Effect of sterol carrier protein-2 gene ablation on HDL-mediated cholesterol efflux from cultured primary mouse hepatocytes

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Sterol carrier protein-2 (SCP-2) facilitates HDL-mediated cholesterol transport across the plasma membrane and intrahepatocyte transfer of HDL-derived free cholesterol. In contrast, sterol carrier protein-3 (SCP-3) overexpression in mice increases, while SCP-2 overexpression decreases intermembrane cholesterol transfer more slowly than SCP-3. Ablation of SCP-2 and L-FABP decreased HDL-mediated NBD-cholesterol efflux. These results indicate that SCP-2 expression plays a significant role in HDL-mediated cholesterol efflux by regulating the size of rapid vs. slow cholesterol efflux pools and/or eliciting concomitant upregulation of L-FABP in cultured primary hepatocytes.

confocal imaging; bile; scavenger receptor B1

EXCESS CHOLESTEROL IS PRIMARILY REMOVED from the body via the liver into bile, and >95% of excreted cholesterol originates from lipoproteins, primarily HDL (9). HDL removes unesterified (free) cholesterol from tissues, free (as well as esterified) cholesterol to the liver, and binds to scavenger receptor class B type 1 (SRB1) at hepatocyte basolateral plasma membranes (9). Hepatic clearance of HDL free cholesterol from blood is rapid [half time (1/2) = 3 min (15, 31), consistent with very rapid (1/2 < 1 min) transmembrane movement of free cholesterol (16, 34). In contrast, HDL cholesterol ester clearance is much slower (1/2 = 1.1 h), consistent with endocytic uptake (15). Although HDL free cholesterol clearance is directly related to SRB1, but not ATP-binding cassette (ABC) A-1 (ABCA-1), expression (15, 31), free cholesterol transfer between HDL and cells is bidirectional (42). While ABCG-5 and ABCG-8 determine directionality of HDL-derived cholesterol transport for efflux into bile, it is unclear how HDL-derived free cholesterol rapidly (1/2 = 1.6 min) traffics by nonvesicular transport within the hepatocyte for efflux into serum or bile (9).

Hepatocytes express multiple membrane-associated proteins involved in cholesterol transport, but must have not been shown to transfer HDL-derived free cholesterol between the plasma membrane and intracellular sites. In contrast, sterol carrier protein (SCP)-mediated intracellular cholesterol transport is sufficiently fast to account for the known rapid intrahepatocyte transfer of HDL-derived free cholesterol. SCP-2 binds cholesterol with high affinity (dissociation constant 4 nM) (19, 22, 32), binds plasma membrane caveolin-1, and enhances rapid (detectable in <1 min) directional cholesterol transfer from the plasma membrane to intracellular sites (13, 28). SCP-2 overexpression in mice increases, while SCP-2 antisense cDNA treatment in rats decreases, excretion of cholesterol into bile (1, 30, 41). In addition, liver cytosol also contains high levels of fatty acid-binding protein (L-FABP), which binds cholesterol with lower affinity than SCP-2, induces intracellular membrane cholesterol transfer more slowly than SCP-2, and facilitates cholesterol transfer from the plasma membrane to intracellular sites much more slowly than SCP-2 (19, 32). Although L-FABP gene ablation does not alter biliary cholesterol secretion or cholesterol saturation index in control chow-fed male mice, in the context of SCP-2 gene ablation the concomitant upregulation of L-FABP increases excretion of cholesterol into bile (12, 20, 38). Surprisingly, in response to a cholesterol-rich or lithogenic diet, the male L-FABP gene-ablated mice also exhibit increased biliary cholesterol and cholesterol saturation index, suggesting other contributing factors (20, 38). While these studies suggest potential role(s) for SCP-2 in facilitating HDL-derived cholesterol intrahepatocyte transport into bile for excretion, the effect of SCP-2 on HDL-mediated cholesterol efflux from hepatocytes remains to be shown. This issue was addressed by laser scanning confocal microscopy of
the fluorescent sterol 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) in cultured primary hepatocytes from wild-type (WT) and SCP-2 gene-ablated (SCP-2/SCPx-null) mice. NBD-cholesterol is readily taken up, poorly esterified, and monitors free cholesterol efflux independent of esterification/hydrolysis (6, 11, 24).

SCP-2 ablation enhanced HDL-mediated cholesterol efflux from cultured primary hepatocytes, consistent with a potential role for SCP-2 in regulating cholesterol rapid efflux, not only into bile, but also back into serum.

**MATERIALS AND METHODS**

**Materials.** Lipid standards and silica gel G thin-layer chromatography plates were obtained from Nu-Chek Prep (Elysian, MN) and Anatech (Newark, DE), respectively; rabbit polyclonal antibodies to ABCG-1, ABCG-5, ABCG-8, acyl coenzyme A:cholesterol acyltransferase (ACAT)-1, P-glycoprotein [Pgp]; recognizes all 3 mouse Pgp isoforms, including multi-drug resistance protein (MDR) 1 (ABCB-1), MDR2, and MDR3 (ABCB-4); fatty acid transport protein-4, L-FABP, goat polyclonal antibodies to lysosomal-associated membrane protein 1, anti-protein disulfide isomerase, anti-formiminotransferase cycloleaminase, and peroxisomal membrane protein 70 from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-heat shock protein 70 from Thermo Fisher Scientific (Rockford, IL); rabbit anti-ABCA1 and anti-SRB1 from Novus Biologicals (Littleton, CO); rabbit anti-ACAT-2 from Cayman Chemical (Ann Arbor, MI); and mouse anti-flootillin-1 from BD Transduction Laboratories, BD Biosciences (Palo Alto, CA). Rabbit anti-paricin-glutamic-oxaloacetic transaminase was prepared as previously described (5). NBD-cholesterol was obtained from Molecular Probes (Eugene, OR); alkaline phosphatase-conjugated rabbit anti-goat IgG and goat anti-rabbit IgG from Sigma-Aldrich (St. Louis, MO); and purified human HDL, LDL, VLDL, and apolipoprotein A1 (apoA1) from CalBiochem, EMD Biosciences (San Diego, CA). HDL was subfractionated to obtain HDL2 and HDL3 (14, 17, 18). [1,2,6,7-3H(N)]cholesterol and methyl-β-cyclodextrin (MβCD) were obtained from NEN/PerkinElmer (Waltham, MA) and Sigma (St. Louis, MO), respectively. BLT-1 (an SRB1 inhibitor) and protocol were obtained from Sigma and ChemBridge (San Diego, CA). All reagents and solvents were highest grade available and cell culture material.**

**Animals.** All animal protocols were approved by the Institutional Animal Care and Use Committee (no. A-4562-01) at Texas A & M University. All mice were on a C57BL/6Ncr background. WT male C57BL/6Ncr mice (8–10 wk old, 20–25 g body wt) were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). SCP-2/SCPx-null mice were generated as described elsewhere (4). SCP-2/SCPx-null mice were crossed with C57BL/6Ncr mice (8–10 wk old, 20–25 g body wt) from the National Cancer Institute. Western blot analysis of hepatocyte proteins. Hepatocyte lysates were resolved by SDS-PAGE and analyzed by Western blotting and densitometry via Scion Image (Scion, Frederick, MD) (2).

**Western blot analysis of hepatocyte proteins.** Hepatocyte lysates resolved by SDS-PAGE were analyzed by Western blotting and densitometry via Scion Image (Scion, Frederick, MD) (2). Western blot analysis of hepatocyte proteins. Hepatocyte lysates were resolved by SDS-PAGE and analyzed by Western blotting and densitometry via Scion Image (Scion, Frederick, MD) (2). The separation of polypeptides is performed by gel electrophoresis, where proteins are separated according to their molecular weight. The proteins are then transferred to a membrane (usually nitrocellulose or polyvinylidene fluoride) using an electroblotting apparatus. This step is known as western blot. The membrane is then incubated with a primary antibody specific for the protein of interest, followed by an secondary antibody conjugated to an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). The enzyme activity is detected using chemiluminescence (CL) or colorimetric methods. The densitometry is performed using software such as Scion Image or ImageJ, where the integrated optical density (IOD) is calculated and normalized to the total protein content in the gel. The results are then analyzed statistically by comparing the expression levels of different proteins or pathways.

**Metabolism of NBD-cholesterol in cultured primary hepatocytes.** Hepatocytes were cultured as described above (4), washed twice with PBS (pH 7), incubated in complete (5% FBS) medium with 0.1 μM NBD-cholesterol for 15 min, washed twice with PBS, and further incubated in PBS for 0–8 h (4 replicates per time point). PBS was removed, and lipids were extracted and analyzed by thin-layer chromatography. Images of NBD-cholesterol in each plate were acquired (IS-500, Alpha Innotech, San Leandro, CA) with UV excitation and analyzed by densitometry of negative images with Scion Image to obtain relative NBD fluorescence in the hepatocytes at each time point.

**Metabolism and HDL-mediated efflux of radiolabeled cholesterol in primary mouse hepatocytes.** [1H]cholesterol complexes with MβCD ([1H]cholesterol-MβCD) were prepared as described elsewhere (40) under conditions similar to those described above for NBD-cholesterol labeling/metabolism, except cells were labeled with [1H]cholesterol-MβCD. For efflux studies, the cells were incubated with HDL (40 μg/ml PBS) for 0 or 60 min at 37°C, lipid was extracted, and cells were analyzed by thin-layer chromatography (7). Cholesterol and cholesterol ester spots were identified by fluorescence relative to known standards visualized under iodine, scraped, and quantified for 3H disintegrations per minute in a liquid scintillation analyzer (Packard Tri-carb 1600TR, PerkinElmer) with 1 ml/sample of ScintiSafe Gel (Thermo Fisher Scientific, Pittsburgh, PA).

**Specificity of lipoprotein-mediated NBD-cholesterol uptake and efflux from cultured primary hepatocytes.** Hepatocytes (1.5 × 10^5) cells were cultured as described above and washed twice with serum-free complete medium. For uptake studies, the cells were then incubated for 0–20 min in PBS containing 0.1 μM NBD-cholesterol without or with added lipoprotein (HDL, LDL, VLDL, or albumin) at 37°C with 5% CO₂, and NBD-cholesterol uptake was monitored by confocal microscopy (see below). Alternately, cells were washed twice in PBS, incubated without or with 100 μM probucol or BLT-1 at 37°C for 30 min, and washed in PBS, and uptake was monitored from culture medium containing 5% FBS and 0.1 μM NBD-cholesterol (see below). For efflux studies, the hepatocytes were incubated for 15 min with complete (5% FBS) medium supplemented with 0.1–1.0 μM NBD-cholesterol in PBS at 37°C with 5% CO₂, washed twice with warm PBS, incubated for an additional 15 min at 37°C with 5% CO₂ in 800 μl of PBS, and transferred to a heated (37°C) tissue culture stage for imaging. Confocal fluorescence imaging was performed without or with lipoprotein (HDL, LDL2, LDL3, LDL4, VLDL, or apoA1) added in 200 μl of PBS (20–80 μg/ml final concentration) on an imaging system (MRC-1024MP LSMC, Bio-Rad, Hercules, CA), as described elsewhere (6). Samples were exposed to the light source for minimal time periods at 1-min intervals after addition of lipoproteins to minimize photobleaching effects. Intensity measurements were used directly for uptake or converted to percentage of total NBD fluorescence remaining in the cell (compared with time 0) for efflux, and average values from multiple replicates were calculated and graphed (Excel, Sigmaplot). Efflux to HDL was best fit to a two-exponential decay curve, \( y = Ae^{-kt} + Ce^{-dt} \), where A and C are percentage of total cellular NBD-cholesterol fluorescence in the two sterol efflux pools, b and d are apparent rate constants, and t is time in minutes (6). Half times for each pool were determined as \( t_{1/2} = \ln(2)/k \), where k is the apparent rate constant for that pool. Initial rates (IR) were obtained by setting t = 0 (IR = Ab + Cd). Half times, k values, and initial rates were apparent values because of the dependence on HDL acceptor concentrations. Efflux data points with HDL subtypes were best fit by a single-exponential, two-parameter exponential curve, \( y = Ae^{-kt} \), where the variables are as described above, with the initial rates calculated as IR = Ab.

**Isolation of basolateral and canalicular plasma membranes from polarized cultured primary hepatocytes.** Basolateral and canalicular plasma membranes were isolated from polarized cultured primary hepatocytes as described elsewhere (10).

**Immunolabelling of fixed cultured primary hepatocytes.** Double-immunofluorescence confocal and electron microscopy were performed to colocalize and/or determine intermolecular distances be-
between SCP-2/SR-B1, SCP-2/ABCG-5, and SCP-2/ABCG-8 as described elsewhere (29).

Cross-linking coimmunoprecipitation. SRB1 was immunoprecipitated from cell lysates after dithiobis (DTSP) cross-linking of cultured primary hepatocytes (8). Immunoprecipitates were isolated, resolved by SDS-PAGE, and probed by Western blotting to detect coimmunoprecipitated SRB1, PDZ domain-containing protein-1 (PDZK1, also called CLAMP/Dipor-1/CAP70/NaPi-Cap1), SCP-2, ABCG-1, ABCG-5, ABCG-8, and Pgp, as described elsewhere (2).

Statistical analysis. Values are means ± SE based on the indicated number of replicates (n). To determine statistical variance between mean values, GraphPad Prism (GraphPad Software, San Diego, CA) was used to perform unpaired t-tests on the selected values. Differences at P < 0.05 were considered statistically significant.

RESULTS

Uptake and metabolism of NBD-cholesterol in cultured primary hepatocytes from WT mice. Confocal imaging showed that NBD-cholesterol uptake from buffer was very slow but was stimulated by serum lipoproteins in the following order: HDL > LDL > VLDL > control (buffer only, which did not differ from buffer with BSA; Fig. 1). The greater effectiveness of HDL was apparent, whether compared on the basis of equivalent of lipoprotein protein (Fig. 1A) or lipoprotein cholesterol (Fig. 1B) added. BLT-1 (an SRB1 inhibitor; Fig. 1D), but not probucol (an ABCA-1 inhibitor; Fig. 1C), abolished NBD-cholesterol uptake, consistent with NBD-cholesterol uptake mediated primarily by the HDL-SRB1 pathway.

Since >96% of NBD-cholesterol taken up was recovered as unesterified NBD-cholesterol, there was little/no esterification or oxidation of the NBD moiety up to 8 h of postincubation (Fig. 1E). In contrast, 15 ± 1 and 93 ± 10% of $[^3]$Hcholesterol taken up was esterified by 15 and 60 min, respectively (not shown). Thus, confocal imaging of NBD-cholesterol in hepatocytes reported on unesterified NBD-cholesterol, thereby avoiding potential complications of intracellular esterification/hydrolysis in measuring HDL-mediated cholesterol efflux.

Optimization of NBD-cholesterol loading and HDL-mediated efflux conditions in cultured primary hepatocytes. NBD-cholesterol was distributed throughout the hepatocyte interior, except for nuclei (Fig. 2A, 0 min). NBD-cholesterol efflux to HDL decreased the intensity of NBD-cholesterol in the hepatocytes without altering this distribution (Fig. 1A, 30 and 60 min vs. 0 min). While increasing medium NBD-cholesterol 10-fold did not change relative intracellular distribution (not shown), the subsequent efflux to HDL was enhanced, reflecting increased initial rate, decreased half time (14.4 ± 0.8 vs. 11.5 ± 0.3 min), and increased pool size (58 ± 2 vs. 79 ± 1%), until it approached a plateau (Fig. 2B). To ensure that quantification of HDL-mediated NBD-cholesterol efflux was not complicated by saturation, efflux was measured after loading with a lower concentration of NBD-cholesterol (i.e., 0.1 μM). Similarly, a low level of HDL (20–40 μg/ml) was used to avoid saturation of NBD-cholesterol efflux (Fig. 2C).

Specificity of NBD-cholesterol efflux from cultured primary hepatocytes: VLDL, LDL, and HDL. Incubation of hepatocytes with buffer alone did not elicit net loss of NBD-cholesterol (Fig. 2D). Lipoproteins increased NBD-cholesterol efflux in the following order: HDL > LDL >> VLDL (Fig. 2D), consistent with known specificity for HDL in eliciting cholesterol efflux.

Effect of SCP-2/SCP-x gene ablation on extent of HDL-mediated sterol efflux from cultured primary hepatocytes. SCP-2/SCP-x gene ablation increased the efflux of NBD-cholesterol to HDL (Fig. 3, A and B). Similarly, SCP-2/SCP-x gene ablation increased $[^3]$Hcholesterol efflux to HDL, as shown by 35 ± 6% less ($P < 0.05$) $[^3]$Hcholesterol remaining in hepatocytes from SCP-2/SCP-x-null than WT hepatocytes. The enhanced $[^3]$Hcholesterol efflux was not due to differences in $[^3]$Hcholesterol esterification after 15 min of loading (17 ± 1 vs. 15 ± 1%) or 60 min of incubation (91 ± 6 vs. 93 ± 10%) in SCP-2/SCP-x-null vs. WT hepatocytes. HDL-mediated efflux of NBD-cholesterol from single nonpolarized hepatocytes did not differ significantly from that of polarized hepatocyte couplets (Fig. 3, F and G). Similarly, HDL-mediated NBD-cholesterol efflux from the cell body (excluding the canaliculus region) did not differ from that from the canaliculus region, where NBD-cholesterol was most intensely distributed (Fig. 3G). SCP-2/SCP-x gene ablation increased NBD-cholesterol efflux to HDL, whether hepatocytes were nonpolarized (Fig. 3C), polarized (Fig. 3, D and E), or measured from the cell body vs. canalicus region (Fig. 3G).
Kinetic analysis of HDL-mediated NBD-cholesterol efflux from WT and SCP-2/SCP-x-null cultured primary hepatocytes. NBD-cholesterol efflux from WT and SCP-2/SCP-x-null hepatocytes best fit two exponentials, indicating two transport pools of NBD-cholesterol. WT hepatocytes exhibited 1) a small pool (6.5 ± 0.6% of total), with a more rapid efflux and shorter half time (2.4 ± 0.6 min), and 2) a larger pool (93.3 ± 0.6% of total), with slower efflux and longer half time (26.5 ± 0.2 min; Table 1).

Fig. 2. Real-time laser scanning confocal microscopy of NBD-cholesterol efflux and lipoprotein specificity from cultured primary hepatocytes. A: representative images of cells with increasing time of NBD-cholesterol efflux to HDL. B: NBD-cholesterol efflux to HDL was measured from hepatocytes prelabeled for 15 min with 0.1–1.0 μM NBD-cholesterol in 5% FBS culture medium. Percent NBD-cholesterol remaining was determined for multiple hepatocytes and expressed as mean ± SE (n = 3–10). C: representative time-course analysis of NBD-cholesterol efflux from hepatocytes incubated with increasing HDL. Percent NBD-cholesterol remaining was determined for multiple hepatocytes and expressed as mean ± SE (n = 3–7). D: representative time-course analysis of NBD-cholesterol efflux from cultured primary hepatocytes incubated with PBS without (control) or with lipoproteins (40 μg/ml). Percent NBD-cholesterol remaining over time was determined for multiple hepatocytes and expressed as mean ± SE (n = 7–12).

Fig. 3. SCP-2/SCP-x-induced alterations in HDL-mediated cholesterol efflux from cultured primary hepatocytes. A: representative time-course analysis of average percent initial NBD fluorescence remaining in wild-type (WT) and SCP-2/SCP-x gene-ablated [knockout (KO)] hepatocytes after addition of PBS without (control) or with HDL (40 μg/ml). Values are means (n = 3–6), expressed as percent NBD-cholesterol fluorescence remaining at each time point. B: percent NBD-cholesterol fluorescence remaining after incubation of WT and SCP-2/SCP-x-KO hepatocytes with increasing HDL in PBS. Values are means ± SE (n = 4–10). *P < 0.05 vs. WT. C–E: representative images of NBD-cholesterol in individual WT cultured primary hepatocytes (C), clustered couples of WT cultured primary hepatocytes (D), and clustered couples of SCP-2/SCP-x KO cultured primary hepatocytes (E). F: percent NBD-cholesterol remaining after efflux to HDL from individual hepatocytes (single cells) or clustered couples as in A. G: as in F, except efflux of NBD-cholesterol to HDL was determined from hepatocytes (except bile canalicular region) and bile (canalicular region only) separately, and differences are plotted as a function of time.
SCP-2/SCP-x-null hepatocytes exhibited increased relative half time and size (9.6-fold) of the rapidly effluxing sterol pool, unaltered half time but decreased size of the slower effluxing pool, and increased overall initial rate (Table 1). Thus, SCP-2/SCP-x gene ablation enhanced HDL-mediated NBD-cholesterol efflux, primarily by increasing the size of the rapidly effluxing pool and the initial rate of efflux.

**HDL subtype specificity of HDL-mediated NBD-cholesterol efflux from cultured primary hepatocytes from WT and SCP-2/SCP-x-null mice.** HDL3-mediated NBD-cholesterol efflux was faster and enhanced greater maximum values than HDL2-mediated efflux (Fig. 4A), consistent with the specificity of these HDL subclasses in cholesterol efflux. The half time of NBD-cholesterol efflux mediated by HDL3 was 51% shorter, i.e., faster (P < 0.05), than that mediated by HDL2 (Table 2). Additionally, the initial rate of NBD-cholesterol efflux mediated by HDL3 was twofold faster (P < 0.05) than that mediated by HDL2 (Table 2). However, the faster efflux kinetics observed with HDL3 acceptors were likely not due to greater transferable cellular NBD-cholesterol pool size, since the efflux pool size of HDL3-mediated NBD-cholesterol was slightly smaller than that of HDL2-mediated NBD-cholesterol (Table 2).

SCP-2/SCP-x-null hepatocytes exhibited increased initial rate and decreased half time of NBD-cholesterol efflux to HDL2 (Fig. 4B, Table 2). In contrast, HDL3-mediated NBD-cholesterol efflux was inhibited in SCP-2/SCP-x-null hepatocytes to decrease initial rate and increase efflux half time without significantly altering the total amount of transferable cellular NBD-cholesterol (Fig. 4C, Table 2). Although both HDL subtypes were able to accept similar amounts of NBD-cholesterol from the labeled hepatocytes, SCP-2/SCP-x gene ablation differentially altered subtype specificity: HDL2-mediated efflux was increased and HDL3-mediated efflux was inhibited. Since HDL2 is the predominant subtype in HDL, the net effect of SCP-2/SCP-x loss was to increase HDL-mediated NBD-cholesterol efflux (Fig. 3, A and B, Table 1).

Although nascent apoA1 is produced in the liver, it is primarily involved in facilitating phospholipid and cholesterol efflux from peripheral cells to HDL for delivery to the liver. On this basis, it was expected that apoA1 would be a poor initiator/acceptor for cholesterol from the cultured primary hepatocytes. Consistent with this expectation and in contrast to HDL, apoA1 had no discernable effect on NBD-cholesterol efflux from WT or SCP-2/SCP-x gene-ablated mice (Fig. 4D).

**Influence of SCP-2/SCP-x on expression of hepatocyte proteins involved in cholesterol metabolism and cellular cholesterol pools.** SCP-2/SCP-x gene ablation did not increase expression of the major liver ACAT isoform, ACAT-2 (Fig. 5A), but increased expression of the minor isoform, ACAT-1 (Fig. 5A). SCP-2/SCP-x-null hepatocytes had less total and esterified, but not free, cholesterol (Fig. 5B). Thus, increased HDL-mediated efflux of NBD-cholesterol and [3H]cholesterol was not due to altered cellular free cholesterol content. Effect of SCP-2/SCP-x gene ablation on hepatocyte expression of cytosolic proteins involved in cholesterol intracellular binding/transport: SCP-2 and L-FABP. WT hepatocytes expressed SCP-2 (0.30 ± 0.03 µg/mg; Fig. 5C) and even more SCP-2/SCP-x-null hepatocytes after addition of HDL3. Values are means ± SE (n = 6–21). Parameters for efflux of NBD-cholesterol to HDL (40 µg/ml) from wild-type (WT) and sterol carrier protein (SCP)-2/SCP-x-null (KO) hepatocytes were derived using the following multiparameter exponential decay equation: \( y = A e^{-bt} + C e^{-ct} \), where A and C represent percentage of maximal 22-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol) remaining in each differentiated pool, b and d are apparent rate constants, and t is time; for calculation of initial rates (IR), \( t = 0 \); thus IR = \( Ab + Cd \). Half times (\( t_{1/2} \)) for pool 1 (\( t'_{1/2} \)) and pool 2 (\( t''_{1/2} \)) were calculated as follows: \( t'_{1/2} = \ln(2)/b \) and \( t''_{1/2} = \ln(2)/d \), where b and d are apparent rate constants. *P < 0.05 and †P < 0.005 vs. WT.

**Table 1. Effect of SCP-2/SCP-x gene ablation on kinetics of HDL-mediated NBD-cholesterol efflux from cultured primary hepatocytes**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Initial Rate</th>
<th>Half Time</th>
<th>Pool Size</th>
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<tbody>
<tr>
<td></td>
<td>( t'_{1/2} ) (s)</td>
<td>( t''_{1/2} ) (s)</td>
<td>A (µg/mg)</td>
</tr>
<tr>
<td>WT</td>
<td>4.4 ± 0.5</td>
<td>2.4 ± 0.6</td>
<td>26.5 ± 0.2</td>
</tr>
<tr>
<td>KO</td>
<td>10.0 ± 0.04†</td>
<td>4.8 ± 0.2*</td>
<td>27 ± 1.2</td>
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</table>

Values are means ± SE (n = 6–21). Parameters for efflux of NBD-cholesterol to HDL (40 µg/ml) from wild-type (WT) and sterol carrier protein (SCP)-2/SCP-x-null (KO) hepatocytes were derived using the following multiparameter exponential decay equation: \( y = A e^{-bt} + C e^{-ct} \), where A and C represent percentage of maximal 22-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol) remaining in each differentiated pool, b and d are apparent rate constants, and t is time; for calculation of initial rates (IR), \( t = 0 \); thus IR = \( Ab + Cd \). Half times (\( t_{1/2} \)) for pool 1 (\( t'_{1/2} \)) and pool 2 (\( t''_{1/2} \)) were calculated as follows: \( t'_{1/2} = \ln(2)/b \) and \( t''_{1/2} = \ln(2)/d \), where b and d are apparent rate constants. *P < 0.05 and †P < 0.005 vs. WT.
L-FABP (18 ± 6 μg/mg; Fig. 5D), SCP-2/SCP-x gene ablation elicited more than twofold upregulation of L-FABP (Fig. 5D). When HDL-mediated NBD-cholesterol efflux was measured in hepatocytes from L-FABP−/−/SCP-2/SCP-x−/− TKO mice, efflux was decreased (Fig. 5E), indicating that concomitant L-FABP upregulation in SCP-2/SCP-x-null hepatocytes more than compensated for loss of SCP-2 and that these proteins may exhibit opposing effects on HDL-mediated NBD-cholesterol efflux.

Effect of SCP-2/SCP-x gene ablation on expression of plasma membrane proteins involved in HDL-mediated cholesterol efflux (ABCA-1, ABCG-1, ABCG-5, ABCG-8, Pgp, and SRB1). WT and SCP-2/SCP-x-null hepatocytes expressed SRB1 (Fig. 6F), the HDL receptor protein involved in bidirectional cholesterol transport across the plasma membrane, but also several other plasma membrane proteins involved in cholesterol efflux, including ABCA-1 (Fig. 6A), ABCG-1 (Fig. 6B), ABCG-5 (Fig. 6C), ABCG-8 (Fig. 6D), and Pgp (Fig. 6E). SCP-2/SCP-x gene ablation did not upregulate any of these proteins but, rather, selectively downregulated SRB1 (Fig. 6F), ABCG-8 (Fig. 6D), and Pgp (Fig. 6E). Downregulation of SRB1 and ABCG-8 would be expected to decrease, rather than increase, cholesterol efflux from SCP-2/SCP-x-null hepatocytes. Although long-chain fatty acids may be an energy source for enhancing cholesterol efflux, SCP-2/SCP-x gene ablation decreased expression of fatty acid transport protein-4 (Fig. 6G), while only slightly increasing expression of glutamic-oxaloacetic transaminase (Fig. 6H).

Potential mechanism of SCP-2-regulated cholesterol efflux to HDL: codistillation and close proximity of SCP-2 for interaction with SRB1. The proximity of SCP-2 to SRB1 at the basolateral and canalicular membrane of cultured primary hepatocytes was examined as follows.

First, in a biochemical approach, plasma membranes were isolated from polarized cultured primary hepatocytes and subfractionated into basolateral and canalicular membrane-enriched fractions, and purity was confirmed by Western blotting of the appropriate protein markers (Fig. 7, A–C). Interestingly, SRB1 was detected in basolateral and canalicular membranes (Fig. 7D), but the scaffolding protein PDZK1 (CLAMP, which interacts with the COOH-terminal domain of SRB1) and the accessory protein membrane-associated protein 17 (which regulates the level of PDZK1) were localized with canalicular membranes (data not shown). Distribution of SRB1 to basolateral (Fig. 7, E and G) and canalicular (Fig. 7, F and G) membranes was confirmed by immunogold electron microscopy of fixed, polarized cultured primary hepatocytes. SCP-2 codistributed with SRB1 in purified basolateral and canalicular membranes (Fig. 7D), suggesting potential interaction in both plasma membrane regions of the hepatocyte.

Second, double-immunolabeling confocal microscopy and electron microscopy imaging further established significant codistribution (yellow colocalized pixels) of SCP-2 with SRB1 at the plasma membrane (Fig. 8B), prominently at the basolateral membrane (Fig. 8A) and microvillar protuberances from

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Table 2. Effect of SCP-2/SCP-x gene ablation on HDL2- and HDL3-mediated NBD-cholesterol efflux from cultured primary hepatocytes

<table>
<thead>
<tr>
<th>Cells</th>
<th>Initial Rate</th>
<th>Half Time</th>
<th>Pool Size (A)</th>
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</thead>
<tbody>
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<td><strong>HDL2-mediated efflux</strong></td>
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<tr>
<td>WT</td>
<td>0.8 ± 0.01</td>
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<td>98.9 ± 0.5</td>
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<td><strong>HDL3-mediated efflux</strong></td>
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<td></td>
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<td>42.3 ± 0.5§</td>
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<tr>
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<td>1.3 ± 0.01†‡</td>
<td>54.2 ± 0.4†‡</td>
<td>98.1 ± 0.4</td>
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Values are means ± SE (n = 6–21). Parameters for efflux of NBD-cholesterol to 20 μg/ml HDL2 or HDL3 from WT and SCP-2/SCP-x null (KO) hepatocytes were derived using the following exponential decay equation: \[ y = Ae^{-bt} \], where \( A \) represents percentage of cellular NBD-cholesterol able to efflux to the lipoproteins, \( b \) is apparent rate constant, and \( t \) is time; for calculation of initial rates, \( t = 0 \); thus IR = Ab. Half time for maximal NBD-cholesterol efflux was calculated as follows: \( t_{1/2} = \ln(2)/b \). † P < 0.005 vs. WT. ‡ P < 0.05 vs. HDL2 kinetic value.

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Fig. 5. Effects of SCP-2/SCP-x gene ablation on hepatocyte expression of proteins involved in cholesterol esterification [acyl CoA:cholesterol acyltransferases (ACAT-1 and ACAT-2)] and intracellular trafficking [SCP-2 and liver fatty acid-binding protein (L-FABP)]. A: Western blot determination of of ACAT-1 and ACAT-2, with β-actin used as loading control. Values are means ± SE (n = 3–6). B: free cholesterol, cholesterol ester, and total cholesterol content after lipid extraction, resolution, and quantification. Total cholesterol represents the sum of free cholesterol and cholesteryl ester values for each cell type. Values are means ± SE (n = 3–6).

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Fig. 8. Effect of SCP-2/SCP-x gene ablation on HDL-mediated cholesterol efflux. (A) Western blot determination of SCP-2 and liver fatty acid-binding protein (L-FABP) in cultured primary hepatocytes isolated from WT, SCP-2/SCP-x KO, and L-FABP/SCP-2/SCP-x triple-null (TKO) mice, with β-actin used as loading control. Values are means ± SE (n = 4–6) after comparison with Western blot bands of known concentrations of the respective pure proteins. C: HDL-mediated NBD-cholesterol efflux from WT and SCP-2/SCP-x/L-FABP TKO hepatocytes. *P < 0.05 vs. WT.
the basolateral membrane (Fig. 8A), less prominently at the canalicular membrane of the biliary vacuole (left edge of Fig. 8A), and somewhat prominently within the cytoplasm, suggesting vesicles (Fig. 8A). In the absence of HDL, much less SCP-2 colocalized with SRB1 in the whole cell (Fig. 8F) or at the plasma membrane (Fig. 8G), and more was visibly distributed in punctate vesicular structures localized throughout the cytoplasm (Fig. 8D). HDL increased codistribution of SCP-2 with SRB1 in hepatocytes (Fig. 8F), especially at the plasma membrane (Fig. 8G), compared with the cell interior (Fig. 8E). Serum also increased SCP-2-SRB1 colocalization at the plasma membrane (Fig. 8A vs. no HDL in Fig. 8D). Double-immunofluorescence-fluorescence resonance energy transfer confocal microscopy indicated a very close intermolecular distance (~57 Å) between SCP-2 and SRB1 (not shown). This was confirmed by double-immunogold electron microscopy, which showed clusters of SCP-2 with SRB1 at basolateral (Fig. 8C, boxed area, Fig. 9A) and canalicular (Fig. 9B) membranes. The intermolecular distance between SCP-2 and SRB1 in representative clusters was ~50 Å (n = 7). However, because of the large size of IgG antibody (i.e., ~150 kDa) and use of an antibody sandwich in the above-mentioned studies, ~50 Å is near the limit of resolution.

Third, chemical cross-linking with DTSP followed by co-immunoprecipitation was used determine whether SCP-2 was in even closer proximity to SRB1 in cultured primary hepatocytes. DTSP is a very small, membrane-permeable, homobifunctional, amine-reactive, cross-linker with 12-Å spacer. SRB1 was cross-linked within 12 Å of SCP-2 (Fig. 9F), as well as the scaffolding protein PDZK1 (Fig. 9E), known to bind SRB1’s cytoplasmic COOH terminus.

Potential mechanism of SCP-2-regulated cholesterol efflux to HDL: codistribution and proximity for interaction with ABCG-5 and ABCG-8. Subcellular fractionation showed that SCP-2 codistributed, not only with SRB1 (Fig. 7D), but also ABCG-5, ABCG-8, and Pgp (Fig. 7C), in the canalicular membrane. Furthermore, double-immunogold labeling detected clusters of SCP-2 with ABCG-8 (Fig. 9C) and SCP-2 with ABCG-5 (Fig. 9D) at the canalicular membrane. Finally, DTSP cross-linked SRB1 with ABCG-5 and ABCG-8 (Fig. 9F), but not ABCG-1 or Pgp (not shown).

DISCUSSION

While many aspects of the reverse cholesterol transport pathway are increasingly well understood, free cholesterol transfer between HDL and cells is bidirectional (42). Thus it is
important to resolve mechanisms whereby HDL free cholesterol, once taken up, rapidly traffics within the hepatocyte for subsequent rapid biliary excretion vs. efflux back to serum HDL (9). Since little is known regarding these mechanisms, especially within living hepatocytes, this investigation addressed the hypothesis that SCP-2 may function in facilitating HDL-derived cholesterol trafficking to retain cholesterol within hepatocytes for excretion into bile.

First, the fluorescent sterol NBD-cholesterol was developed as a useful probe for real-time imaging of cholesterol dynamics in living hepatocytes. Studies with lipoproteins and selective...
inhibitors indicated that NBD-cholesterol uptake and efflux exhibited SRB1-dependent preferential specificity for HDL (and HDL subspecies) similar to that established for cholesterol. While there are several HDL receptors in hepatocytes (SRB1, CD36, and glycosylphosphatidylinositol-anchored HDL-binding protein-1), SRB1 accounts for most of the selective uptake of cholesterol (9, 27, 39). Although apoA1 is known to be secreted by hepatocytes and facilitate the ABCA-1-mediated efflux of phospholipid and cholesterol from peripheral cells, apoA1 did not enhance NBD-cholesterol efflux from cultured primary hepatocytes, even though the hepatocyte plasma membrane expresses high amounts of ABCA-1 transporter (4). Consistent with the lack of effect of apoA1 on NBD-cholesterol efflux, hepatic clearance of HDL free cholesterol was also not affected by apoA1 knockout (15). Furthermore, unlike [3H]cholesterol, the real-time HDL-mediated efflux kinetics of NBD-cholesterol from hepatocytes were independent of potential complication from intracellular esterification/hydrolysis of the probe. Finally, the preferential intracellular distribution of NBD-cholesterol in the canalicular region of polarized cultured primary hepatocytes reflected that of cholesterol and other naturally occurring sterols (23, 37). Canalicular membrane NBD-cholesterol (like naturally occurring sterols) was also transported for efflux back to HDL at the basolateral membrane as rapidly as from the rest of the hepatocyte cell body.

Second, real-time confocal imaging of HDL-mediated NBD-cholesterol efflux for the first time resolved rapidly (detectable in 1–2 min) from a more slowly effluxing pool in living hepatocytes. While kinetic analysis alone does not establish the identity of these two NBD-cholesterol pools, neither pool was attributed to spontaneous diffusion from intracellular sites through the cytosol to the plasma membrane, a much slower process ($t_{1/2} = 3$ h to days) (37). Since the half time of transbilayer cholesterol movement is <1 min (16), the half times of the rapid (2.4 min) and slowly (26 min) effluxing NBD-cholesterol pools were in the range of those for protein-mediated molecular transfer and vesicular transfer, respectively (6, 37).

Third, SCP-2 gene ablation enhanced HDL-mediated NBD-cholesterol and [3H]cholesterol efflux from hepatocytes. In contrast, SCP-2 overexpression inhibits HDL-mediated efflux of NBD-cholesterol, dansyl-cholesterol, and [3H]cholesterol from fibroblasts (6, 33), while SCP-2 overexpression in rodents inhibits VLDL cholesterol secretion (1, 41). SCP-2’s role(s) in facilitating retention of HDL-derived cholesterol for biliary cholesterol secretion is supported by rodent studies, where SCP-2 overexpression and SCP-2 antisense treatment increased and decreased, respectively, biliary free cholesterol excretion (1, 30, 41). While the exact mechanism(s) whereby SCP-2 elicits these effects is not completely clear, the studies presented here (biochemical fractionation, double immunolabeling, fluorescence resonance energy transfer, and chemical cross-linking) suggest that SCP-2 is in sufficiently close proximity for direct interaction with SRB1, a receptor whose activity is known to be regulated by interaction with another protein (PDZK1) at the SRB1 cytoplasmic COOH terminus (36). Furthermore, the finding that SRB1 (especially in the presence of HDL) was also in close proximity to ABCG-5 and ABCG-8 was consistent with SCP-2’s known ability to enhance biliary cholesterol efflux (1). Finally, the time frame of HDL-derived cholesterol clearance ($t_{1/2} = 3$ min) and transhepatocyte nonvesicular cholesterol transfer ($t_{1/2} = 1–2$ min) (15, 31) is consistent with the known properties of SCP-2 in vitro and in cultured transformed cells (reviewed in Ref. 13).
Second, increased, rather than decreased, HDL-mediated NBD-cholesterol efflux from SCP-2 gene-ablated hepatocytes was attributed in part to concomitant upregulation of L-FABP, another cytosolic cholesterol-binding protein present at high level in hepatocytes (19). The role of concomitant L-FABP upregulation in counteracting the loss of SCP-2 in SCP-2/SCP-x-null hepatocytes was confirmed by significantly slower HDL-mediated NBD-cholesterol efflux from SCP-2/SCP-x-L-FABP TKO hepatocytes. Similarly, higher levels of L-FABP increase hepatic VLDL secretion, thereby counteracting the effect of SCP-2, the increased expression of which inhibits VLDL secretion (1, 26, 41). The net effect of these opposing influences in SCP-2/SCP-x-null mice is to increase serum triglyceride, suggesting that increased VLDL secretion is attributable to the concomitant upregulation of L-FABP (12, 35).

In summary, the data reported here are consistent with important, but opposing, roles of SCP-2 and L-FABP in HDL-mediated cholesterol efflux from hepatocytes. In the absence of SCP-2, but concomitant L-FABP upregulation, HDL-mediated NBD-cholesterol efflux was enhanced. In contrast, when both SCP-2 and L-FABP were ablated, the HDL-mediated NBD-cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x NBD-cholesterol efflux was enhanced. In contrast, when both SCP-2 and L-FABP were ablated, the HDL-mediated NBD-cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased. The fact that SCP-2/SCP-x gene ablation also altered the HDL subclass specificity of cholesterol efflux was decreased.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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