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Streptococcal Serum Opacity Factor Increases the Rate of Hepatocyte Uptake of Human Plasma High-Density Lipoprotein Cholesterol[†]

Baiba K. Gillard,*[,‡] Corina Rosales,[‡] Biju K. Pillai,[‡] Hu Yu Lin,[‡] Harry S. Courtney,[§] and Henry J. Pownall*[,‡]

[‡]*Section of Cardiovascular Research, Department of Medicine, Baylor College of Medicine, Houston, Texas 77030, United States, and* [§]*Veterans Affairs Medical Center and Department of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38104, United States*

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ABSTRACT: Serum opacity factor (SOF), a virulence determinant of *Streptococcus pyogenes*, converts plasma high-density lipoproteins (HDL) to three distinct species: lipid-free apolipoprotein (apo) A-I, neo HDL, a small discoidal HDL-like particle, and a large cholesteryl ester-rich microemulsion (CERM) that contains the cholesterol esters (CE) of up to ~400000 HDL particles and apo E as its major protein. Similar SOF reaction products are obtained with HDL, total plasma lipoproteins, and whole plasma. We hypothesized that hepatic uptake of CERM-CE via multiple apo E-dependent receptors would be faster than that of HDL-CE. We tested our hypothesis using human hepatoma cells and lipoprotein receptor-specific Chinese hamster ovary (CHO) cells. The uptake of [^{3}H]CE by HepG2 and Huh7 cells from HDL after SOF treatment, which transfers >90% of HDL-CE to CERM, was 2.4 and 4.5 times faster, respectively, than from control HDL. CERM-[^{3}H]CE uptake was inhibited by LDL and HDL, suggestive of uptake by both the LDL receptor (LDL-R) and scavenger receptor class B type I (SR-BI). Studies in CHO cells specifically expressing LDL-R and SR-BI confirmed CERM-[^{3}H]CE uptake by both receptors. RAP and heparin inhibit CERM-[^{3}H]CE but not HDL-[^{3}H]CE uptake, thereby implicating LRP-1 and cell surface proteoglycans in this process. These data demonstrate that SOF treatment of HDL increases the rate of CE uptake via multiple hepatic apo E receptors. In so doing, SOF might increase the level of hepatic disposal of plasma cholesterol in a way that is therapeutically useful.

Serum opacity factor (SOF), a virulence determinant of *Streptococcus pyogenes*, converts HDL[1] to lipid-free (LF) apo A-I, neo HDL, which is a small HDL-like particle, and a large cholesteryl ester-rich microemulsion (CERM) that contains the cholesteryl esters (CE) of ~400000 HDL particles and monomeric apo E and its heterodimer with apo A-II as its sole apos (*1*−*6*). Recombinant SOF (rSOF) is potent and catalytic; rSOF (1 μg/mL) quantitatively converts HDL to CERM, neo HDL, and LF apo A-I with a half-time of ~30 min (*4*, *5*). On the basis of the reaction products and kinetics, we proposed a model for the rSOF reaction in which rSOF is a heterodivalent fusogenic protein that uses a docking site to displace apo A-I and bind to exposed CE surfaces on HDL (*4*). The initial rSOF−HDL complex recruits additional HDL with its binding delipidation site and through multiple fusion steps forms large CERM and releases neo HDL and LF apo A-I (*4*). Importantly, CERM contains apo E. We hypothesized that with its high apo E and CE contents, CERM could transfer large amounts of cholesterol to the liver for disposal via the LDL receptor (LDL-R) or other apo E receptors (*4*).

CERM and chylomicron remnants (CR) share some properties, suggesting that they might be cleared via similar pathways. Both contain apo E as a major protein that could mediate cellular uptake via multiple apo E-dependent receptors. Both have large neutral lipid cores that comprise a mixture of cholesteryl esters and triglycerides. Hepatic CR undergo sequestration within the perisinusoidal space, where locally secreted apo E enhances binding and uptake (*7*). Heparan sulfate proteoglycans (HSPG) within the hepatic perisinusoidal space can also bind apo E, which anchors the CR for hydrolysis by lipases, subsequently promoting its uptake. The final endocytic step is mediated by the low-density lipoprotein receptor (LDL-R) and by the low-density lipoprotein receptor-related protein (LRP). Cell-surface HSPG are important in the initial sequestration in the perisinusoidal space and integral to HSPG/LDL-R and LRP pathways. HSPG also function as independent receptors. Given the high CE content of HDL and its complement of apo E, conversion of HDL to CERM via the rSOF reaction might enhance hepatic CE uptake via multiple apo E-dependent receptors. In contrast, most hepatic HDL-CE uptake of CE occurs via the selective pathway mediated by SR-BI, the mouse ortholog of

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*To whom correspondence should be addressed: One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4160. Fax: (713) 798-9005. E-mail: baibag@bcm.tmc.edu or hpownall@bcm.tmc.edu.

[1]Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; apo, apolipoprotein; Bodipy-CE, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-*s*-indacene-3-dodecanoate; BSA, bovine serum albumin; C, cholesterol; CE, cholesteryl ester; CERM, cholesteryl ester-rich microemulsion; CETP, cholesteryl ester transfer protein; CHO, Chinese hamster ovary; CR, chylomicron remnants; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; HDL, high-density lipoproteins; HSPG, heparan sulfate proteoglycans; LDL, low-density lipoproteins; LDL-R, low-density lipoprotein receptor; LF, lipid-free; LRP, low-density lipoprotein receptor-related protein; MEM, minimum essential medium; rSOF, recombinant serum opacity factor; SEC, size exclusion chromatography; SR-BI, scavenger receptor class B type I; RAP, receptor-associated protein; RCT, reverse cholesterol transport; TBS, Tris-buffered saline; TLP, total lipoproteins.

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human CLA-1 (*8*, *9*). Here we show that rSOF converts human HDL to CERM in whole plasma and that uptake of CERM-CE by human hepatoma cells occurs via multiple apoE-dependent lipoprotein receptors and is faster than that of the HDL-CE from which the CERM were formed.

MATERIALS AND METHODS

Materials. HDL were isolated from normal human plasma obtained from The Methodist Hospital Blood Donor Center by sequential flotation at densities of 1.063 and 1.21 g/mL. KBr was removed by dialysis against Tris-buffered saline [TBS, consisting of 10 mM Tris, 100 mM NaCl, and 1 mM EDTA (pH 7.4)], which was used as the standard buffer except where noted otherwise. Lipoprotein purity was verified by SDS−PAGE and size exclusion chromatography (SEC) over two Superose HR 6 columns (GE Healthcare) in tandem (*4*). SEC was also used to obtain lipoprotein profiles according to size. HDL-[^{3}H]CE was prepared from HDL and [^{3}H]cholesterol (Amersham/GE Healthcare) by biological labeling as described previously (*4*). Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-*s*-indacene-3-docecanoate (Bodipy-CE), a fluorescent CE analogue, was from Invitrogen/Molecular Probes (Seattle, WA). Buffer salts were from Thermo/Fisher Scientific, Inc. (Rockville, MD). A recombinant polyhistidine-tagged, truncated form of *sof2*, encoding amino acids 38−843 (rSOF), was cloned and expressed in *Escherichia coli* and purified by metal affinity chromatography as described previously (*6*). Protein concentrations were determined using the DC Protein Kit (Bio-Rad). Immunoblots were created as described previously (*4*) with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare).

Cell Culture. HepG2 cells and wild-type CHO cells, CHO-K1 (LDL-R-positive), were from American Type Culture Collection (Manassas, VA); CHO-ldlA7 cells (LDL-R-negative) and the latter transfected to express large amounts of mouse SR-BI (CHO-SR-BI) were provided by M. Krieger (*10*). Huh7 cells were provided by Y. Xu and B. Yoffe (*11*). Huh7 and HepG2 cells were cultured as described previously (*12*) in minimal essential medium (MEM) with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and penicillin and streptomycin antibiotics (10 units/mL and 10 μg/mL, respectively). CHO cell lines were cultured in Hams F-12 with 5% FBS, 1 mM sodium pyruvate, and penicillin and streptomycin antibiotics (10 units/mL and 10 μg/mL, respectively). The CHO-SR-BI medium also contained G418 (300 μg/mL). Tissue culture reagents were from Invitrogen (Carlsbad, CA).

CE Uptake. The CE uptake was assayed as described previously (*8*). The day prior to the assay, HDL-[^{3}H]CE (1 mg/mL) was incubated overnight at 37 °C without (control) or with (+SOF) rSOF (2 μg/mL) in cell culture medium with 0.5% fatty acid-free bovine serum albumin (BSA) [medium B (*8*)]. SEC analysis of the reaction mixtures confirmed that rSOF treatment transferred >90% of the [^{3}H]CE into CERM-[^{3}H]CE. Uptake was initiated by addition of 20 μL aliquots of control (HDL-[^{3}H]-CE) or CERM-[^{3}H]CE (HDL-[^{3}H]CE +rSOF) reaction mixture to cells in 1 mL of medium B. Cells were incubated for 0−3 h at 37 °C in a 5% CO_2 incubator. Uptake was stopped when the cells were placed on ice. Cells were washed, and [^{3}H]CE taken up by the cells was determined by extraction with 2-propanol and β-counting. Inhibitors of uptake, unlabeled HDL and LDL, RAP (Innovative Research Low Endotoxin Human RAP) (*13*−*15*), and heparin (Sigma heparin sodium from porcine intestinal mucosa) (*13*, *16*), were diluted in medium B and added as 100 μL aliquots to washed cells in medium B just prior to addition of the radiolabeled reaction mixture aliquots, to give a final volume of 1 mL. The effect of Simvastatin (Sigma, St. Louis, MO) on CE uptake was determined as described previously (*17*, *18*). In brief, Simvastatin was activated to the acid form according to the Sigma protocol. Huh7 and HepG2 cells were pretreated with 0.01−10 μM Simvastatin for 18 h in medium with 10% lipoprotein deficient plasma prior to the start of the uptake assay.

Bodipy-CE Labeling of Lipoproteins. Bodipy-CE (1 mg) was dried from chloroform onto the bottom of a conical glass test tube under a stream of N_2. Human plasma HDL (12.5 mg of protein/2.5 mL) was added and sonicated (40 W) on ice for 40 min. The labeled HDL was filtered (0.45 μm), giving HDL-Bodipy-CE. HDL-Bodipy-CE (5 mg/mL) was incubated overnight with rSOF (10 μg/mL), giving CERM-Bodipy-CE, which was isolated by SEC. To prepare LDL-Bodipy-CE, Bodipy-CE (1 mg) was dried onto the bottom of a glass test tube as described above, human plasma LDL (~5 mg/mL) was added in TBS, and the mixture was incubated for 24 h at 37 °C with continuous low-speed vortexing. Unbound Bodipy-CE was removed by filtration (0.45 μm). The incorporation of the label into lipoproteins was assessed fluorometrically.

Confocal Microscopy. Cells grown in 35 mm Glass Bottom Dishes No. 1.5 (MatTek Corp., Ashland, MA) were used for live cell imaging. Huh7 cells were incubated with HDL-, CERM-, or LDL-Bodipy-CE (50 μg/mL in complete medium) for 60−90 min at 37 °C. Cells were washed three times with phosphate-buffered saline (PBS) and placed on the microscope stage. In some experiments, after the lipoprotein uptake, cells were counterstained with Hoechst dye to label nuclei and Lysotracker DND-99 (Lysotracker Red) to label lysosomes (both from Molecular Probes/Invitrogen). For nuclear staining, cells were incubated for 5 min at 37 °C with ~2 mL of 2 μg/mL Hoechst dye in Hank's balanced salt solution (HBSS), prepared from a 1 mM stock, and then washed twice with HBSS. For lysosomal labeling, cells were incubated for 2 min at 25 °C with 2 mL of 100 nM Lysotracker DND-99 in HBSS and washed twice with HBSS. A Zeiss LSM 510 laser scanning two-photon confocal microscope was used to collect images, which were captured using a 63× oil immersion objective with 512 pixel × 512 pixel (8 bit) image size and a scan rate of 26 μs/image. Excitation was provided by an argon ion laser and the emission passed through a bandpass filter and a main dichroic filter to reduce excitation light. The detector gain was between 700 and 900 with an amplifier offset of 0.1 and an amplifier gain of 1. Images were analyzed with the Zeiss software package designed for the LSM 510 microscope.

RESULTS

rSOF Is Active in the Presence of Total Lipoproteins in Vitro. In previous work, we showed that rSOF opacifies HDL but not LDL or VLDL (*3*). In the reaction of rSOF with HDL, three products are formed, CERM, neo HDL, and lipid-free apo A-I (*4*). We now report that these same products are formed in the presence of total lipoproteins. Total lipoproteins (TLP, 0.5 mg/mL protein) from a 1.21 g/mL KBr flotation of human plasma were incubated with rSOF (1 μg/mL) overnight at 37 °C. As shown in Figure 1 A (gray fill), SEC separates the major lipoproteins. Moreover, all of the products of the rSOF reaction against HDL are formed in the presence of LDL and VLDL, i.e., CERM, neo HDL, and LF apo A-I (Figure 1 A, black curve).

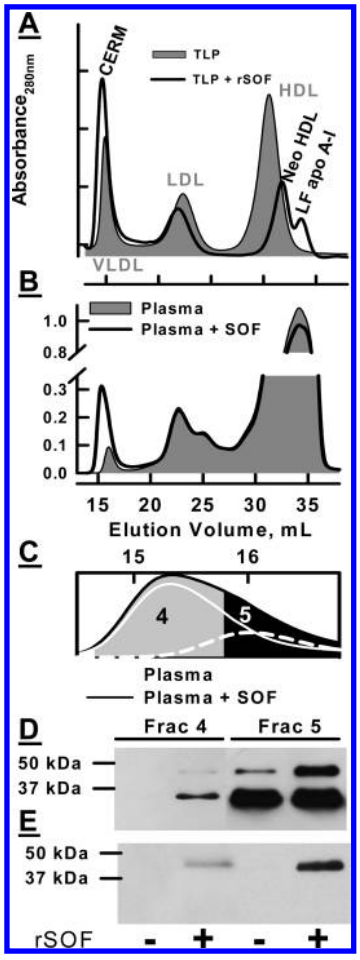

FIGURE 1: (A and B) SEC of TLP and plasma before (gray fill) and after (black curve) incubation with rSOF, respectively. (C) Expansion of the SEC profile to show separation of VLDL and CERM in rSOF-treated plasma: white dashed line for plasma VLDL and white line for CERM, the difference between plasma and rSOF-treated plasma. (D and E) Immunoblots of fractions 4 and 5 probed with anti-apo E and A-II, respectively.

There is a small shift in the LDL peak to a larger particle size. These data show that most of the observations made with isolated HDL for mechanistic studies (*4*, *5*) are preserved in the more physiological setting of TLP.

rSOF Transfers Apo E to CERM in Whole Plasma. Although rSOF treatment of isolated HDL produces CERM that contain apo E (*4*), its effect on the distribution of apo E in whole plasma was not known. To address this, whole plasma was treated with rSOF. As found with TLP, rSOF treatment of whole plasma produces CERM; observation of neo HDL and LF apo A-I in the plasma SEC profile is obscured by other plasma proteins (Figure 1 B). VLDL elutes near the void volume (~16 mL), while the peak corresponding to CERM elutes at 15.3 mL. Figure 1C shows an expansion of this region and the fractions that were collected for immunoblot analysis with anti-apo E and anti-apo A-II (Figure 1D,E) according to the method described in ref *4*. Fraction 4 includes the peak tube for the CERM of the rSOF-treated plasma and only the leading edge of plasma VLDL in the untreated sample. Fraction 5 contains the peak for the VLDL of whole plasma and the trailing edge of the CERM. The reproducibility of the CERM elution volume in 14 consecutive injections was 15.300 ± 0.016 mL. Figure 1D shows that fraction 4 of the untreated plasma contains no detectable apo E whereas the same fraction for the treated plasma contains a strong apo E-positive band as well as a band with a molecular mass of ~45 kDa, which corresponds to the apo E−apo A-II heterodimer (*12*). Although fraction 5 of the untreated plasma contains the same pair of bands, they are much more intense for the rSOF-treated sample. Immunoblotting with anti-apo A-II supports our assignment of the 45 kDa band as the apo E−apo A-II heterodimer (Figure 1E). The untreated samples contain no apo A-II, whereas fractions 4 and 5 from the rSOF-treated samples contain an apo A-II-positive band with a migration distance identical to that of the higher-molecular mass band visualized by anti-apo E. These data clearly show that apo E is present on CERM formed in whole plasma as it is on CERM obtained by rSOF treatment of isolated HDL (*4*). These data also show that the rSOF reaction in TLP and whole plasma emulates that of rSOF against isolated HDL.

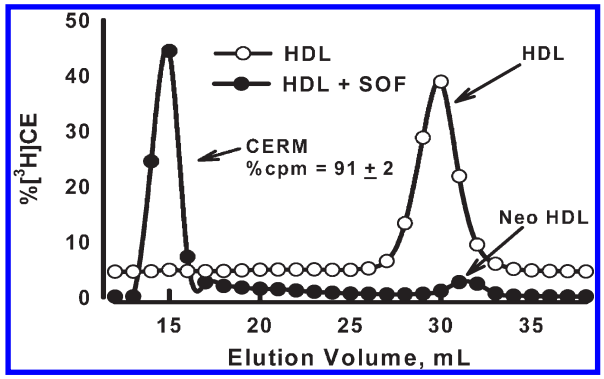

FIGURE 2: SEC profiles of control HDL-[^{3}H]CE (○) and rSOF-treated HDL-[^{3}H]CE (●) reaction mixtures. rSOF treatment transfers 91 ± 2% of the total [^{3}H]CE to the CERM fraction. Aliquots of the control and rSOF reaction mix were used for [^{3}H]CE uptake experiments. The SEC trace for the control HDL-[^{3}H]CE begins at zero percent but in this figure is offset from that of HDL-[^{3}H]CE with SOF to permit better comparison.

rSOF Transfers HDL-CE to CERM. SEC analysis of HDL that was biologically labeled with [^{3}H]CE according to the method described in ref *4* showed that the HDL is a single radiolabeled species (Figure 2). Treatment of the HDL-[^{3}H]CE with rSOF transferred >90% of the [^{3}H]CE to CERM and the balance to neo HDL. We compared the uptake of [^{3}H]CE from HDL-[^{3}H]CE and from the rSOF product, CERM-[^{3}H]CE, in hepatic cell lines and in CHO cells expressing specific lipoprotein receptors.

rSOF Treatment Increases the Rate of Hepatic HDL-CE Uptake. The kinetics of cellular uptake of HDL-[^{3}H]CE and its product CERM-[^{3}H]CE were tested in two human hepatoma lines, Huh7 and HepG2. In all cases, the rates were linear with time up to 3 h (Figure 3A,B). Comparison of the slopes of the rate curves showed that rSOF treatment increased the rate of [^{3}H]CE uptake to levels 4.5- and 2.4-fold greater than the control rate of HDL-[^{3}H]CE uptake for Huh7 and HepG2 cells, respectively (Table 1).

Kinetic constants were also determined from [^{3}H]CE uptake measured as a function of HDL-protein concentration (Figure 3C,D). These data, summarized in Table 2, show that for hepatoma cells the maximum uptake V_{max} was not changed by rSOF treatment. In contrast, the HDL protein concentration at which uptake was 50% of the maximum (B_m) was reduced to 7 and 44% of control by rSOF treatment for Huh7 and HepG2 cells, respectively, indicating that uptake of CE from CERM occurs by higher-affinity interaction than uptake from HDL. On the basis of the kinetic parameters listed in Table 2, rSOF treatment of HDL increased the catalytic efficiency Cat_{eff}

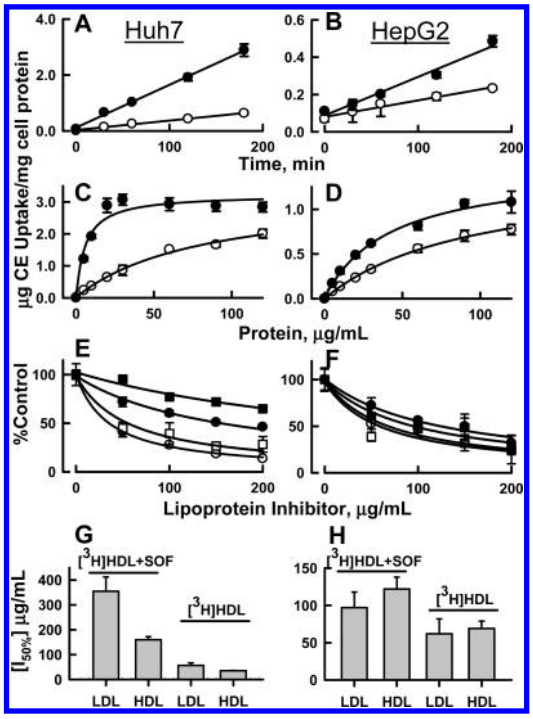

FIGURE 3: Uptake of [^{3}H]CE from HDL with or without rSOF by human hepatoma cells, with data for Huh7 cells in the left panels and data for HepG2 cells in the right panels. (A and B) Time course. (C and D) Dose response. Empty circles (○) denote uptake from HDL-[^{3}H]CE, and filled circles (●) denote uptake from HDL-[^{3}H]-CE pretreated with rSOF to form CERM-[^{3}H]CE. (E and F) Inhibition of uptake by HDL (circles) or LDL (squares) from HDL-[^{3}H]CE (empty symbols) or CERM-[^{3}H]CE (filled symbols) at 20 μg/mL. (G and H) Comparison of the concentration of LDL or HDL lipoprotein inhibitors (indicated on the *x*-axis) needed to reduce the rate of CE uptake by 50%, calculated from panels E and F according to the equation % control = $(a + I_{50\%})/(I_{50\%} + [I])$, where $I_{50\%}$ is the concentration of lipoprotein inhibitor required for 50% inhibition, [I] is the inhibitor concentration (micrograms per milliliter), and a is the initial % uptake of radiolabeled CERM-CE (left two bars) or HDL-CE (right two bars).

Table 1: Rates of Uptake of Cholesterol Ester by Hepatoma and CHO Cell Lines

	CE uptake rate[a]			r^2	
cell type	control HDL-CE	with SOF CERM-CE	with SOF/ control ratio	control	with SOF
Huh7	3.39 ± 0.10	15.32 ± 0.40	4.52	0.99	0.99
HepG2	0.88 ± 0.09	2.07 ± 0.20	2.35	0.97	0.97
CHO-K1	1.14 ± 0.09	3.03 ± 0.48	2.66	0.98	0.93
ldl-A7	0.64 ± 0.22	1.64 ± 0.20	2.56	0.73	0.97
SR-BI	14.20 ± 1.20	14.10 ± 1.80	0.99	0.97	0.96

[a]Nanograms of CE per milligram of cell protein per minute, from Figures 3A,B and 4A,B.

for [^{3}H]CE uptake by 14.4- and 2.4-fold for Huh7 and HepG2 cells, respectively.

To identify the receptors mediating uptake of CERM-CE, we determined the effects of unlabeled HDL and LDL on the uptake of CE from HDL-[^{3}H]CE and CERM-[^{3}H]CE (Figure 3E,F) and the concentration of lipoprotein inhibitor required to reduce the rate of [^{3}H]CE uptake by 50% ($I_{50\%}$). These data, summarized in Figure 3G,H, showed the following. In Huh7 cells, uptake of CE from HDL-[^{3}H]CE was well inhibited by both HDL and LDL, while uptake from CERM-[^{3}H]CE was poorly inhibited, requiring 5 and 6 times more HDL and LDL for 50% inhibition, and LDL was a much weaker inhibitor of uptake from CERM-[^{3}H]CE. Similar studies in HepG2 cells showed smaller differences for HDL and LDL inhibition of uptake from CERM-[^{3}H]CE versus HDL-[^{3}H]CE, with ~2 times more HDL and LDL required for 50% inhibition of CERM-[^{3}H]CE than HDL-[^{3}H]CE uptake. For both cell types, uptake of CE from CERM was less inhibited by HDL or LDL than uptake from HDL.

rSOF Treatment of HDL Increases the Rate of [^{3}H]CE Uptake by Receptor-Specific CHO Cells. The results with Huh7 and HepG2 cells suggested that both LDL and HDL receptors mediate CERM-[^{3}H]CE uptake. Thus, we compared the uptake of CE from control HDL with that of rSOF-treated HDL in CHO cells expressing different levels of lipoprotein receptors. These cells were CHO-K1, which express LDL-R and low levels of SR-BI, CHO-ldl-A7, in which LDL-R has been ablated but which still have a low level of endogenous SR-BI, and CHO-SR-BI, CHO-ldl-A7 transfected to express high levels of SR-BI (*8*). rSOF treatment of HDL-[^{3}H]CE increased rates of [^{3}H]CE uptake by CHO-K1 and CHO-ldlA7 cells to 2.7 and 2.6 times the control rate of HDL-[^{3}H]CE uptake, respectively. With CHO-SR-BI cells, CE uptake rates were 5−20 times faster than with CHO-K1 and CHO-ldlA7 cells, whereas the rates were similar for HDL-[^{3}H]CE and CERM-[^{3}H]CE (Figure 4A−C and Table 1).

Dose−response kinetic constants for HDL-[^{3}H]CE with or without rSOF were determined by measuring CE uptake as a function of lipoprotein concentration (Figure 4D−F). In CHO-K1 cells, V_{max} was higher and B_m lower, leading to a 13.7-fold higher Cat_{eff} for uptake of CERM-[^{3}H]CE than for uptake of HDL-[^{3}H]CE (Table 2). In CHO-ldlA7 cells, rSOF treatment of HDL-[^{3}H]CE decreased V_{max} slightly and reduced B_m 8-fold, which led to a 6.6-fold higher Cat_{eff}. Although V_{max} was higher for control than for rSOF-treated HDL-[^{3}H]CE, the much lower B_m associated with rSOF treatment again led to an increase in Cat_{eff} with rSOF treatment. As expected, the rate of uptake of CE from HDL-[^{3}H]CE by SR-BI-overexpressing cells was much higher than for CHO-K1 and CHO-ldlA7 cells (*8*). In contrast to the other cell lines, rSOF treatment did not have a profound effect on the kinetic parameters for CE uptake in cells over-expressing SR-BI; rSOF treatment increased both V_{max} and B_m, resulting in Cat_{eff} values that were the same with and without rSOF treatment (Table 2).

The effects of lipoprotein inhibitors on the uptake of HDL-[^{3}H]CE with or without SOF by the CHO cells were compared (Figure 4G−L). For all three CHO cell lines, both LDL and HDL reduced the rate of uptake of [^{3}H]CE from HDL-[^{3}H]CE (Figure 4G−I). Lipoprotein inhibition of uptake of [^{3}H]CE from CERM-[^{3}H]CE was weaker than from HDL-[^{3}H]CE. In particular, in CHO-K1 cells, uptake from CERM-[^{3}H]CE was not inhibited by a 20-fold excess of HDL or LDL (Figure 4J). The ratio of uptake of [^{3}H]CE from CERM-[^{3}H]CE to that of HDL-[^{3}H]CE in the presence of the lipoprotein inhibitors illustrates the magnitude of this effect for all three CHO cells (Figure 4J−L; values above bars). Thus, the CERM-[^{3}H]CE rSOF products have a higher binding affinity for cells than do the HDL-[^{3}H]CE products from which they were formed, especially for the CHO-K1 cells.

Inhibition of Uptake of HDL-[^{3}H]CE and CERM-[^{3}H]-CE by RAP and Heparin. The resistance of CERM-CE uptake

Table 2: Kinetic Parameters for Uptake of CE from Control and rSOF-Treated HDL by Hepatoma and CHO Cell Lines[a]

	V_{max} [ng of CE (mg of cell protein)$^{-1}$ min^{-1}]		B_m (μg of HDL protein/mL)		$Cat_{eff} = V_{max}/B_m$			r^2	
cell type	control HDL-CE	with SOF CERM-CE	control HDL-CE	with SOF CERM-CE	control HDL-CE	with SOF CERM-CE	with SOF/ control ratio	control	with SOF
Huh7	3300 ± 30	3300 ± 40	82 ± 18	5.7 ± 2.0	40.2	579	14.4	0.92	0.99
HepG2	1400 ± 80	1500 ± 80	96 ± 12	42 ± 8	14.6	35.7	2.44	0.99	0.99
CHO K1	259 ± 62	526 ± 11	42 ± 21	6.2 ± 0.6	6.2	84.8	13.7	0.92	0.99
ldl-A7	228 ± 21	183 ± 6	37 ± 7	4.5 ± 0.7	6.2	40.6	6.60	0.98	0.98
SR-BI	7047 ± 316	11866 ± 1800	47 ± 5	79 ± 22	150	152	0.99	0.99	0.98

[a]Based on data in Figures 3C,D and 4D−F.

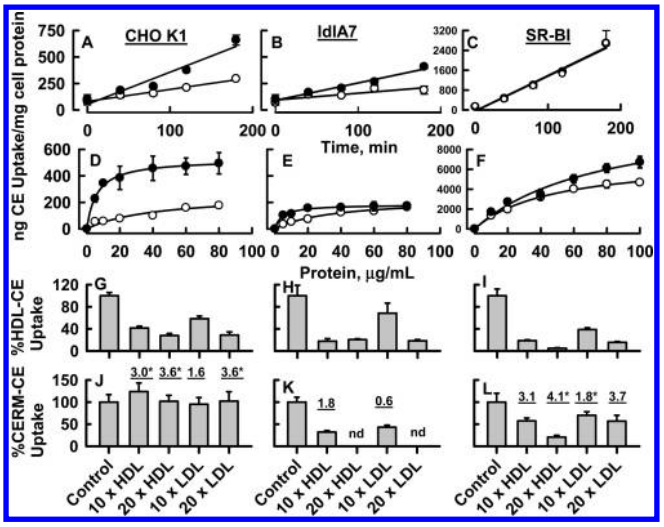

FIGURE 4: Uptake of [^{3}H]CE from HDL with or without rSOF treatment by CHO cell variants: CHO-K1 (left), CHO-ldlA7 (middle), and CHO-SR-BI (right). (A−C) Time course and (D−F) dose response: (○) HDL-[^{3}H]CE and (●) HDL-[^{3}H]CE pretreated with rSOF to form CERM-[^{3}H]CE. (G−L) Inhibition of CHO cell uptake of [^{3}H]CE from HDL with or without rSOF treatment by unlabeled lipoproteins. CHO cell variants were incubated with 10 μg/mL HDL-[^{3}H]CE with or without rSOF pretreatment in the presence or absence of a 10- or 20-fold excess (100 or 200 μg/mL, respectively) of human plasma HDL or LDL. *$p < 0.05$, with rSOF vs control. Numbers above bars are the ratios of % CERM-CE uptake to % HDL-CE uptake in the presence of the various lipoprotein inhibitors.

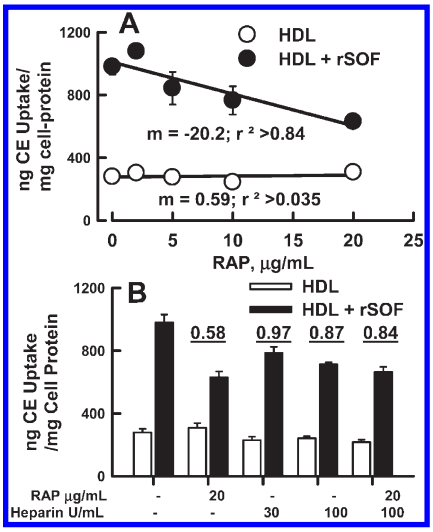

FIGURE 5: RAP and heparin inhibition of uptake of HDL-[^{3}H]CE with or without SOF by Huh7 cells. (A) Dose-dependent effect of RAP on the uptake of [^{3}H]CE from HDL-[^{3}H]CE (○) and HDL-[^{3}H]CE pretreated with rSOF to form CERM-[^{3}H]CE (●). (B) Effects of heparin and RAP on the uptake of [^{3}H]CE from HDL-[^{3}H]CE (empty bars) and CERM-[^{3}H]CE (filled bars). Numbers above bars are the ratio of % CERM-CE uptake to % HDL-CE uptake in the presence of the various inhibitors.

to inhibition by HDL and especially LDL indicated that receptors in addition to LDL-R and SB-BI were involved in uptake of CE from CERM. RAP (*13*−*15*) and heparin (*13*, *16*) inhibit lipid uptake mediated by the LRP receptor and by heparan sulfate proteoglycans (HSPG), respectively. Thus, we tested the effects of RAP and heparin on the uptake of HDL-[^{3}H]CE with or without SOF by Huh7 cells. Addition of RAP to cell media just prior to the start of the uptake assay had no effect on the uptake of [^{3}H]CE from HDL-[^{3}H]CE (Figure 5A) but produced a dose-dependent reduction in the rate of uptake of [^{3}H]CE from rSOF-treated HDL-[^{3}H]CE. The effects of heparin alone or in combination with RAP were also tested (Figure 5B). Heparin alone did not significantly affect uptake from HDL-[^{3}H]CE. However, the combination of 100 units/mL heparin and 20 μg/mL RAP reduced the rate of uptake of [^{3}H]CE from HDL-[^{3}H]CE by 22% ($p = 0.018$). Heparin alone reduced the rate of uptake of CE from CERM-[^{3}H]CE (28% inhibition at 100 units/mL; $p = 0.006$), and the combination of 100 units/mL heparin and 20 μg/mL RAP produced a 32% inhibition ($p = 0.001$), similar to that with RAP alone (36% inhibition; $p = 0.004$). To compare RAP and heparin inhibition of uptake from HDL-[^{3}H]CE and CERM-[^{3}H]CE, we calculated the ratios of the percent activity in the presence of the inhibitors versus the control (Figure 5B, values above bars). These values show the greatest difference for RAP (0.58) and RAP with a high dose of heparin (0.84). Thus, both LRP and cell surface HSPG promote uptake of CE from CERM.

Statins upregulate LDL-R expression in hepatocytes (*17*, *18*). We tested the effect of Simvastatin (0.01−1.0 μM) pretreatment of Huh7 cells on CE uptake and found only a slight effect, with the rate of CE uptake increased by 10−20% for HDL-[^{3}H]CE and CERM-[^{3}H]CE (data not shown).

Hepatic Uptake of Bodipy-CE. The subcellular localization of a fluorescent CE analogue, Bodipy-CE, after uptake from CERM, was compared to subcellular localization after uptake from HDL or LDL by confocal fluorescence microscopy. Huh7 cells were incubated with Bodipy-CE-labeled CERM, HDL, or LDL (Figure 6, green fluorescence). Cell lysosomes were visualized with LysoTracker Red (red fluorescence) and nuclei with Hoechst stain (blue fluorescence). Numerous perinuclear Bodipy-CE-positive vesicles were seen after a 90 min incubation of cells with Bodipy-CE-labeled HDL (Figure 6, top green panel). Lysosomes were primarily perinuclear (Figure 6, top red panel), and

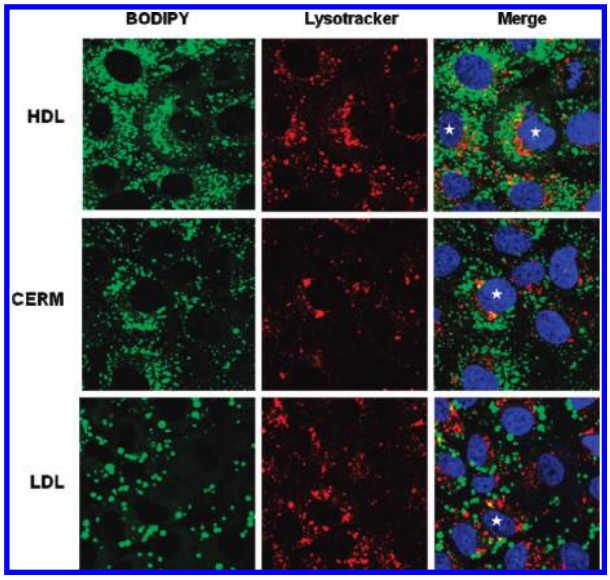

FIGURE 6: Confocal fluorescence microscopy of the accumulation of Bodipy-CE in Huh7 cells incubated with Bodipy-CE-labeled HDL, CERM, and LDL (left panels, green). Cells were also stained with LysoTracker Red to label lysosomes (middle panels, red) and with Hoeschst (blue) to label nuclei. The merged images (right panels) show distinct green labeling of intracellular vesicles, as well as some colocalization with lysomes (yellow) as seen especially in the cells noted with the stars.

merged images showed that a portion of the Bodipy-CE label colocalized with lysosomes (Figure 6, top left panel, yellow vesicles in the merged image). Thus, by 90 min, some of the Bodipy-CE taken up by cells from HDL-Bodipy-CE had reached the lysosomes. rSOF treatment of HDL-Bodipy-CE formed CERM-Bodipy-CE. Incubation of cells with CERM-Bodipy-CE gave a similar pattern for Bodipy-CE fluorescence, a portion of which also colocalized with LysoTracker Red (Figure 6, middle panels). Uptake of LDL-Bodipy-CE showed numerous bright Bodipy-CE-positive spherical bodies and some overlap with lysosomes (Figure 6, bottom panels). Many of these spherical bodies colocalize with phase dense lipid droplets in Huh7 cells (Figure 1 of the Supporting Information). Thus, confocal microscopic imaging indicates vesicular CERM-CE uptake by hepatocytes and at least a portion of the CE traffics to lysosomes, which occurs for uptake of Bodipy-CE from HDL and LDL.

DISCUSSION

SOF is produced by *S. pyogenes*, a human pathogen that causes a variety of diseases ranging from infections of the pharynx and skin to highly invasive infections that have high degrees of morbidity and mortality (*1*). This raises an interesting question of whether there is a reduction in the plasma level of cholesterol during an infection by *S. pyogenes*. However, we do not know of any study in which cholesterol levels have been measured in patients in conjunction with *S. pyogenes* infection. This is not meant to suggest that an infection by *S. pyogenes* would be beneficial as the consequences of infection can be too severe. However, our recent studies support the concept that purified rSOF may have a beneficial effect of lowering the plasma level of cholesterol in vivo (*19*) without the ramifications of an infection by a highly virulent organism. Toward establishing the efficacy of an rSOF-based therapy to reduce the plasma level of cholesterol, we report here that rSOF enhances the uptake of HDL cholesterol by hepatocytes and elsewhere (*20*) that rSOF treatment of HDL increases macrophage cholesterol efflux and reduces the macrophage inflammatory response.

The data of Figure 1 show that rSOF acts on HDL in a mixture of total plasma lipoproteins and in whole plasma, as previously reported for HDL alone (*4*), to form an apo E-containing CERM that contains small amounts of an apo E heterodimer with apo A-II. These data suggest that conclusions based on the effects of rSOF treatment of HDL would be applicable to the more complex setting of whole plasma, perhaps even in vivo. Treatment of [^{3}H]CE-labeled HDL with rSOF transfers nearly all of the [^{3}H]CE to CERM (Figure 2), so that uptake of [^{3}H]CE from labeled HDL after rSOF incubation is essentially CERM-[^{3}H]CE uptake as discussed below.

Our data demonstrate that hepatocyte uptake of CE from CERM occurs faster than from the parent HDL and is inhibited by both HDL and LDL, suggesting that both SR-BI and LDL-R mediate CERM-CE as well as HDL-CE uptake. However, inhibition of CERM-CE uptake is less profound than that of HDL-CE uptake, indicating that CERM has a higher-affinity interaction with these cell surface receptors. To demonstrate that LDL-R and SR-BI receptors are involved in CERM-CE uptake, we utilized CHO cells expressing various lipoprotein receptors. Ablation of LDL-R in CHO-ldlA7 cells compared to wild-type CHO-K1 decreases the rate of uptake of CE from both HDL and CERM, with a larger 66% reduction in V_{max} of CERM-CE than HDL-CE uptake (Figure 4, panel A vs panel B and panel D vs panel E, and Tables 1 and 2). Loss of the LDL-R does not affect the Cat_{eff} for HDL-CE but decreases that for CERM-CE uptake by half. Thus, LDL-R is a major receptor for CERM in these cells, but not the only one. A similar comparison of the uptake of CE by CHO-ldlA7 versus CHO-SR-BI cells shows that the SR-BI receptor mediates the uptake of CE from both HDL and CERM with essentially equal efficiency (Figure 4, panel B vs panel C and panel E vs panel F, and Tables 1 and 2).

These data are complemented by studies of CE uptake inhibited with excess unlabeled HDL and LDL. As with hepatocyte uptake, the uptake of CERM-CE by CHO cells was more resistant to inhibition by HDL and LDL than the uptake of HDL-CE. Even for the CHO-SR-BI cells, which showed similar rates of uptake of CE from CERM and HDL (Figure 4C), the uptake inhibition data (Figure 4I,L) show that CERM has a higher affinity for SR-BI than does HDL. The most profound differences in HDL and LDL inhibition of CE uptake were seen in the CHO-K1 cells (Figure 4G,J). While HDL-CE uptake was robustly inhibited by both HDL and LDL, neither lipoprotein inhibited CERM-CE uptake. This implicates other receptors in CERM-CE uptake.

In addition to LDL-R, apo E is a ligand for LRP-1 and cell surface heparan sulfate proteoglycans (*7*). Given the high apo E content of CERM, we evaluated the role of these receptors in CERM-CE uptake using their respective inhibitors, RAP and heparin (Figure 5), and found that both receptors promote hepatocyte CE uptake from CERM but not from HDL. Thus, hepatocyte uptake of CE from CERM occurs by interaction with the apo E receptors LDL-R and LRP-1 and is facilitated by binding to cell surface heparan sulfate proteoglycans, as well as via the HDL receptor SR-BI. As shown in Figure 6, CERM-CE is taken up and internalized by hepatocytes with a subcellular distribution similar to that for the uptake of CE from HDL and LDL. Importantly, CERM-CE traffics to lysosomes where it can be catabolized.

In summary, the uptake data and inhibition studies indicate that multiple apo E-dependent receptors are involved in the

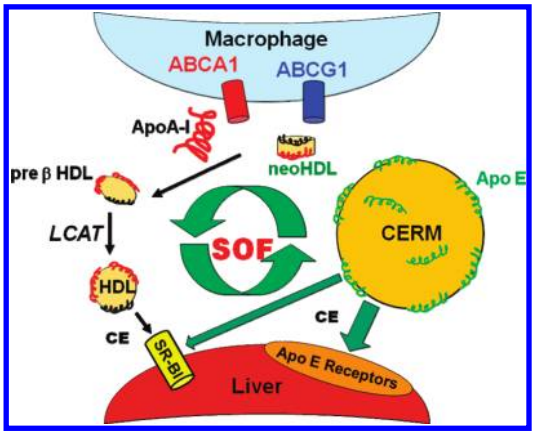

FIGURE 7: Model for promotion of RCT by the action of rSOF. rSOF activity transfers CE from HDL to form CERM, a cholesterol ester-rich micoemulsion with surface apo E. CERM CE is taken up by liver cells via various apo E receptors, including LDL-R, LRP, and HSPG, as well as the HDL receptor SR-BI. rSOF activity also produces lipid-free apo A-I and neo HDL, both of which can remove additional cholesterol from macrophages to form pre-β HDL. After LCAT esterification and remodeling, the mature HDL can enter another cycle of the SOF reaction.

uptake of CERM-CE and that hepatocyte CERM-CE uptake is more robust than HDL-CE uptake. This effect is likely mediated by apo E and not the apo E heterodimer with apo A-II because of the lower abundance of the latter in the CERM and the lower affinity of the heterodimer for the LDL-R (*21*). Comparative affinities for LRP-1 and proteoglycans are not known.

Figure 7 is a model of how the rSOF reaction could improve RCT. rSOF activity converts HDL to neo HDL, lipid-free apo A-I, and CERM (*2*, *4*). With its low cholesterol content, the neo HDL could remove additional cholesterol from macrophages, with a higher activity than the parent HDL from which neo HDL was formed (*20*), after which lecithin:cholesterol acyltransferase activity (LCAT) esterifies and remodels the particle to its more mature form (*22*), which could enter another cycle of the rSOF reaction. Lipid-free apo A-I could interact with ABCA1 producing additional early forms of HDL that also mature to rSOF targets, although excess lipid-free apo A-I is removed by the kidney cubilin receptor (*23*). However, the key step for rSOF-driven RCT is the formation of CERM with apo E and the clearance of cholesterol via multiple hepatic apo E-dependent receptors in addition to the SR-BI receptor, a process that gives directionality to the overall rSOF reaction as an agent of enhanced RCT.

Mechanisms that enhance RCT are thought to be anti-atherogenic (*24*). The conventional view is that high plasma levels of HDL-C correlate with efficient RCT. On the other hand, other evidence suggests that future therapies might better focus on enhancing RCT irrespective of the effects on HDL-C. Several observations support this view. First, a common CETP gene mutation that lowers plasma CETP activity is associated with a high level of HDL-C and possibly with increased coronary heart disease in men with hypertriglyceridemia (*25*, *26*). Second, in murine models of atherosclerosis, hepatic overexpression of SR-BI, the HDL receptor that "pulls" cholesterol out of extrahepatic spaces, decreases plasma levels of HDL-C (*27*−*29*) and increases the rate of HDL-CE clearance (*28*−*30*), biliary cholesterol, and its transport into bile (*27*, *29*, *31*) and reduces the incidence of atherosclerosis (*32*−*34*). Conversely, ablated or attenuated hepatic SR-BI expression elevates the plasma level of HDL-C, reduces selective HDL-CE clearance, and is atherogenic (*35*−*37*). Thus, enhancement of the final RCT step, hepatic removal of lipoprotein cholesterol and its conversion to bile salts that are excreted, is likely to have therapeutic value.

Like the genetic changes that lower the level of HDL-C but decrease the incidence of atherogenesis, our studies show that rSOF could potentially increase the rate of transfer of cholesterol to the liver via apo E-dependent receptors even while likely reducing plasma HDL-C levels. Indeed, recent studies have shown that injection of rSOF in mice rapidly and profoundly reduces plasma cholesterol levels via hepatic uptake (*19*), so we are interested in identifying the mechanisms and receptors that mediate this process in vivo. Studies of the transfer of cholesterol between cells and rHDL containing various amounts of cholesterol have shown that above ~15 mol %, rHDL is a cholesterol donor rather than acceptor (*38*). Given that cholesterol constitutes ~14 mol % of the surface lipid of mature HDL (*39*), there is likely little reserve for accepting more cholesterol from cells and thus for increasing the amount of RCT mediated directly by mature plasma HDL. Rather, transfer of the HDL-CE by action of rSOF to CERM, a new lipid particle that contains apo E, might enhance hepatocyte CE clearance and thus RCT. This activity is similar to that of CETP, which exchanges HDL-CE for TG of triglyceride-rich lipoproteins, with subsequent clearance by liver receptors (*40*−*42*). Our future studies of subcellular trafficking and metabolism of CERM-CE will determine whether the higher rate of uptake of cholesterol from CERM is followed by higher rates of conversion of cholesterol to bile salts and their excretion. If successful, these studies would provide a compelling rationale for pursuing other tests of the rSOF reaction as a modality for promoting RCT and reversing atherosclerosis in mouse models of dyslipidemic atherosclerosis.

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SUPPORTING INFORMATION AVAILABLE

LDL-Bodipy-CE uptake labels phase dense lipid droplets of Huh7 cells (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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