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Streptococcal Serum Opacity Factor promotes Cholesterol Ester Metabolism and Bile Acid Secretion In Vitro and In Vivo

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Abstract

Plasma high density lipoprotein-cholesterol (HDL-C) concentrations negatively correlate with atherosclerotic cardiovascular disease. HDL is thought to have several atheroprotective functions, which are likely distinct from the epidemiological inverse relationship between HDL-C levels and risk. Specifically, strategies that reduce HDL-C while promoting reverse cholesterol transport (RCT) may have therapeutic value. The major product of the serum opacity factor (SOF) reaction versus HDL is a cholesteryl ester (CE)-rich microemulsion (CERM), which contains apo E and the CE of ~400,000 HDL particles. Huh7 hepatocytes take up CE faster when delivered as CERM than as HDL, in part via the LDL-receptor (LDLR). Here we compared the final RCT step, hepatic uptake and subsequent intracellular processing to cholesterol and bile salts for radiolabeled HDL-, CERM- and LDL-CE by Huh7 cells and *in vivo* in C57BL/6J mice. In Huh7 cells, uptake from LDL was greater than from CERM (2-4×) and HDL (5-10×). Halftimes for [¹⁴C]CE hydrolysis were 3.0 ± 0.2, 4.4 ± 0.6 and 5.4 ± 0.7 h respectively for HDL, CERM and LDL-CE. The fraction of sterols secreted as bile acids was ~50% by 8 h for all three particles. HDL, CERM and LDL-CE metabolism in mice showed efficient plasma clearance of CERM-CE, liver uptake and metabolism, and secretion as bile acids into the gall bladder. This work supports the therapeutic potential of the SOF reaction, which diverts HDL-CE to the LDLR, thereby increasing hepatic CE uptake, and sterol disposal as bile acids.

Abstract Graphic

Keywords

HDL function; Reverse cholesterol transport; Serum opacity factor; Cholesterol ester metabolism; Bile acid secretion; Huh7 human hepatocytes

1.0 Introduction

1.1

Atherosclerosis is the underlying cause of most cardiovascular diseases (CVD), which lead to stroke and heart attacks. By inhibiting cholesterol biosynthesis, statins lower CVD risk and reduce the incidence of cardiovascular disease by reducing the plasma low density lipoprotein (LDL) cholesterol concentrations. Nevertheless, there remains a need for other therapies that reduce CVD risk that include improved high density lipoprotein (HDL) function (Reviewed) [1, 2]. These include improved reverse cholesterol transport (RCT) as assessed by enhanced macrophage cholesterol efflux to plasma, and more efficient hepatic disposal of cholesterol, which is trafficked to the intestine for excretion.

1.2

Streptococcus Pyogenes express a surface protein, serum opacity factor (SOF), that clouds human plasma via its activity against HDL [3]. The SOF-HDL reaction produces three products—lipid-free apolipoprotein (apo) AI, a neo HDL that is smaller than native HDL, phospholipid- and apo AII-rich, and cholesteryl ester poor, and a large cholesteryl ester-rich microemulsion (CERM) that is responsible for the clouding [4]. Apo E is the major CERM-protein, and CERM cholesteryl ester (CERM-CE) is taken up by HepG2 and Huh7 human hepatocytes 2 – 5 times faster than HDL-CE, by multiple hepatic apo E receptors, including LDL receptor (LDLR), lipoprotein receptor-related protein-1 (LRP-1), cell surface proteoglycans, and the scavenger receptor class B type I (SR-BI) [5]. Low dose SOF injection into mice reduces their plasma cholesterol levels ~40% in 3 h, an effect that is due to the apo E-mediated CERM uptake by hepatic LDLR [6]. However, it is not known whether CERM-CE is converted to bile acids for excretion or recycled back to plasma with no net contribution to RCT. Here we further test the therapeutic potential of SOF by comparing the uptake and metabolism of HDL-, CERM- and LDL- radiolabeled cholesteryl

ester by the human hepatoma cell line, Huh7 as well as *in vivo* in mice. We compared the metabolic fate of CE taken up from CERM particles, with those for HDL-CE and LDL-CE. Since CERM-CE uptake is mediated by SR-BI, the HDL receptor, and the LDLR and other apoE-dependent receptors [5], we hypothesized that CERM-CE metabolism after uptake would be intermediate between those for HDL- and LDL-CE; our data support this hypothesis.

2.0 Materials and Methods

2.1 Materials

LDL and HDL were isolated from normal human plasma by sequential flotation at 1.006, 1.063, and 1.21 g/mL. A polyhistidine-tagged, truncated recombinant form of *sof2* (rSOF) encoding amino acids 38–843 was cloned and expressed in *Escherichia coli* and purified by metal affinity chromatography as described [7]. HDL was labeled with [³H]- or [¹⁴C]-cholesterol (Perkin Elmer, Boston MA), which was esterified using the lecithin:cholesterol acyltransferase (LCAT) activity of human lipoprotein-deficient serum; free cholesterol was removed by multiple exchanges with LDL, as described [4]. Alternatively, [¹⁴C] cholesteryl oleate was synthesized from [¹⁴C] cholesterol and oleic acid by the method of Patel *et al.* [8] and incorporated into HDL and LDL by the method of Roberts *et al.* [9]. The HDL- and LDL-[¹⁴C]CE were further purified by size exclusion chromatography (SEC) over two Superose HR6 columns in tandem [5]. Radiolabelled lipoprotein stocks were dialyzed into PBS and sterile filtered prior to incubation with cells. Commercial kits were used to determine the protein (DC protein assay, Bio-Rad, Hercules, CA) and lipid composition (total cholesterol, free cholesterol, phospholipid and triglyceride, WAKO Life Sciences, Richmond, VA) of lipoprotein stocks. The specific activities of the lipoproteins were calculated from the radioactivities and CE concentrations of aliquots of each. CERM-[¹⁴C]CE was prepared from HDL-[¹⁴C]CE by overnight incubation of HDL-[¹⁴C]CE (1–2.5 mg HDL protein/ mL) with rSOF (2–5 µg/mL) at 37°C. Completeness of the CERM reaction was monitored by SEC as described [5]. Representative SEC chromatograms of HDL-[¹⁴C]CE, CERM-[¹⁴C]CE and LDL-[¹⁴C]CE appear in Supplemental Figure 1.

2.2 Cell Culture

Huh7 cells were kindly provided by Drs. Yumin Xu and Boris Yoffe (Baylor College of Medicine, Houston, TX). Hepatocytes were cultured as described [10] in a 50:50 mix of DMEM and Opti-MEM with 10% fetal bovine serum (FBS), 1× GlutaMax, 1 mM sodium pyruvate, 10 U/mL penicillin and 10 g/mL streptomycin (Life Technologies/Gibco, Grand Island, NY). For uptake and metabolic studies, cells were seeded into multi-well plates or culture flasks and grown to confluence in complete media, washed and transferred to serum-free medium immediately before the start of experiments.

2.3 CE Uptake and Metabolism

CE uptake was assayed as described [5, 11]. Confluent Huh7 cells grown in complete medium with 10% FBS were washed twice in serum free medium (Medium A) and put into serum-free medium containing 5 mg/mL bovine serum albumin (Medium B). Uptake was initiated by adding radiolabelled lipoproteins to cells in Medium B. The time course and

dose response for CE uptake from HDL-, CERM- and LDL-[¹⁴C]-CE were determined by incubating each lipoprotein in triplicate with Huh7 cells for 0 – 3 h.

To study post-uptake CE metabolism, cells were pulse-labelled with HDL-, CERM- and LDL-[¹⁴C]CE in Medium B for 2 hours, washed, and chased in serum containing medium for 0, 2, 4 or 6 hours. After the chase, media were collected, cells washed, cell lipids extracted with 2-propanol containing 1% acetic acid, and residual cell protein solubilized with 0.1M NaOH. Aliquots of cell lipid, protein and media were β-counted; solubilized protein aliquots contained no radioactivity. Cell lipid extracts were desalted on C18-BondElut columns (Agilent Technologies, Santa Clara, CA) [12] and lipids separated by high performance thin layer chromatography (HPTLC) on HPTLC Silica Gel 60 plates (EMD Chemicals, Darmsted, Germany) by a modification of the multi-one-dimensional procedure of White *et al.* [13-15]. Separation of CE, free cholesterol (FC), and the bile salts chenodeoxycholate, cholate, glycochenodeoxycholate, glycocholate, taurochenodeoxycholate and taurocholate was obtained by developing the plate to 70% with Solvent A:chloroform:methanol:water:glacial acetic acid 70:25:0.5:0.5 (v:v:v:v), drying the plate, and then developing the entire plate in Solvent B: hexane:diethylether:water:glacial acetic acid, 75:35:0.5:0.5 (v:v:v:v). Radioactivity in the HPTLC bands was quantified using a Typhoon FLA 7000 imaging system (GE Healthcare). Endogenous cell lipids were detected by primuline spray reagent [13] and imaged on the ImageQuant LAS 400 system (GE Healthcare).

2.4 Bile Salt Formation

To determine the fraction of total sterol converted to bile salts, cell lipid and media samples were solvent-partitioned [16]. Huh7 cells were pulse labeled as above with HDL-, CERM-, and LDL-[¹⁴C]CE (32 - 61 nmoles CE /mL) for two hours after which cells were chased for 2 and 6 hours. Cell lipids were extracted into 2-propanol, which was evaporated and the lipid residue sonicated into 87 mM aqueous KCl for partitioning. Media aliquots collected at t = 0, at the end of the 2 hour pulse, and the 2 and 6 hour chase were solvent-partitioned. Prior to partitioning, cold carrier lipids were added to minimize non-specific loss of the radioactive sterols - cholate, glycocholate, taurocholate, chenodeoxycholate, glycochenodeoxycholate and taurochenodeoxycholate (2 µg/mL each) were added to both cell and media samples, while cholesteryl ester and cholesterol (5 µg/mL each) were added to media samples. Samples were partitioned between an upper aqueous phase, containing bile acids, and a lower organic phase containing neutral sterols as follows: The aqueous samples (1 mL) were combined with 3 mL of chloroform:methanol (1:2), 2 mL chloroform and 1 mL of water, with vortexing after each addition. The mixtures were centrifuged, the volumes of the phases recorded, aliquots of the upper and lower phases evaporated, and the radioactivity in each determined by β-counting. Bile acid formation was expressed as the ratio of bile acid- to total sterol-radioactivity.

2.5 Huh7 endogenous cell lipid, oxysterol and bile acid quantitation

Huh7 cells were harvested at low (3.4×10^4 cells/cm²) and high (21.0×10^4 cells/cm²) density, washed, and suspended in PBS containing 0.1% butylated hydroxytoluene. Cell homogenates were assayed for protein, total cholesterol, free cholesterol, phospholipid and

triglycerides. Samples for oxysterol and bile acid assays were lipid-extracted after addition of seven deuterium labeled oxysterol internal standards (Avanti Polar Lipids, Alabaster, AL), and processed for analysis as trimethylsilyl ether derivatives by gas chromatography-mass spectrometry (GC-MS) using a modification [17] of the isotope dilution method [18-20]. Ions used for analysis (m/z) appear in Supplemental Table 1.

2.6 Lipoprotein-Induced Changes in Gene Expression

Confluent Huh7 cells in 6-well plates were treated with HDL or the equivalent amount of CERM or LDL as CE (11 nmoles/mL) for 0 to 5 h as above for the pulse label, in serum-free Medium B. To control for the effect of transferring cells from complete media to the serum-free Medium B used for the lipoprotein pulse, control cells were treated with just the lipoprotein buffer, PBS. Total RNA was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA), and reverse transcribed using 1 µg of RNA with the iScript cDNA Synthesis kit from Bio-Rad Laboratories (Cat# 170-8890). The resulting cDNA was diluted 10-fold, and analyzed for gene expression by quantitative RT-PCR (ABI Viia7) using SYBR green detection or Taqman gene expression assays. To confirm detection within the log-linear range of the assay, cDNA was pooled from all samples and 1:4 serially diluted 8 times to generate standard curves for each primer set. SYBR green reagents were from Bio-Rad laboratories, iTaq Universal SYBR green supermix (Cat# 172-5121). SYBR green primers were from Sigma Genosys; Taqman primers were from Life Technologies. A complete list of the realtime primers used is given in Supplemental Table 2. Relative mRNA expression levels were quantified using Beta-actin as the housekeeping gene, and calculated by the

Ct method normalized to the amount of mRNA present in cells at time zero. Data are expressed as the mean ± standard error for biological replicates.

2.7 CE Metabolism In Vivo

[Cholesteryl-1,2-³H(N)]-oleate (Perkin Elmer) was used to prepare high specific activity HDL-, CERM and LDL-[³H]CE by the method of Roberts et al. [9]. Lipoproteins were purified and concentrated by ultracentrifugation and dialyzed into PBS. Trace amounts of lipoproteins of high specific activity (0.8 – 1 million dpm/200 µL) were injected into mice by tail-vein injection. Timed plasma samples were obtained by retro-orbital bleeds. Mice were sacrificed at 6 h, perfused with saline, and tissues were harvested for lipid analyses as described [6]. Experiments were approved by the Baylor College of Medicine IACUC committee, protocol #AN-6243.

2.8 Data analysis

All values are expressed as mean ± SD. Data were compared using ANOVA followed by Dunn's Method or Holm-Sidak analysis based on failure or pass of the normality test. Student's *t* test was used for pairwise comparisons. Statistical analyses and calculation of regression curves were performed using Sigma Plot 12.3 (SyStat Software, Inc.). Rate constants were calculated from regression fits of the data points vs. time.

3.0 Results

3.1 Kinetics of CE Uptake

The kinetics of CE uptake from HDL-, CERM-, and LDL- ^{14}C CE were compared by incubating cells with CE equivalents of HDL, CERM or LDL, and measuring cell-associated radioactivity over time (Figure 1A). CE uptake increased as HDL- ^{14}C CE < CERM- ^{14}C CE < LDL- ^{14}C CE with respective rate constants of 0.54 ± 0.11 , 1.10 ± 0.02 , and 2.46 ± 0.19 nmoles CE uptake/mg cell protein/h. Rates for CE uptake from HDL, CERM and LDL were all significantly different from each other, $p < 0.001$. When cells were treated with equivalent amounts of CE/mL as HDL, CERM or LDL, uptake from LDL was 2 and 5 times faster than uptake from CERM and HDL particles respectively.

The dose dependence for CE uptake from HDL- ^{14}C CE, CERM- ^{14}C CE, and LDL- ^{14}C CE also differed (Figure 1B). Maximum uptake ($B_{\max}$) increased as HDL- ^{14}C CE < CERM- ^{14}C CE $\ll$ LDL- ^{14}C CE, respectively $B_{\max} = 1.9 \pm 0.1$, 4.9 ± 0.3 , and 82 ± 13 nmoles CE uptake/mg cell protein. Respective cell-associated radioactivities at 50% of $B_{\max}$ occurred at media CE concentrations of 1.9 ± 0.8 , 7.0 ± 1.6 , and 111 ± 25 nmoles CE/mL. According to their $B_{\max}$ values, LDL-CE uptake was highest—16- and 40-fold greater than that of CERM- and HDL-CE respectively.

3.2 CE Metabolism

The kinetics of CE metabolism after cell uptake from HDL- ^{14}C CE, CERM- ^{14}C CE, and LDL- ^{14}C CE for conversion to ^{14}C FC and ^{14}C oxysterols were determined by pulsing the cells for 2 h with the respective ^{14}C CE lipoprotein, to allow for ^{14}C CE uptake, and then chasing for up to 6 h (total time up to 8 h) and measuring the disappearance of ^{14}C CE and the appearance of the products ^{14}C FC and ^{14}C oxysterols over time by HPTLC. HPTLC, which shows good separation of ^{14}C CE, ^{14}C FC and ^{14}C oxysterols (Figure 2 A—C), revealed differences in rates of metabolism (Figure 2 D—F; Table 1). A larger rate constant, k_d , was observed for disappearance for HDL- ^{14}C CE vs. CERM- and LDL- ^{14}C CE, with $t_{1/2} = 3.0$, 4.4 and 5.4 h respectively. The rate constants k_f for ^{14}C FC formation from HDL- and CERM- ^{14}C CE were similar and twice that of ^{14}C FC formation from LDL- ^{14}C CE. Notably, the larger standard error (SE) for the LDL data reflect a poorer fit to an exponential rise ($r^2 = 0.97$) compared to a sigmoidal fit ($r^2 = 0.99$), consistent with a lag time of ~ 1 h for formation of FC from the CE taken up from LDL. Oxysterols are formed at the rate of 1 – 2% of the total sterol/h, with formation from CERM-CE slower than from LDL-CE ($p = 0.037$) but not significantly different than from HDL-CE. ^{14}C Bile acids in cell lipid extracts were below the HPTLC detection limit.

3.3 Sterol and Bile Acid Secretion

Pulse-chase studies also revealed the rates of sterol secretion. Secretion of ^{14}C sterols was determined from the radioactivity in the media collected during chase. Between 15 and 20% of the ^{14}C sterol taken up during the 2 h pulse was secreted into the media at 4 to 8 h (Figure 3A). This proportion was the same for cells treated with HDL-, CERM- and LDL- ^{14}C CE, even though absolute CE uptake from LDL-CE was several-fold greater than that

from HDL- and CERM-CE, indicating that even the higher amount of uptake from LDL-CE did not alter the relative rates of sterol secretion.

To determine the fraction of sterols converted to bile acids, media and cell lipids were partitioned into organic and aqueous phases to separate bile salts from neutral sterols [16]. At 4 h, ~40% of secreted sterols occurred as bile acids, rising to ~50% at 8 hours (Figure 3B). Again, these ratios were the same for cells treated with HDL, CERM or LDL even though uptake from these three CE-containing particles differed more than seven-fold. The bile acid fraction of total sterols was significantly higher at 8 vs 4 hours for the CERM treated samples ($p = 0.009$); the HDL-treated samples show the same trend, but the means were not significantly different. Essentially no bile acid secretion occurred during the initial 2 hour pulse, as partitioning of the pulse media from the HDL-[^{14}C]CE-, CERM-[^{14}C]CE-, or LDL-[^{14}C]CE-treated cells showed <0.2%, <0.1% and <0.6% of the counts respectively recovered in the aqueous phase of the partitions. Cell bile acid content was determined by partitioning cell lipids (Figure 3C). In cells treated with HDL-[^{14}C]CE and CERM-[^{14}C]CE, less than 0.7% of intracellular [^{14}C]sterols were present as bile acids, while in LDL-[^{14}C]CE treated cells 4.2% were bile acids ($p=0.03$) by 4 h. The greater CE uptake from LDL than from CERM and HDL may be driving this conversion to bile acids.

3.4 Endogenous Huh7 Cellular Lipids

Cellular cholesterol homeostasis is highly regulated. To address the effect of the mass of [^{14}C]CE uptake in these experiments on cell-CE homeostasis, the endogenous lipid contents of Huh7 cells were determined (Table 2). In the course of growing Huh7 cells, we noted that as cells approached confluence, the mass of phase-dense lipid droplets increased (Supplemental Figure 2). Therefore cells were harvested for lipid analysis both during the growth phase (3.4×10^4 cells/cm²) and at confluent density (21.0×10^4 cells/cm²). Both total cholesterol and triglyceride were significantly higher in cells harvested at high density ($p < 0.007$), with 150 nmoles total cholesterol/mg cell protein. Confluent Huh7 cells contain considerable oxysterol, 1.2 nmoles/mg cell protein, consistent with the HPTLC autoradiogram patterns after [^{14}C]CE metabolism (Figure 2A). Two of the three most abundant oxysterols are the initial products in the classical and alternative bile acid synthesis pathways: 27-hydroxycholesterol is the Cyp27A1 product, the first in the alternative pathway that leads to chenodeoxycholate, and 7 α -hydroxycholesterol is the Cyp7A1 product, the first product in the classic pathway that leads to cholic acid.[21] Only two bile acids, cholate and chenodeoxycholate, were detected in the cell samples, at low amounts, 0.20 and 0.26 pmoles/mg cell protein. This is consistent with the low amounts of cellular bile acids found in the cell-lipid partitioning experiments (Figure 3C), with detectable cell [^{14}C]bile acids only in the LDL-treated cells, consistent with a rapid secretion of bile acids from Huh7 cells.

3.5

CE uptake as a fraction of endogenous cell cholesterol was calculated from the rate of uptake of CE (Figure 1) and the endogenous cell cholesterol content (Table 2). At 2 h, CE uptake from HDL-, CERM- and LDL-[^{14}C]CE was 1.3, 2.3 and 5.5 nmoles CE/mg cell protein, and this was 0.9%, 1.5% and 3.7% of the endogenous total cholesterol/mg cell

protein, and 4.4%, 7.6% and 18.4% of the endogenous cholesteryl ester/mg cell protein. We hypothesized that the amount of CE uptake, especially from LDL, might perturb cellular cholesterol homeostasis, and therefore probed cells for changes in mRNA expression of genes of cholesterol and bile acid metabolism as a function of CE uptake from HDL, CERM and LDL.

3.6 Effect of CE uptake on mRNA expression

Given the tight regulation of cell cholesterol content, we tested whether CE uptake from CERM vs. HDL and LDL was different in its effect on mRNA expression of key genes in cholesterol and bile acid metabolism. Given that much more CE was taken up from LDL, we hypothesized that this would have the greatest effect on mRNA levels. The maximum effect observed was on *SREBF1* mRNA, which increased 23-fold in control PBS treated cells at 1 h, but less (15-fold) in cells treated with HDL, CERM or LDL, suggesting that the lipoproteins partially compensate for the change to serum-free media. (Figure 4A). *SREBF1* mRNA remained elevated at 2.5 and 5 h, but PBS and lipoprotein levels were no longer different. Thus, the change from 10% FBS media to serum free media results in a large and persistent increase in *SREBF1* mRNA, that is only partially compensated for by the addition of HDL, CERM and LDL. *CYP7A1* mRNA levels increased 6-12-fold by 1 hour of treatment, but PBS and lipoprotein values were not significantly different (Figure 4B), suggesting that *CYP7A1* mRNA levels are sensitive to the serum content of the media but the amount of added lipoproteins had no additional effect. By 5 hours, all treated-cell *CYP7A1* mRNA levels had returned to baseline. Interestingly, we observed no significant differences between control PBS and lipoprotein treatment in the expression of *HMGCR*, *CYP8B1*, *CYP7B1*, *CYP27A1*, *ABCG8*, *ABCB11*, *ABCA1*, *LIPA*, *CES1*, *NCEH1*, *LDLR*, *LRP1*, *SCARB1*, *VLDLR*, or *SDC1* during 5 hours of treatment (Supplemental Figure 3). However, the expression of these genes in response to the media change did vary, with some increasing, some not changing and some (*VLDLR* and *SDC1*) decreasing.

3.7 In vivo metabolism of CERM-CE

Comparison of HDL-, LDL- and CERM-CE was extended to in vivo studies with C57BL/6J mice. Plasma clearance and metabolism of HDL-, LDL- and CERM-[3H-CE] was followed for 6 hours after tail vein injection. Initially plasma CERM-[3H-CE] clearance was intermediate between that for HDL- and LDL-[3H-CE], but while the latter slowed after 200 min, the CERM-[3H-CE] clearance continued to decrease to 7% of the injected dpm by 6 h (Figure 5A). A three-parameter exponential fit of the data gave rate constants of $(14.0 \pm 2.5) \times 10^{-3}$, $(5.2 \pm 1.0) \times 10^{-3}$, and $(6.3 \pm 1.0) \times 10^{-3} \text{ min}^{-1}$ for the disappearance of [3H]CE in HDL-, CERM, and LDL, which correspond to respective halftimes 50, 135, and 111 min. Importantly, CERM-CE had the smallest fraction of injected dose remaining in the plasma compartment of all lipoproteins at the terminal 6 hour time point: CERM-CE (7 ± 2)% vs HDL-CE (24 ± 1)% and LDL-CE (34 ± 5)%, $p < 0.001$ (Figure 5A, 5B). Analysis of mouse tissues at 6 hours showed that liver uptake predominated, with comparable uptake of HDL- and CERM-CE, (47 ± 6)% and (39 ± 9)% percent of the injected dpm), while liver uptake from LDL-CE was less, (20 ± 3)%, $p < 0.001$. Output of sterols to bile was greatest from HDL-CE (4.9 ± 1.2)%, $p < 0.05$ compared to CERM-CE (0.9 ± 0.1)% and LDL-CE (1.2 ± 0.2)%. The extent of CE metabolism in liver harvested at 6 hours was analyzed by thin layer

chromatography, and was comparable for HDL-, CERM- and LDL-CE, by which time 70-80% of the CE was hydrolyzed to free FC, with 2 – 4% present as oxysterols and bile acids. (Figure 5C). Partitioning of liver lipids and of bile to separate bile acids from neutral sterols showed less than one percent of liver sterols were bile acids (Figure 5D), but greater than 85% of secreted sterols were bile acids (Figure 5E). Thus in both Huh7 cells and mouse hepatocytes, bile acids are rapidly secreted after synthesis.

4.0 Discussion

4.1 SOF has profound physiological effects that might contribute to improved RCT

SOF lowers plasma cholesterol in mice by diverting HDL-CE to hepatic apo E-dependent receptors, especially the LDL receptor. [5, 6] SOF activity also makes plasma a better acceptor of macrophage cholesterol efflux, an effect that reduces macrophage inflammation; the mechanistic basis for this is the SOF-mediated formation of neo HDL, which is more cholesterol-poor than HDL. [22] rSOF also enhances hepatic HDL-cholesterol uptake [5]. Whereas hepatic CERM-CE uptake is known, the subsequent intrahepatic itinerary of the CERM-CE is not. Conversion to bile acids is one of the last steps in the RCT pathway, and manipulations that drive bile acid synthesis and secretion should promote RCT. To establish that hepatic CERM-CE uptake promotes this step, we compared the hepatic metabolism of CERM-CE with those of HDL- and LDL-CE. Importantly, the “good stuff”—lipid free apo A-I and neo HDL, which support macrophage cholesterol efflux, are not rapidly removed.

4.2 Multiple lipoprotein receptors mediate hepatic CE uptake and its attendant kinetics

The LDLR mediates LDL-CE endocytic uptake [23], while SR-BI selectively removes HDL-CE with the concurrent exclusion of protein and some polar lipids. [11, 24, 25]. CERM-CE uptake is mediated by the SR-BI as well as LDLR and other apoE-dependent receptors [5]. When Huh7 cells are treated with equivalent amounts of CE as HDL, CERM and LDL, both the rate and mass of CE uptake (dose response) from CERM is between those for HDL and LDL (Figure 1). *In vitro* CE uptake from HDL, LDL and CERM were compared at below physiological CE concentrations: in normolipidemic human plasma with lipoprotein concentrations of 40 mg/dL HDL-CE and 97 mg/dL LDL-CE [26], the HDL-CE and LDL-CE concentrations are respectively about 0.65 and 1.57 μ moles HDL- and LDL-CE/mL plasma, an order of magnitude higher than that tested here (Figure 1B). Still, the kinetic and dose response curves of Figure 1 indicate that under physiological conditions, hepatic CE uptake from LDL would be several-fold greater than that from HDL. SOF-mediated conversion of HDL-CE to CERM-CE increases both the rate and capacity of uptake to values that are closer to those for LDL-CE. Whereas apo AI and HDL are the initial acceptors of free cholesterol from macrophages, the initiating RCT step, in plasma, cholesterol readily transfers to the apo B-containing lipoproteins, LDL and very low density lipoproteins, as well as red blood cells [22, 27, 28]. In addition to these steps, CETP activity transfers HDL-CE to the apo B-containing lipoproteins so that in human RCT, IDL and LDL likely play a more important quantitative role in delivery of excess cholesterol to the liver for disposal. SOF activity emulates these consequences of CETP by transferring HDL-CE to large apo E-containing lipid particles that interact with LDLR and other apo E-dependent receptors thereby enhancing hepatic lipid uptake [5].

4.3 The metabolic fates of LDL- and HDL-CE are distinct

Following LDL uptake, LDL-CE are hydrolyzed by a lysosomal acid lipase (LAL), which is encoded by the *LIPA* gene [29, 30]. In contrast, HDL-CE, which enter cells via the selective SR-BI pathway, [11] are hydrolyzed by cytoplasmic neutral CE hydrolases [31-33], which in liver is primarily the neutral CE hydrolase encoded by *CES1* [34, 35], while in macrophages two neutral CE hydrolases, encoded by *LIPE* and *NCEH1*, account for most of the activity [33, 36-38]. CERM-CE enters cells via multiple receptors [5], so that some of the CE is hydrolyzed in the endosomal/lysosomal LDLR pathway, and the remainder is hydrolyzed by neutral cholesteryl ester hydrolases in the SR-BI pathway. Consistent with this, the rate constants for CERM-CE hydrolysis and the appearance of FC were between the faster and slower rate constants for HDL-CE and LDL-CE respectively (Figure 2 and Table 1). While these values are significantly different, the absolute values are within a 2-fold range, i.e. are not profoundly different. Thus, while various intracellular pathways and compartments—plasma membrane, endosomes and lysosomes, and neutral and acidic cholesteryl ester hydrolases catalyze CE hydrolysis, the overall rates for FC formation via these various pathways in hepatocytes occur at rates that differ several fold but not by orders of magnitude.

4.4

Sterol secretion was proportional to the mass of HDL-CE, CERM-CE and LDL-CE taken up by Huh7 cells (Figure 3A), indicating that the under our experimental conditions, the capacity of the Huh7 cells to metabolize CE after internalization, even in the instance of much higher CE uptake from LDL, was not exceeded. Consistent with this, the fractions of sterol secreted as bile acids were also similar for HDL-CE, CERM-CE and LDL-CE (Figure 3B). We conclude that CERM-CE uptake was greater than that of HDL-CE, and subsequent metabolism to FC, oxysterols and bile acids occurs at rates similar to those observed for both HDL- and LDL-CE. In effect, SOF-treatment of HDL to form CERM promotes the final RCT step, hepatocyte CE uptake, and its metabolism and secretion into bile. *In vitro* and *in vivo* experiments in mice have shown that selective uptake of CE from HDL by the SR-BI receptor is efficiently coupled to bile acid secretion [34, 39]. Similarly, *in vivo* experiments in rats comparing liver uptake and biliary secretion of HDL-[³H]CE to LDL HDL-[³H]CE showed efficient coupling of selective uptake of HDL-CE to bile acid secretion, with greater uptake and biliary secretion from HDL-[³H]CE than LDL-[³H]CE, as shown here in our mouse study (Figure 5B); treatment of rats with oestradiol to upregulate LDLR equalized the rates of biliary secretion [40]. Thus the main determinant of bile sterol secretion both *in vitro* and *in vivo* is the array and activity of cell surface hepatic lipoprotein receptors. CERM, with apo E, is targeted to multiple apo E-dependent receptors [5].

4.5

As the Huh7 cells approached confluence, their lipid content increased and lipid droplets appeared more prominent (Table 2, Supplemental Figure 3 and [41]). The cholesterol and triglyceride contents of subconfluent Huh7 cells were similar to those previously reported [42]. Our report of Huh7 oxysterol content, which is the first, shows that these cells produce measurable amounts of oxysterols. Two of the three most abundant oxysterols, 7 α -

hydroxycholesterol and 27-hydroxycholesterol, are respectively the initial products of the classical and alternative pathways for bile acid synthesis [21]. The Huh7 cells contained small amounts of intracellular cholate and chenodeoxycholate. Human HepG2 cells also synthesize and secrete cholic and chenodeoxycholic acid, but contain only traces as intracellular bile acids. [43, 44]

4.6 Effect of CE uptake on cholesterol homeostasis/ mRNA results

Despite marked differences in the rates of uptake of HDL-CE, CERM-CE, and LDL-CE by the hepatocytes, there were no differences among these lipoproteins in gene expression. We observed no significant differences in mRNA for the bile acid synthetic genes *CYP7A1*, *CYP27A1*, *CYP8B1*, or *CYP7B1*. Likewise, expression of genes involved in lipoprotein uptake (*LDLR*, *SCARB1*, *VLDLR*), CE hydrolysis (*LIPA*, *CES1*, *NCEH1*), cholesterol synthesis (*HMGCR*) and HDL biogenesis and cholesterol and bile acid efflux (*ABCA1*, *ABCG8*, *ABCB11*) were also unaffected. This may be due to the low lipoprotein dose used in these experiments (11 nmoles/mL). While well-suited to trace the intracellular fate of lipoprotein CE, it appears this dose did not substantially impact the regulatory pool of cholesterol. It is however noteworthy that *SREBF1* was upregulated at one hour after the change to serum free media, and that the addition of HDL-, CERM and LDL-CE each suppressed this increase. The mechanistic basis for the increase in *SREBF1* mRNA levels is not known. Future studies *in vivo* will allow us to more accurately assess the impact of CERM on hepatic gene expression, cholesterol and bile acid metabolism, and reverse cholesterol transport.

4.7

In our study of the various cholesterol metabolism genes, we discovered that the change of media from complete 10% serum-containing media for cell culture to the serum free medium used for the pulse of cells for lipoprotein CE uptake [11] elicited significant increases in mRNA expression in 3 of 4 bile acid synthesis genes. (Figure 4 and Supplemental Figure 3). Work by others [45, 46] revealed that changing cell medium can produce transient 10-fold changes in sphinganine and sphingosine. Our results suggest that bile acid synthesis is also sensitive to changes in cell culture media.

4.8

To further validate the efficacy of CE clearance by CERM particles, we extended our study of CERM-CE metabolism to include *in vivo* studies in mice (Figure 5). Our results demonstrate that in C57BL/6J mice, HDL-, LDL-, and CERM-[³H]CE were similarly cleared from plasma and after six hours, most ³H accumulated in the liver as free cholesterol; traces (<0.5%) of [³H]bile acids remained in liver while nearly all [³H]sterol in bile occurred as bile acids. The mouse data contrasts with those obtained with Huh7 cells in that hepatic uptake of LDL-CE in mice was less than that from HDL- and CERM-CE, in contrast to the greater uptake in Huh7 cells (Figure 1), perhaps due to the different array and activity of cell surface hepatic lipoprotein receptors on the human vs mouse hepatocytes.

5.0 Conclusion

5.1

We identified the metabolic fate of CE taken up from CERM particles, and compared it to that for CE taken up from HDL and from LDL. Since CERM-CE uptake is mediated by the HDL receptor SR-BI as well as the LDLR and other apoE receptors [5], we hypothesized that its metabolic pathway after uptake would be intermediate between that for HDL-CE and LDL-CE, and our results confirm this. These results provide further support for the therapeutic potential of rSOF as an agent that promotes RCT from the initial step of CE efflux from macrophages to the final secretion of excess sterol by hepatocytes as bile acids (Figure 6). Repeated administration of rSOF may generate neutralizing antibodies or elicit an immune response that could potentially ameliorate further use. Therefore we have in progress work on the X-ray crystal structure of rSOF to guide in the development of smaller, non-immunogenic functional rSOF mimetics.

5.2

Our previous studies *in vitro* and *in vivo* showed that SOF converted HDL to several products, one of the them being CERM, which contains apo E as a major protein. Our cell studies showed that CERM were ligands for several apo E dependent receptors [5] and this study as well as previous *in vivo* studies showed that CERM were rapidly cleared from plasma by the liver. [6] However, these data did not reveal the metabolic fate of the CERM-CE. If the CERM-CE were recycled back to plasma, there would be no net contribution to RCT. However, our data shows that the CERM-CE enters the intracellular RCT pathway that terminates in the transfer of CERM-CE to bile as cholesterol, oxysterols, and bile acids. Liver uptake of CE from CERM occurs via multiple receptors. Moreover, upon entering the hepatocyte, CERM-CE transfers to bile so that as with CE uptake, the amount of sterol secreted to bile increases in proportion to liver uptake. The SOF-mediated diversion of CE to the LDL receptor and the greater uptake of CERM- vs. HDL-CE support the previously proposed hypothesis, that the SOF reaction may improve RCT, something that remains to be shown *in vivo*.

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Abbreviations

B_{max}	maximum uptake
C	cholesterol
CE	cholesteryl ester
CERM	cholesteryl ester rich microemulsion
FBS	fetal bovine serum
FC	free cholesterol
GC-MS	gas chromatography-mass spectrometry
HDL	high density lipoprotein
HPTLC	high performance thin layer chromatography
LCAT	lecithin: cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LRP-1	lipoprotein receptor related protein-1
PBS	phosphate buffered saline, pH 7.2

RCT	reverse cholesterol transport
SEC	size exclusion chromatography
SOF	serum opacity factor
SR-BI	scavenger receptor class B member 1
TG	triglyceride
TLC	thin layer chromatography

Highlights

Background

New strategies that promote clearance of excess cholesterol by reverse cholesterol transport (RCT) are needed.

Results

We investigated the therapeutic potential of serum opacity factor to promote the final steps of RCT-hepatic CE uptake, metabolism and secretion as bile acids *in vitro* in Huh7 human hepatocytes and *in vivo* in C57BL/6J mice.

Conclusion

The rates of hepatic uptake and metabolism of the SOF product CERM-CE were intermediate between those for HDL-CE and LDL-CE. Both *in vitro* and *in vivo*, CERM-CE is metabolized by hepatocytes to bile acids and secreted.

Significance

This work indicates that the CERM particle is competent for delivery of cholesterol to the liver, supporting it as a therapeutic strategy to promote RCT.

Fig. 1.

Uptake kinetics. A. CE uptake from HDL, CERM, and LDL, as a function of time as labeled; data are plotted according to a first order linear polynomial from which calculated slopes yielded rates. B. CE uptake from HDL, CERM, and LDL as a function of dose as labeled. Kinetic constants, given in the text, were calculated from a two parameter hyperbolic function. For the time course, cells were incubated with 11.1 nmol CE/mL as HDL-[^{14}C] CE, CERM-[^{14}C] CE or LDL-[^{14}C] CE. For the dose-response, cells were incubated with the indicated amounts of CE for 3 h and then harvested for analysis of CE uptake. Data points are the mean $\pm$ SD of triplicate samples.

Fig. 2.

CE metabolism in Huh7 cells. A–C: HPTLC autoradiograms for time course for Huh7 cell metabolism of ^{14}C CE taken up from HDL, CERM and LDL. Cells were pulsed for 2 h with the respective ^{14}C CE radiolabeled lipoprotein at 14.3 nmol CE/mL, and chased for 0, 2, 4 or 6 h prior to harvest of cells for analysis. Total time (pulse + chase) is indicated, and triplicate samples are shown for each time point. The lower panels, labeled CE, show the primuline stain of the endogenous CE present in the samples. D–F: Kinetic curves for cellular CE hydrolysis and cellular FC and oxysterol formation, respectively. Three oxysterol bands were detected, and the sum of the volume of these was plotted as the rate of formation of oxysterols. Amounts at each time point were calculated by phosphor image analysis of the autoradiograms, using ImageQuant software. The data are the average of two independent experiments, each done in triplicate. Significant differences ($p < 0.05$) at each time point are indicated by symbols: α , HDL vs CERM; β , HDL vs LDL; χ , CERM vs LDL. Kinetic constants were determined for CE and FC according to exponential decay and rise to maximum, respectively. The rate of oxysterol appearance was calculated from a first order linear polynomial. Rate constant values are given in Table 1.

Fig. 3.

Sterol secretion and bile acid formation in Huh7 cells. Cells were pulsed for 2 h with HDL-, CERM- or LDL-¹⁴C]CE at 32–61 nmol CE/mL media, washed, and harvested (2 h) or chased in fresh media and harvested at 4, 6 or 8 h. Chase media was collected for analysis of secreted C-sterols. Cell lipids and media were partitioned to determine the ratios of bile acids to neutral sterols. A. Secreted sterol as a fraction of the total sterol in cells plus media. B. Bile acid percent of the secreted ¹⁴C-sterols. C. Bile acid percent of the cell ¹⁴C- sterols. HDL and CERM were tested at 2, 4 and 8 h, LDL was tested only at 4 h. Asterisks indicate significant differences, *p* < 0.05, between the bracketed groups.

Fig. 4.

Effect of CE uptake from HDL-, CERM- or LDL on mRNA levels of (A) SREBF1 and (B) CYP7A1. Double asterisk in panel A at 1 h indicates all samples are elevated relative to 0 h control, and the PBS treated cells are greater than the lipoprotein treated cells. Single asterisks in panels A and B indicate all treatments at that time point are greater than that at 0 h, but do not differ from each other (Kruskal–Wallis one way analysis of variance on ranks). Values for SREBF1 are mean $\pm$ SE, values for CYP7A1 are median and 25–75% range. Results for other genes of cholesterol and bile acid metabolism are in Supplemental Fig. 3.

Fig. 5.

In vivo metabolism of HDL-, CERM- and LDL-CE. A. Plasma [³H]CE clearance after injection of the indicated radiolabelled lipoprotein. B. Tissue uptake of sterols at 6 h. HDL-CE uptake was greatest in all tissues except spleen, where CERM-CE uptake was greatest (ANOVA, *p* < 0.05). C. Liver CE metabolism at 6 h. D. Bile acids as portion of total liver sterols at 6 h. E. Bile acids as a portion of total bile sterols at 6 h.

Fig. 6.

Therapeutic potential of SOF. SOF or its analogs may have therapeutic potential as agents that enhance cholesterol uptake by the liver and promote its clearance into bile. rSOF acts on HDL to form neoHDL, lipid free apo AI and CERM. The first two products promote macrophage cholesterol efflux. CERM promotes hepatocyte CE uptake by interaction with several liver receptors. After uptake, CERM-CE is metabolized and secreted as bile acids.

Table 1
Kinetic Constants for HDL-, CERM-, and LDL-[¹⁴C]CE Metabolism^a

Precursor	k_d , h^{-1} , ($t_{1/2}$, h)	k_r , h^{-1} , (max %)	m (%/h)
	[¹⁴ C]CE	[¹⁴ C]FC	[¹⁴ C]oxysterol
HDL-[¹⁴ C]CE	0.23 ± 0.01 , (3.0)	0.26 ± 0.09 , (78 $\pm$ 12)	1.9 ± 0.3
CERM-[¹⁴ C]CE	0.15 ± 0.02 , (4.4)	0.27 ± 0.03 , (65 $\pm$ 3)	0.9 ± 0.5^b
LDL-[¹⁴ C]CE	0.13 ± 0.02 , (5.4)	0.14 ± 0.12 , (72 $\pm$ 37)	2.1 ± 0.2

^aValues are calculated kinetic constants $\pm$ SE from Figure 2 D – F, fitting the data to an exponential decay (k_d), exponential rise (k_r) or linear fit (m).

^bOxysterol rate of formation from CERM-CE is significantly different from LDL-CE ($p=0.037$) but not HDL-CE ($p=0.102$)

Table 2
Lipid content of Huh7 cells

Harvest Density, 10 ⁴ cells/cm ²	3.4	21.0	<i>p</i> ^b
Lipids ^a nmoles/mg cell protein, mean ± SD			
Total cholesterol	91 ± 6	150 ± 15	0.007
Cholesteryl Ester	16 ± 4	30 ± 8	0.088
Free Cholesterol	75 ± 3	120 ± 7	0.001
Triglyceride	80 ± 8	132 ± 4	0.000
Phosphatidyl Choline	132 ± 2	179 ± 18	0.026
Oxysterols pmoles/mg cell protein, mean ± SD			
27-Hydroxycholesterol		379 ± 87	
5β,6β-Epoxycholestanol		239 ± 92	
7α-Hydroxycholesterol		228 ± 51	
7β-Hydroxycholesterol		106 ± 36	
4β-Hydroxycholesterol		74 ± 23	
25-Hydroxycholesterol		59 ± 25	
24(S)-Hydroxycholesterol		26 ± 9	
7-Ketocholesterol		26 ± 10	
5α,6α-Epoxycholestanol		13 ± 8	
3β,5α,6β-Trihydroxycholestane		7 ± 5	
Total oxysterols		1157 ± 273	
Bile Acids ^c pmoles/mg cell protein, mean ± SD			
Cholate		0.20 ± 0.10	
Chenodeoxycholate		0.26 ± 0.08	
Total bile acids		0.46 ± 0.14	

^aTwo (low density) or four (high density) dishes of Huh7 cells were harvested for lipid and protein assays. Oxysterols and bile acids were determined for the high density harvested cells. Each assay was done in triplicate.

^bStudent t-test p value comparing lipid content at high and low density.

^cGlycocholate, taurocholate, glycochenodeoxycholate and taurochenodeoxycholate were not detected in the cell samples.