40% of HDL cholesterol normally resides (57). This redistribution may occur in TG-enriched HDL because the increase in TG-CE ratio markedly lowers the order state of the core lipids (11), thus increasing the solubility of cholesterol in the core (58), and the increase in the size of HDL provides a larger core volume to accommodate cholesterol. Additionally, TG, which is more soluble in the surface phospholipids than CE (59), may reduce the effective surface concentration of cholesterol when the TG-CE ratio rises. Together, these changes would result in an HDL particle that has increased capacity to take up cholesterol, but a larger portion of this cholesterol is excluded from the surface, where it can be esterified by LCAT.

We recently reported that HDLs modified by CETP and VLDL (i.e., in the absence of LDL) are ineffective donors of CE to cells via the SR-BI (24). This finding was confirmed here with HDL enriched in TG under more physiological conditions. Others have reported that HDL size is an important determinant of CE selective uptake (60). However, since we also found deficient CE selective uptake from TG-enriched HDL subfractions of similar size, it appears that changes in lipoprotein composition are the most influential determinant of uptake in this instance. This supports our earlier hypothesis that CE selective uptake from HDL is strongly influenced by the TG-CE ratio in the HDL core.

In summary, CETP incubated with lipoproteins, combined to simulate ratios present in mildly hyperlipidemic plasma, forms LDL and HDL containing TG-CE ratios that are commonly observed in hypertriglyceridemic plasma (7, 10). Unlike other studies that have modified LDL or HDL in the presence of VLDL alone (16, 18, 19), no apparent change in apolipoprotein content occurred. TG-enriched LDL interacts with the LDL receptor on fibroblasts with slightly lower affinity, but is taken up by these cells normally. TG-rich LDL, due to its reduced cholesterol content, aberrantly regulates sterol biosynthesis.

Also, cells poorly degrade apoB in TG-enriched LDL. This processing defect may lead to the lysosomal accumulation of apoB, as occurs in foam cells induced by oxidized LDL (61). TG-enriched HDL, while mediating greater cholesterol efflux from macrophages, is unable to support normal LCAT activity. It is likely that this increased capacity to accept cholesterol would be quickly exhausted in vivo without efficient conversion to CE by LCAT. In terms of mechanisms facilitating CE removal from HDL, it has been previously shown that TG-enriched HDLs are less effective donors of CE to VLDL via CETP (8, 47, 62). We report here that TG enrichment also reduces CE clearance by a second pathway mediated by the SR-BI. These findings collectively indicate that elevations in HDL TG-CE can lead to impairment of almost every step in reverse cholesterol transport. Together, these studies suggest that TG enrichment of LDL and HDL by CETP enhances their atherogenic potential. These results extend previous reports (6, 10, 11, 53) that core lipid composition (TG-CE ratio), independent of lipoprotein size, can modulate lipoprotein structure and function.

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