Sterol Carrier Protein-2 Alters HDL-Mediated Cholesterol Efflux

by

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FOOTNOTES:

Abbreviations: SCP-2, sterol carrier protein-2; HDL, high density lipoprotein; ACAT, acyl-CoA:cholesterol O-acyltransferase; RCT, reverse cholesterol transport; NBD-cholesterol, [22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3b-ol], ADRP, adipose differentiation-related protein.

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ABSTRACT

Although sterol carrier protein-2 (SCP-2) participates in the uptake and intracellular trafficking of cholesterol, its effect on ‘reverse cholesterol transport’ has not been explored. As shown herein, SCP-2 expression inhibited HDL-mediated efflux of \[^3\text{H}\]cholesterol and fluorescent NBD-cholesterol up to 61 and 157\%, respectively. Confocal microscopy of living cells allowed kinetic analysis of two intracellular pools of HDL-mediated NBD-cholesterol efflux: the highly fluorescent lipid droplet pool and the less fluorescent pool outside the lipid droplets, designated the cytoplasmic compartment. Both the whole cell and the cytoplasmic compartment exhibited two similar kinetic pools, the half-times of which were consistent with protein (t\(^b_{1/2}\) near 1 min) and vesicular (t\(^d_{1/2}\)=10-20 min) mediated sterol transfer. While SCP-2 expression did not alter cytoplasmic sterol pool sizes, the rapid t\(^b_{1/2}\) decreased 36\% while the slower t\(^d_{1/2}\) increased 113\%. Lipid droplets also exhibited two kinetic pools of NBD-cholesterol efflux, but with half-times over 200\% shorter than those of the cytoplasmic compartment. The lipid droplet slower effluxing pool size and t\(^d_{1/2}\) were increased 48\% and 115\%, respectively, in SCP-2 expressing cells. Concomitantly, the level of the lipid droplet-specific adipose differentiation related protein (ADRP) decreased 70\%. Overall, HDL-mediated sterol efflux from L-cell fibroblasts reflected that of the cytoplasmic, rather than lipid droplet, compartment. SCP-2 differentially modulated sterol efflux from the two cytoplasmic pools. However, net efflux was determined primarily by inhibition of the slowly effluxing pool, rather than by acceleration of the rapid protein-mediated pool. Finally, SCP-2 expression also inhibited sterol efflux from lipid droplets, an effect related to decreased ADRP, a lipid droplet surface protein that binds cholesterol with high affinity.
INTRODUCTION

While the HDL-mediated steps of cholesterol transfer from the cell surface membrane and subsequent fate of cholesterol in the vasculature have been extensively studied, much less is known about intracellular components of cholesterol efflux (rev. in (1-5)). Plasma membrane cholesterol is distributed into multiple pools or domains (rev. in (6,7)). It is now recognized that there may be a connection between such domains and HDL-receptor mediated reverse cholesterol transport (rev. in (7-9)). The transbilayer distribution of cholesterol in plasma membranes is asymmetric, with the cholesterol enriched 400% in the cytofacial leaflet vs exofacial leaflet (rev. in (10-14)). Transbilayer movement of cholesterol across the plasma membrane appears fast, $t_{1/2}$ 1-6 min (rev. in (10)). Plasma membrane cholesterol is also distributed into lateral cholesterol-rich and -poor membrane domains (rev. in (6,10)). Most of the cholesterol in the plasma membrane is localized in lateral domains that are, for the most part, relatively inert in terms of transfer kinetics (i.e. $t_{1/2}$ = hours to days) and movement between such domains is also slow. However, a small pool of plasma membrane cholesterol appears highly dynamic (rev. (6) and is associated with cholesterol-rich, HDL-receptor containing microdomains called caveolae (rev. in (8,9,15,16)). Molecular details of cholesterol entry/exit, cholesterol organization, and mechanism(s) of cholesterol transbilayer movement in caveolae remain to be determined. Likewise, the relationships between caveolae, ‘rafts’, and other cholesterol rich plasma membrane microdomains is not yet clear (rev. in (9)).

The intracellular steps preceding cellular cholesterol efflux include transfer of cholesterol from the Golgi, endoplasmic reticulum, and lipid droplets to the plasma membrane (rev. in (1,9,17)). The time-frame of bidirectional vesicular transfer of cholesterol between plasma membranes and Golgi has a $t_{1/2}$ of 10-20 min (rev. in (8,18,19)). Alternately, molecular cholesterol transfer, mediated by cholesterol binding proteins in the cytoplasm, occurs much faster ($t_{1/2}$ near 1-2 min) from the lysosome (exogenous
cholesterol) to the plasma membrane (20,21) and from the endoplasmic reticulum (newly synthesized cholesterol) to the plasma membrane (rev. in (7,8,22). Which one or both processes participate in cholesterol trafficking from lipid droplets, a primary site of cholesterol storage, is presently unknown.

The purpose of this investigation was to visualize three aspects of cholesterol efflux in intact cells: First, to determine the role of SCP-2 in HDL-mediated cholesterol efflux. Previously it was shown that SCP-2 stimulates biliary cholesterol secretion (23-25) but inhibits lipoprotein secretion (22). Second, to address the lack of information regarding the lipid droplet as a source for cholesterol efflux via the HDL pathway. Lipid droplets contain a surface coat of cholesterol and phospholipid surrounding a cholesterol ester/triglyceride core (26,27). Further, the lipid droplet surface monolayer contains unique proteins including perilipin and adipose differentiation related protein (ADRP), neither of whose function is well understood (28). Since ADRP binds cholesterol, this suggests a potential role of this protein in cholesterol trafficking from the lipid droplet (29). Third, to examine the effect of SCP-2 expression on HDL-mediated cholesterol efflux from the lipid droplet. This possibility was suggested by the fact that SCP-2 stimulates cholesterol transfer from lipid droplets to mitochondria in adrenal cells (rev. in (27). The results presented herein provide fresh insights into the efflux process and for the first time establish new information demonstrating that SCP-2 expression: (i) inhibits HDL-mediated cholesterol efflux and (ii) inhibits cholesterol efflux from a little understood, subcellular compartment, i.e. lipid droplets.

**MATERIALS AND METHODS**

*Materials.* [³H]cholesterol was from Dupont New England Nuclear (Boston, MA). Bovine serum albumin (BSA, essentially fatty acid free) and fetal bovine serum (FBS) were from Sigma (St. Louis, MO). Human high density lipoprotein (HDL) was from Calbiochem (San Diego, CA). Lab-Tek Coverglass slides
were from Fisher (Pittsburgh, PA). NBD-cholesterol [22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-
23,24-bisnor-5-chole-3b-ol] and Nile red were from Molecular Probes (Eugene, OR). Sandoz 58-035 was
a gift from T.Y. Chang (Dartmouth Medical School, Hanover, NH). Rabbit polyclonal antisera to ADRP
were prepared (30) using ADRP generously provided by Dr. G. Serrero (University of Maryland,
Baltimore, MD). Monoclonal anti-caveolin-1 was from Transduction Laboratories (Lexington, KY).
Rabbit polyclonal anti-scavenger receptor B1 antisera were from Novus Biologicals (Littleton, CO). All
reagents and solvents used were of the highest grade available and were cell culture tested.

**L-Cell culture.** Murine L-cells (L arpt’ik’) were grown to confluency in Higuchi medium (31)
supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) as described (30). Development of
mock-transfected cells (designated as control) and cells transfected with cDNA corresponding to the 15
kDa pro-SCP-2 protein was as described earlier (30,32). In high expression cells, SCP-2 comprised
0.036±0.002 % of the total cytosolic proteins while levels in the control and mock transfected cells were
below the level of detectability. Cellular cholesterol and cholesterol ester mass were increased 20 and
100%, respectively, in the SCP-2 expression clones. For radiolabeled experiments, control and SCP-2
expressors were plated to 24-well plates at approximately 5 x 10^5 cells/well. For fluorescence imaging
experiments, cells were seeded at a density of 50,000 cells/chamber onto Lab-Tek Chamber Coverglass
slides (Nunc, Naperville, IL). To ensure a monolayer, samples analyzed by imaging were examined within
20 hours of seeding. Cells for radiolabeled experiments were treated as described below.

**Measurement of Cellular[^3]H]cholesterol efflux.** Cholesterol efflux was done as described earlier
(33). Briefly, mock-transfected and SCP-2 expressors were plated in medium containing 1 μCi/ml of
[^3]H]cholesterol and 2.5 % fetal bovine serum (1ml/well) overnight. The medium was then replaced with
medium containing 0.3% bovine serum albumin (BSA) to allow equilibration between different cholesterol
pools. After 24 hours, cells were washed 2x with phosphate buffered saline (PBS). HDL at indicated concentrations was then added and radiolabeled aliquots (50 µl) were removed at indicated time points and counted. At the end of the time course, the monolayers were washed 3x with PBS, cells were dissolved in 0.1 N NaOH (500 µL) overnight, and protein was determined (34). Unless otherwise stated, Sandoz 58-035 was included during labeling and equilibration to eliminate competition by intracellular [³H]-cholesterol esterification. Extra wells without acceptor were harvested at the beginning of the time course to determine the initial [³H]cholesterol for each cell line.

Efflux was determined from the fraction of [³H]-cholesterol remaining in the cell at different time points. Data points were fitted to the single exponential decay curve $y = Ae^{-kt} + C$ where $y$ is the fraction of initial cellular [³H]-cholesterol remaining in the cells at time $t$; $A$ is the cholesterol pool size available for efflux; and $k$ is the apparent rate constant. Half-times ($t_{1/2}$) were determined from the equation $t_{1/2} = \ln 2/k$. Half-time and $k$ values were apparent values due to dependence on HDL acceptor concentrations.

**Laser scanning confocal microscopy.** Confocal fluorescence imaging was performed on a Bio-Rad (Hercules, CA) MRC-1024 Laser Scanning Confocal Imaging System. Confocal and co-localized images were acquired using multiple photomultiplier tubes under the control of LaserSharp v.3.2 software (Bio-Rad). For probe excitation, the MRC-1024 system utilized a 15 mW krypton-argon laser (American Laser Corp., Salt Lake City, UT) with a 5 mW output. Cells cultured on cover slips were placed on the stage of a Zeiss Axiovert 135 inverted epifluorescence microscope (Zeiss, Thornwood, NY) and examined with a 63X oil-immersion, infinity objective (numerical aperture 1.4). Fluorescence emission was detected using 488 nm excitation and a 540/32 band path emission filter for NBD-cholesterol (green channel) and 568 nm excitation with a HQ598/40 band path emission filter for Nile red (red channel). Samples were exposed to the light source for minimal time periods to minimize photobleaching effects. Image files were analyzed...
using either Metamorph software (West Chester, PA) or NIH Image, a program written by W. Rasband and available by anonymous FTP from zippy.nimh.nih.gov.

**NBD-cholesterol efflux measurement by laser scanning confocal microscopy.** To perform the efflux experiments, cells were first loaded with NBD-cholesterol (0.35 µM) in medium containing 2.5% FBS for 1 hour in a 37°C CO₂ incubator. Since only 7.8% of NBD-cholesterol is esterified at 24 h(29), the ACAT inhibitor was not used for the fluorescence experiments for the comparatively short (40 min) incubation time used herein. After loading, the cells were washed 2x with Pucks buffer (1 mM Na₂HPO₄, 0.9 mM H₂PO₄, 5.0 mM KCl, 1.8 mM CaCl₂, 0.6 mM MgSO₄, 6 mM glucose, 138 mM NaCl and 10 mM HEPES) and placed in serum-free medium. A medial section passing through 5-10 cells was selected, and the section was scanned for initial fluorescence intensity of NBD-cholesterol. HDL was added to start the efflux experiment. The fluorescence in the total area of each cell was measured over time and was used to gauge the extent of NBD-cholesterol efflux from the cell.

**NBD-cholesterol co-localization with lipid droplets.** Three types of co-localization studies were performed in order to determine the identity of high intensity regions of NBD-cholesterol visible within the cell: First, NBD-cholesterol was co-localized with nile red, a lipid stain. Second, nile red was co-localized with ADRP, a protein closely associated with lipid droplets. Third, NBD-cholesterol was co-localized with ADRP. Cells overexpressing SCP-2 were incubated with both NBD-cholesterol (0.35 µM) and nile red (0.4 µM) for 30 minutes in a 37°C CO₂ incubator. While nile red is known to stain other non-lipidic structures, care was taken to titer both nile red and NBD-cholesterol to low levels to give a low cytoplasmic background with emphasis on lipid droplet staining in the co-localization experiments. However, it should be noted that nile red and NBD-cholesterol are also located in sites outside lipid droplets where they also co-localize to a large degree. After incubation, cells were washed with Puck’s
buffer and then simultaneously imaged for NBD-cholesterol (488 nm excitation, 540/30 emission, green channel) and nile red (568 nm excitation, HQ598/40 emission, red channel). The confocal images from the green and red channels were merged and appeared yellow where superimposition occurred (red and green are additive and yield yellow to orange in RGB color space). Pixel fluorograms were constructed and correlation coefficients generated from the fluorograms were derived from the following equations as described earlier (35):

\[ C_{\text{red}} = \frac{\sum R_{i,\text{coloc}}}{\sum R_i} \]
\[ C_{\text{green}} = \frac{\sum G_{i,\text{coloc}}}{\sum G_i} \]

where \( R_{i,\text{coloc}} \) is the sum of intensities of all red pixels which also have a green component; \( R_i \) is the sum of intensities of all red pixels in the image; \( G_{i,\text{coloc}} \) is the sum of intensities of all green pixels which also have a red component; and \( G_i \) is sum of intensities of all the green pixels in the image.

To co-localize ADRP with nile red or with NBD-cholesterol, SCP-2 expressors were plated on 8 well Lab-Tek chamber slides (Nunc, Naperville, IL) at subconfluency. The cells were washed with serum free medium and incubated with 0.005% of either nile red or NBD-cholesterol in serum free medium for 30 minutes, at 37\(^\circ\)C. Cells were washed with Hank’s solution, fixed with 70:30 (v/v) acetone:ethanol for 10 min. at 4\(^\circ\)C, permeabilized with 0.05% saponin in Hank’s for 5 min at room temperature, and then blocked with 10% FBS in Hank’s for 1 hour at room temperature. Anti-ADRP (rabbit polyclonal anti-mouse ADRP, affinity purified, concentrated to 0.5 mg/ml, dilution 1:20) was incubated in 5% FBS in Hank’s for 1 hour at room temperature, followed by extensive washing with 2% FBS in Hank’s buffer. Next, the cells were incubated with either goat anti-rabbit IgG conjugated to fluorescein (for the ADRP/nile red colocalization) or goat anti-rabbit IgG conjugated to Texas red (for the ADRP/NBD-cholesterol colocalization) for 1 hour at room temperature. After a final wash with Hank’s, cells were mounted in...
SlowFade (Molecular Probes) according to the manufacturer’s procedure and were visualized with the MRC1024 laser scanning system as described above.

**NBD-cholesterol efflux from subcellular compartments: cytoplasm and lipid droplets.** NBD-cholesterol efflux from the whole cell was compared to that arising from two compartments designated as lipid droplets (based on the above colocalization data) or cytoplasmic (defined as the non-lipid droplet, lower-intensity, diffuse pattern of the remaining NBD-cholesterol fluorescence in the cell). Cells were imaged and analyzed by Metamorph software to graphically determine the separate contributions of the efflux process. Data points were fitted to either a biexponential \( y = Ae^{-bt} + Ce^{-dt} \) or multiparameter \( y = (Ae^{-bTo} + Ce^{-dTTo})e^{-h(t-To)} \) exponential decay equation where \( y \) was the fraction of initial cellular NBD-cholesterol remaining in the cells at time \( t \); \( A \) and \( C \) were the size of the cholesterol pools available for efflux; \( b, d, \) and \( h \) were the apparent rate constants; and \( To \) was the time at which depletion of the pools increased to completion. The multiparameter equation was employed to adjust for an inflection point observed in the time course data obtained from both control and SCP-2 overexpressors. It appeared that at some time \( T_o \), the rate of efflux was altered which effected a decrease in the half-life. In order to compare the data, a model was developed which allowed for the initial decay curve \( (Ae^{-bt} + Ce^{-dt}) \) to be modified at some \( T_o \) to become \( (Ae^{-bTo} + Ce^{-dTTo})e^{-h(T-To)} \). Using a nonlinear least squares routine in Sigma Plot 4.0, all parameters were obtained simultaneously from the fitted data. Unique fittings were obtained individually for each time course involving individual cells. This produced excellent results for the cytoplasmic compartment contribution from the cell. Statistical methods involving the paired t-test were used to compare the control and SCP-2 expressors. However, the lipid droplet contribution was quite “noisy” and an inflection point could not be determined for this data which was subsequently fit to the equation \( Ae^{-bt} + Ce^{-dt} \) for all times. This also contributed some complication in determining the inflection point of the whole
cell since it is the combination of lipid droplet plus cytoplasm. In order to overcome this difficulty, the time courses of N individual cells for the specified concentration and cell type were averaged to produce a single time course which was then fitted to the multiparameter equation.

**Isolation of mouse lipid droplets from L-cell fibroblasts.** Lipid droplets from control cells and SCP-2 expressors were isolated as described by Chanderbhan et al. (36). Briefly, cells scraped from 10 confluent trays (245 x 245 x 25 mm each, Nunc, Naperville, IL) were homogenized in 50 mM NaH$_2$P0$_4$ buffer, pH 7.4, containing 154 mM NaCl and 5mM MgCl$_2$ and centrifuged at 800 x g for 10 min. The supernatant was then centrifuged at 5,000 x g for 20 min to sediment mitochondria. This was followed by centrifuging at 35,000 rpm in a SW 40.1 rotor for 2 hours at 4°C. The lipid droplet fraction, forming a distinct, white band on the surface of the preparation, was removed and protein was quantified (34).

**Quantitative Western blotting for ADRP, caveolin, and SRB1.** Lipid droplets and cell homogenates were analyzed by Western blot analysis to determine ADRP content in control and SCP-2 expressors. Cell homogenates were analyzed for presence of caveolin and SRB1. For all quantitative Western blots, protein samples (17-20 µg) were run on tricine gels (12%) and transferred to nitrocellulose membranes. The blots were blocked in 3 % gelatin in TBST (10 mM Tris-HCl, pH 8, 100 mM NaCl, 0.055 Tween-20) before incubation with the polyclonal rabbit antibodies against either ADRP, caveolin, or SRB-1. Alkaline-phosphatase conjugates of goat anti-rabbit IgG and Sigma Fast 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolim tablets (Sigma, St. Louis, MO) were used to visualize and quantitate bands of interest (37).

**Statistics.** All values were expressed as the mean ± SEM with n and P indicated in the Results section. Statistical analyses were performed using Student's t-test (GraphPad Prism, San Diego, CA). Values with P<0.05 were considered statistically significant.
RESULTS

**Dependence of the extent of HDL-mediated efflux of [³H]cholesterol on HDL concentration of HDL and on SCP-2 expression.** When L-cell clones were prelabeled with [³H]cholesterol followed by incubation in medium without HDL, very little [³H]cholesterol effluxed from either mock transfected control cells (Fig. 1, solid circles) or SCP-2 expressing cells (not shown). While HDL (98 µg HDL protein/ml) dramatically stimulated efflux of [³H]cholesterol from both mock transfected control (Fig. 1, closed triangles) and SCP-2 overexpressing cells (Fig. 1, open triangles), SCP-2 expression decreased the extent of HDL-mediated [³H]cholesterol efflux by 61% (P<0.015, n=4). However, this effect was highly dependent on the HDL concentration. In mock transfected control cells, the extent of HDL-mediated [³H]cholesterol efflux was increased proportional to medium HDL concentration up to a maximum at 98 µg HDL protein/ml (Fig. 2A, open bars). Further increasing the medium HDL concentration to 175 µg HDL protein/ml did not additionally increase [³H]cholesterol efflux, while higher HDL concentrations actually inhibited, rather than increased, [³H]cholesterol efflux. In contrast, in SCP-2 expressing cells the extent of HDL-mediated [³H]cholesterol efflux was increased in proportion to medium HDL concentration up to a maximum at 175 µg HDL protein/ml (Fig. 2A, solid bars) and then decreased at 250 µg HDL protein/ml. Thus, the HDL-concentration required to achieve maximal HDL-mediated [³H]cholesterol efflux was increased 78% in SCP-2 expressing cells. In summary, SCP-2 expression decreased the extent of HDL-mediated [³H]cholesterol efflux and shifted the concentration of HDL-required to achieve maximal efflux of [³H]cholesterol without further stimulating maximal HDL-mediated [³H]cholesterol efflux above the level observed in mock transfected controls.

**Effect of SCP-2 expression on kinetics of HDL-mediated efflux of [³H]cholesterol.** In the absence of HDL, the initial rate of [³H]cholesterol efflux from both mock transfected cells (Fig. 1, solid circles) and
SCP-2 expressing cells (not shown) was essentially near zero. While addition of HDL (98 µg protein/ml) increased the initial rate of [3H]cholesterol efflux dramatically from both mock transfected and SCP-2 overexpressing cells (Fig.1), analysis of multiple experiments revealed that SCP-2 expression decreased the initial rate of HDL-mediated [3H]cholesterol efflux by 20% (P<0.05, n=4) as compared to mock transfected control cells. In mock transfected control cells, the initial rate of HDL-mediated [3H]cholesterol efflux increased in near linear (slope 0.65) proportion with increasing medium HDL concentration up to 250 µg HDL protein /ml (Fig. 2B, open bars). In SCP-2 expressing cells, the initial rate of HDL-mediated [3H]cholesterol efflux similarly increased with medium HDL, except that the slope of the increase was 40% higher (Fig. 2B, closed bars). Thus, SCP-2 expression inhibited the initial rate of HDL-mediated [3H]cholesterol efflux at low, but not high, HDL concentration. However, even at 250 µg HDL/ml the initial rate of HDL-mediated [3H]cholesterol efflux in SCP-2 expressing cells was not increased over that in mock transfected controls.

Further analysis of the HDL-mediated [3H]cholesterol efflux curves in Fig.1 revealed that the efflux curve fit single exponential kinetics which allowed resolution of two parameters: the time at which half the cholesterol pool was depleted (t 1/2) and the pool size of HDL-mediated [3H]cholesterol (A). SCP-2 expression increased the t 1/2 of HDL-mediated [3H]cholesterol efflux by 61% (P<0.019, n=4). The longer half-time was consistent with the inhibitory effect of SCP-2 on the initial rate of cholesterol efflux (Figs.1 and 2). Finally, SCP-2 expression significantly reduced the pool size of HDL-mediated [3H]cholesterol efflux by 23% (P<0.03, n=3) as compared to that in mock transfected control cells.

**Direct visualization of HDL-mediated cholesterol efflux from living cells by laser scanning confocal microscopy: effect of SCP-2 expression on extent of HDL-mediated NBD-cholesterol efflux.** While the above studies with [3H]cholesterol indicated that HDL-mediated [3H]cholesterol efflux was
inhibited by SCP-2 expression, radiolabeled studies did not provide direct visualization of this process. Therefore, cells were preloaded with NBD-cholesterol, washed, and NBD-cholesterol was imaged by laser scanning confocal microscopy as described in Methods. The total fluorescence intensity/cell was then determined individually for a large number of cells. The time dependent disappearance of NBD-cholesterol fluorescence of each cell was then determined in the presence and absence of HDL (see Methods). As with \[^3\text{H}\]cholesterol efflux, NBD-cholesterol fluorescence intensity/cell (corrected for a small amount of photobleaching over the time period studied) was essentially zero in the absence of HDL for both mock transfected control and SCP-2 expressing cells (not shown). The addition of HDL dramatically stimulated NBD-cholesterol efflux from both the SCP-2 expressor and mock transfected control cells (Fig. 3).

However, the extent of this effect was highly dependent on the expression of SCP-2 and the HDL concentration in the medium. HDL-mediated NBD-cholesterol efflux from mock transfected control cells was greater than 90% by 40 min (Fig. 3, solid triangles). In contrast, SCP-2 expression decreased the extent of HDL-mediated NBD-cholesterol efflux over the same time period studied (Fig. 3, open triangles). Examination of a large number of cells showed that the extent of HDL-mediated NBD-cholesterol efflux was 40% less than that from mock transfected control cells (p<0.006, n=11-14).

The extent of NBD-cholesterol efflux was dependent on the HDL-concentration. The extent of efflux increased proportional to medium HDL concentration in near linear fashion for both mock transfected control (slope = 0.74) (Fig. 4A, open bars) and for SCP-2 expressing cells (slope = 1.04) (Fig. 4A, solid bars). SCP-2 expression inhibited HDL-dependent NBD-cholesterol 157% (p<0.006, n=6-9) at low HDL concentration (Fig. 4A), but not at high HDL-concentration. However, even when the SCP-2 mediated inhibition was abolished at high HDL concentration, the extent HDL-mediated NBD-cholesterol efflux in SCP-2 expressing cells was not increased above that exhibited by mock transfected control cells (Fig. 4A).
Comparison of the effect of SCP-2 expression on extent of HDL-mediated NBD-cholesterol and \[^3\text{H}\text{]cholesterol efflux.}\] In order to compare the results from efflux experiments using either \[^3\text{H}\text{]cholesterol or NBD-cholesterol, the amount of HDL added to initiate efflux was adjusted with regard to the number of cells present during the experiment. The number of cells present in the \[^3\text{H}\text{]cholesterol experiments was approximately 10 times greater than with the NBD-cholesterol probe. However, the amount of HDL used to initiate efflux was also 10 times greater than with the \[^3\text{H}\text{]cholesterol experiments so that the ratio of HDL to cell number remained constant regardless of which probe was used. The results from Fig. 5 show that HDL-mediated efflux of \[^3\text{H}\text{]cholesterol and NBD-cholesterol were inhibited to the similar degree at the same ratios of HDL / # of cells. Thus, even though HDL was more effective in mediating efflux of NBD-cholesterol (Fig. 4A) than \[^3\text{H}\text{]cholesterol (Fig. 2A), SCP-2 expression inhibited HDL-mediated sterol efflux at low HDL levels to the same degree (65-75%) and this inhibition was abolished at similar high ratios of HDL / # of cells.\]

**Effect of SCP-2 expression on kinetics of HDL-mediated NBD-cholesterol efflux in living cells.**

SCP-2 expression decreased the initial rate of HDL-mediated NBD-cholesterol efflux as much as 220% at low HDL-concentration in the medium (Fig. 4B). Increasing HDL concentration abolished the inhibitory effect of SCP-2 expression on HDL-mediated NBD-cholesterol efflux. Visual examination of the curves in Fig. 3 indicated that the kinetics of HDL-mediated NBD-cholesterol efflux were not simple exponentials. Attempts at fitting these curves to simple exponential kinetics for either mock transfected controls or SCP-2 expressing cells were unsuccessful (see Methods). In contrast, the efflux curves in Fig. 3 fit very well (\(R^2 = 0.999\)) to a multiparameter exponential decay equation, \(y = (Ae^{-bT_0} + Ce^{-dT_0}) (e^{-b(t-T_0)})\) (see Methods). This equation describes two pools of cholesterol (a large, slow pool and a smaller, faster pool) showing increased efflux after some time point \(T_0\). In mock transfected control cells, the respective
half-times of these cholesterol pools ($t_{1/2}^b$ and $t_{1/2}^d$) were 1.2 and 13.1 min, respectively (Table 1). The longer half-time was increased 100% ($p<0.0025$) in SCP-2 expressing cells. In mock transfected control cells, the pool sizes (A and C) corresponding to these half times were 0.23 and 0.77, respectively (Table 1). Although the slowly effluxing NBD-cholesterol pool was 230% larger than the rapidly effluxing pool, SCP-2 expression did not affect the respective pool sizes. Overexpression of SCP-2 resulted in a 59% decrease in the half-life associated with “h”. $T_0$ was not affected by SCP-2 expression. In summary, these data suggested that SCP-2 expression inhibited NBD-cholesterol efflux from the cells primarily by inhibiting the half-time, rather than altering the pool size of NBD-cholesterol.

**Intracellular localization of the NBD-cholesterol: confocal laser scanning microscopy of L-cells expressing SCP-2.** While the NBD-cholesterol fluorescence in cells preloaded with NBD-cholesterol appeared localized throughout the cell, distinct highly fluorescent regions resembling lipid droplets in size and shape were also prominent (Fig. 6). To establish whether the intensely fluorescent regions were indeed lipid droplets, three colocalization experiments were performed.

First, cells preloaded with NBD-cholesterol were stained with nile red, a selective lipid droplet stain, and emission of the two fluorophores was simultaneously determined through separate photomultipliers: NBD-cholesterol (green), nile red (red). Upon superposition of such images (Fig. 7A), co-localization appeared as yellow/orange areas (red plus green = yellow/orange) while lack of co-localization appeared as separate green and red areas. A graphical representation of the superposition is shown in the form of a pixel fluorogram (Fig. 7B). High co-localization in pixel fluorograms is indicated both by the localization of many pixels along the diagonal, the yellow/orange color, and the “green” and “red” correlation coefficients approaching values near 1.0. The “green” correlation coefficient (0.81 in Fig. 7B) is the ratio of all the green (NBD-cholesterol) intensities which showed a red (nile red) component divided by the sum
of all the green (NBD-cholesterol) intensities. The “red” correlation coefficient (0.92 in Fig. 7B) is ratio of all the red (nile red) intensities showing a green (NBD-cholesterol) component divided by the sum of all the red intensities. It should be noted, however, that while a significant population of data points fell along the diagonal line, NBD-cholesterol also appeared throughout the cell (green in the superimposed image Fig. 7A). Morphometric analysis on intensity measurements using Metamorph software (Methods) revealed the percentage of NBD-cholesterol outside the lipid droplets (i.e. designated as the cytoplasmic compartment of the cell) ranged from 80-96% for both control and SCP-2 expressors. While the correlation coefficients given in Fig. 7B determined the degree of co-localization of Nile red in NBD-cholesterol (and vice-versa), they did not measure the amount of NBD-cholesterol in the lipid droplets versus that in the rest of the cell.

Second, the lipid droplet specific stain, nile red, was co-localized with ADRP, a protein closely associated with lipid droplets. Cells were simultaneously labeled with nile red (red) and anti-ADRP antibodies (green). Confocal fluorescence images for ADRP (Fig. 8B) highly resembled those obtained for nile red (Fig. 8A), the lipid droplet specific stain. Magnification of a representative lipid droplet showed uniform staining with nile red throughout (Fig. 8A, lower left). In contrast, magnification of the same lipid droplet stained with anti-ADRP showed much more intense staining at the lipid droplet surface (Fig. 8B, lower left). Superposition of the two images (Figs. 8A and 8B) yielded a yellow-orange color where co-localization occurred. While both stains co-localized to lipid droplets, the magnified image of the lipid droplet revealed that ADRP (green) was present on the surface of the lipid droplet while the nile red stain was seen both at the surface (orange/yellow) and throughout (red) the lipid droplet (Fig. 8C).

Third, NBD-cholesterol was co-localized with ADRP by simultaneous labeling with anti-ADRP antibodies (red) and NBD-cholesterol (green). Simultaneous acquisition of confocal images for ADRP
(Fig. 8D) and NBD-cholesterol (Fig. 8E) showed that ADRP and NBD-cholesterol were both present in intense staining areas, i.e. lipid droplets shown in superposed images (Fig. 8F). Magnified images of representative lipid droplets (Fig. 8F, inset) showed that ADRP again appeared on the lipid droplet surface (distinct red region on left side of lipid droplet) while NBD-cholesterol was present at the surface co-localized with a portion of ADRP (yellow) as well as throughout the interior of the lipid droplet (green).

**Effect of SCP-2 expression on ADRP levels in transfected L-cell fibroblasts.** Since ADRP binds cholesterol and NBD-cholesterol (29) and is significantly co-localized with ADRP, SCP-2’s inhibition of HDL-mediated cholesterol efflux may occur in part by altered level of ADRP. Since the mock-transfected and untransfected cell lines had similar levels of ADRP (not shown), Western blots of cell homogenates and lipid droplets, isolated as described in Methods, were run for control and SCP-2 expressing cells samples (Fig. 9). Although SCP-2 was not detected in lipid droplets, ADRP was enriched 140% in lipid droplets as compared to cell homogenates for both control (Fig. 9, lanes 2 vs 1) and SCP-2 expressing cells (Fig. 9, lanes 4 vs 3). SCP-2 expression reduced the level of ADRP by 60% (+0.36, n=11) in the cell homogenate and by 70% (+0.08, n=11) in the isolated lipid droplets. These data suggest an important role for ADRP as well as SCP-2 in regulating cellular cholesterol levels.

**Effect of SCP-2 expression on scavenger receptor B1 and caveolin levels in transfected L-cells.** It is possible that SCP-2 inhibition of HDL-mediated cholesterol efflux was due to alterations in plasma membrane components of the cholesterol efflux pathway, caveolin and the HDL-receptor (scavenger receptor B1, SRB1). However, Western blotting of cell homogenates Fig. 9B,C) from control and SCP-2 expression clones showed no significant change in the levels of caveolin (panel B) or SRB-1 (panel C).

**Relative contributions of the intracellular compartments to HDL-mediated cholesterol efflux kinetics.** The relative contributions of the low-intensity, diffuse pattern typical of distribution throughout
the cytoplasm, and the high-intensity pattern localized to lipid droplets, to HDL-mediated cholesterol efflux kinetics, were examined over time: 2 min (Fig. 6, panel A), 28 min (panel B), and 58 min (panel C). After 58 min, the lipid droplet NBD-cholesterol intensity (indicated by arrows) was still visible, while that in cytoplasmic areas of the cell was almost depleted. Since cholesterol was retained in the lipid droplets, this suggested either that the rate limiting step of NBD-cholesterol efflux from the cell was efflux from lipid droplets or that the concentration of NBD-cholesterol in the lipid droplets was higher than in the cytoplasm. To resolve these possibilities, cells were imaged and graphically partitioned (see Methods) to separate the lipid droplet contribution from the cytoplasmic component of the cell. Surprisingly, NBD-cholesterol efflux from lipid droplets (Fig. 10B, solid triangles) was initially faster than from cytoplasm (Fig. 10A), but quickly reached an equilibrium level. Multicomponent kinetic analysis of NBD-cholesterol fluorescence decrease confirmed this observation. The data points derived from the lipid droplet compartment (Fig. 10B, solid triangles) best fit ($R^2=0.99$) to a biexponential decay equation while the cytoplasmic compartment (Fig. 10A, solid triangles) best fit to a multi-parameter decay equation ($R^2=0.99$) suggesting multiple pools of NBD-cholesterol were available for efflux from each compartment. In control cells, the half-times of NBD-cholesterol efflux from the lipid droplets were $t_{1/2}^b=0.66$ and $t_{1/2}^d=8.4$ min, significantly smaller ($p<0.005$) than the respective half time from the cytoplasm $t_{1/2}^b=1.9$ and $t_{1/2}^d=14.7$ min (Table 2). Since Pool A, the rapid efflux component, was nearly 200% larger than that of cytoplasm (0.56 vs 0.18), the larger pool size further contributed to the more rapid efflux of NBD-cholesterol from the lipid droplet than from the cytoplasm. Thus, the efflux of NBD-cholesterol from the lipid droplet was not the rate limiting step in HDL-mediated cholesterol efflux from the cell and efflux kinetics from the two components in the whole cells (Table 1) basically reflected those from the two cytoplasmic components rather than the lipid droplets (Table 2). Lower apparent loss of NBD-cholesterol from the lipid droplets vs
cytoplasm was apparently due to a higher concentration of NBD-cholesterol in the lipid droplets than in the cytoplasm, rather than a slower rate of NBD-cholesterol efflux from the lipid droplet.

**Effect of SCP-2 expression on HDL-mediated cholesterol efflux kinetics from cytoplasm and lipid droplets.** In SCP-2 expressing cells, efflux of NBD-cholesterol from lipid droplets (Fig. 10B, open triangles) was initially faster than from cytoplasm, but did not reach an equilibrium level in either cytoplasm or lipid droplets as quickly as observed with control cells. Multicomponent kinetic analysis of NBD-cholesterol fluorescence decrease showed that efflux from the lipid droplet compartment of SCP-2 expressing cells (Fig. 10B, open triangles) again best fit ($R^2=0.99$) to a biexponential decay equation while from the cytoplasmic compartment (Fig. 10A, open triangles) best fit to a multi-parameter decay equation ($R^2=0.99$). Examination of the relative half-times and pool sizes of NBD-cholesterol efflux from cytoplasm and lipid droplets of SCP-2 expression showed: (i) The half-times of NBD-cholesterol efflux from the lipid droplets of SCP-2 expressing cells were shorter (faster) than those from cytoplasm (Table 2), consistent with the NBD-cholesterol efflux from the lipid droplet not being the rate limiting step in HDL-mediated NBD-cholesterol efflux from SCP-2 expressing cells. (ii) In SCP-2 expressing cells the half-times and pool sizes of NBD-cholesterol efflux from the whole cells (Table 1) reflected those of the cytoplasmic compartment (Table 2), rather than those observed for the lipid droplets (Table 2).

SCP-2 expression differentially affected the two half-times of NBD-cholesterol efflux from the cytoplasm as compared to those in the mock transfected control cells. The half-time $t^{b}_{1/2}$ for rapid NBD-cholesterol efflux from the cytoplasmic compartment was significantly shorter (faster) in SCP-2 expressing cells than that observed for control cells, $1.4\pm0.1$ vs $1.9\pm0.1$ min, $p<0.005$ (Table 2). In contrast, the slower half-time $t^{d}_{1/2}$ was 113% longer in SCP-2 expressing cells as compared to control cells ($31.4\pm2.0$ vs $14.7\pm0.8$ min, $p<0.005$) (Table 2). While SCP-2 expression altered the half-times of NBD-cholesterol
efflux from the cytoplasmic compartment, there was little effect of SCP-2 expression on the respective pool sizes. The pool size C associated with the slower half-time $t^{d}_{1/2}$ was 460% greater than that of pool size A associated with the rapid half-time $t^{b}_{1/2}$ (Table 2). However, neither pool size differed significantly from those in mock transfected control cells. In summary, SCP-2 expression enhanced efflux from the rapidly transferring NBD-cholesterol pool in the cytoplasm. Concomitantly, SCP-2 expression inhibited the efflux from the slowly transferring, but much larger, NBD-cholesterol pool in the cytoplasm.

SCP-2 expression also altered NBD-cholesterol efflux from lipid droplets. SCP-2 expression did not alter the rapid half-time of NBD-cholesterol efflux from the lipid droplets, $t^{b}_{1/2}$ = 0.56, but decreased (slowed) the half-time for the slower efflux pool, $t^{d}_{1/2}$ = 18.1 min, by 220% fold (Table 2). SCP-2 expression increased the pool size C (0.65 vs 0.44, $p<0.005$) of the slower effluxing component, while at the same time decreasing pool size A (0.34 vs 0.56, $p<0.005$) of the more rapidly effluxing component.

**DISCUSSION**

Because cholesterol accumulation inside mammalian cells is detrimental to cell survival and contributes significantly to cholesterol deposition in atherosclerosis, cellular cholesterol homeostasis is tightly regulated by balancing of the influx and efflux of cholesterol (38). Influx of exogenous unesterified and esterified cholesterol occurs by both the classic LDL-receptor pathway (39) as well as by the more recently demonstrated HDL-receptor pathway (rev. in (1,29,38,40). In contrast, efflux of cholesterol occurs by the HDL-receptor, but not LDL-receptor, pathway. HDL-mediated cholesterol efflux utilizes unesterified cholesterol, but not cholesterol ester, transferred from intracellular sites to the plasma membrane by both vesicular and cytosolic pathways (rev. in (1,8,38). Despite the presence of the HDL-mediated cholesterol efflux pathway, some tissues retain cholesterol for steroidogenesis.
(adrenal, testis, ovary), for secretion as VLDL or Chylomicrons (liver, intestine), or for secretion as biliary cholesterol (liver). Because of the paucity of knowledge regarding the sources of intracellular cholesterol and the pathways for transfer of intracellular cholesterol to the cell surface, it is not clear whether cholesterol retention is due to intracellular mechanisms opposing HDL-mediated cholesterol. The data herein show fundamental new observations regarding the role of SCP-2 in cholesterol efflux.

First, overexpression of SCP-2 in transfected L-cell fibroblasts inhibited the extent and rate of HDL-mediated $[^3]$H-cholesterol and NBD-cholesterol efflux. SCP-2 expression inhibited the maximal extent of HDL-mediated $[^3]$H-cholesterol and NBD-cholesterol efflux to the same degree at equivalent, nonsaturating ratios of HDL/cell in the culture medium. SCP-2 expression also increased half-times (longer) and decreased cholesterol pool size available for HDL-cholesterol efflux.

Second, SCP-2 expression within the cell opposed the effects of extracellular HDL on cholesterol efflux. The major effect of SCP-2 was to retain cholesterol in the cell while HDL, an excellent cholesterol acceptor, promoted cholesterol efflux. At non-saturating conditions of HDL, the predominant effect was that of inhibition by SCP-2 expression while at higher HDL level maximal efflux (but not above controls) occurred and overcame inhibition by SCP-2 expression. Substantial evidence in vivo and in vitro is consistent with the interpretation that SCP-2 promotes retention of cholesterol in cells (7,22,30,41-50) for utilization in steroidogenesis (27,51), secretion in bile (rev. in (23-25,52,53) or secretion as lipoproteins (22).

Third, confocal imaging of living cells allowed graphic delineation of HDL-mediated cholesterol efflux into at least two components: lipid droplets and outside lipid droplets. Because of its diffuse distribution, the latter was defined as cytoplasmic. Efflux curves of NBD-cholesterol were best fit to bi-exponential and multi-parameter exponential decay equations indicating the presence of a small,
rapid pool with half-time near 1 min and a larger, slow effluxing pool with half time near 13 min. These data were consistent with data reported elsewhere wherein two pools of intracellular cholesterol transfer with similar half-times were observed and attributed to protein-mediated and vesicular cholesterol transfer, respectively (18,20,21,50,50,53). Furthermore, the efflux kinetics from the cytoplasmic compartment largely reflected that of the whole cell. This suggested that transfer of cholesterol through the cytoplasm, rather than desorption from lipid droplets, was the rate limiting step in HDL-mediated cholesterol efflux.

Fourth, SCP-2 expression differentially affected cholesterol efflux from the cytoplasmic compartment: (i) SCP-2 stimulated the smaller, fast pool of protein-mediated cholesterol through the plasma membrane to the HDL acceptor. In the absence of an extracellular cholesterol acceptor, SCP-2 expression in transfected L-cells was shown to stimulate the rapid transfer of plasma membrane derived cholesterol to the endoplasmic reticulum for esterification by ACAT (49). This was consistent with data from transfected hepatoma cells overexpressing SCP-2 wherein rapid cholesterol cycling of plasma membrane and intracellular cholesterol was observed (22). Also pertinent were observations that the fast pool of protein-mediated cholesterol transfer was abolished by treatment of human fibroblasts with antisense DNA to SCP-2 (50). We speculate that this fast pool represents, at least in part, SCP-2 bound NBD-cholesterol as SCP-2 has been shown to bind NBD-cholesterol with high affinity (7,54,55). Parallel studies on the intracellular diffusion of NBD-stearic acid, a ligand which SCP-2 also binds with high affinity, also showed that SCP-2 expression increased the rapid diffusion coefficient of this ligand in the cytoplasm (30,56,57). Lipid binding proteins enhance the intracellular diffusion of poorly water soluble molecules (i.e. lipids) by increasing the desorption membranes and increasing their aqueous solubility (rev. in (58,59). (ii) SCP-2 expression increased the half-time of the
slow vesicular pool of cholesterol transfer. In contrast, treatment of human fibroblasts with antisense DNA to SCP-2 stimulated the slower, vesicular component of cholesterol transfer through the cytoplasm (50). Likewise, HDL cholesterol secretion (mediated by vesicular transport) was decreased in transfected rat hepatoma cells overexpressing SCP-2 (22).

Fifth, cholesterol efflux from the lipid droplet was characterized by both fast and slow components that were distinct from those observed in the cytoplasm. The data presented herein and earlier (29) demonstrated that lipid droplet cholesterol is present in at least three forms: esterified and localized primarily in the core of the lipid droplet; unesterified and localized in the surface lipid of the lipid droplet; unesterified and bound to ADRP, a cholesterol binding protein present in the surface of the lipid droplet. The effect of SCP-2 expression on lipid droplet cholesterol pool size, taken together with the observation that ADRP binds cholesterol, suggests that the slowly effluxing component was the unesterified lipid. Pool sizes in lipid droplets were dramatically affected by SCP-2 expression where the larger cholesterol pool (C) was increased while that of the smaller rapidly transferable cholesterol pool (A) was decreased. The 60% decrease in the rapidly effluxing pool size in the lipid droplet of SCP-2 expressing cells correlated with a 70% decrease in ADRP level in lipid droplets from SCP-2 expressing cells as compared to the control cells. It should be noted that the two pools were not due to ADRP bound vs unbound NBD-cholesterol that was not esterified. While NBD-cholesterol is esterified at least as well as cholesterol in L-cells, CaCO2 cells, and hamster intestinal enterocytes (29,40), after 24 hours less than 8% of the NBD-cholesterol was observed to be esterified in L-cells (29). Since the time frame of the efflux experiments using NBD-cholesterol was less than an hour, little to no esterification of the probe was expected. Thus, the fact that ADRP binds cholesterol with nM K_d (29) taken together with data showing NBD-cholesterol colocalizes with ADRP, suggests a role for ADRP
in determining the lipid droplet cholesterol pool size available for efflux. Since this observation might be correlative, work is in progress to further delineate the role of ADRP in efflux and other intracellular processes in cells overexpressing ADRP.

In summary, NBD-cholesterol proved a useful probe for monitoring the relative effects of SCP-2 expression on HDL-mediated cholesterol efflux. However, this does not imply that the behavior of NBD-cholesterol is identical to that of cholesterol. One important distinction is that NBD-cholesterol efflux achieved equilibrium in 20-40 min, significantly faster than $^{3}$H-cholesterol (20-24 h). Conversely, HDL-mediated uptake of NBD-cholesterol is also faster than that of $^{3}$H-cholesterol in L-cells, hamster intestinal enterocytes, and CaCO2 cells (29,60). While the mechanism by which HDL facilitates the removal of cholesterol from the cell is not yet fully resolved, the more rapid dynamics of NBD-cholesterol efflux/uptake may simply reflect the higher aqueous solubility of NBD-cholesterol vs cholesterol (55). For example, in the sterol efflux pathway HDL interacts with the scavenger receptor (SRB1) at the plasma membrane surface. The existing evidence favors a mechanism whereby plasma membrane cholesterol then desorbs anddiffuses through the aqueous medium to be taken up by the acceptor HDL (rev. in (61). Thus, the higher aqueous solubility of NBD-cholesterol favors an increased rate of HDL-mediated efflux or, in the reverse direction, uptake. Substantial evidence suggests that NBD-cholesterol undergoes metabolism and utilizes the same intracellular trafficking pathways as cholesterol. NBD-cholesterol distributes in L-cells similarly as dehydroergosterol, a naturally occurring fluorescent sterol (29). NBD-cholesterol is esterified similarly as cholesterol and dehydroergosterol in L-cells, intestinal enterocytes, and CACO2 cells (29,60). Finally, NBD-cholesterol trafficks by similar uptake, intracellular, and secretory pathways as was shown herein and previously by others in studies with L-cell fibroblasts and CaCo-2 cells (29,60). However, there are limitations of the use of NBD-
cholesterol which include the inability to incorporate to as high a degree as cholesterol in some membranes (such as the plasma membrane) (62). Notwithstanding, while NBD-cholesterol does not accurately reflect all aspects of cholesterol, the above studies taken together with those presented herein indicate that NBD-cholesterol is an acceptable probe for examining relative effects of factors such as HDL concentration and SCP-2 expression on cholesterol uptake, efflux, and metabolism.

The physiological importance of the findings reported herein is that SCP-2 may participate in maintaining high intracellular pools of cholesterol. This is especially significant to tissues such as liver, intestine, and steroidogenic tissues wherein cholesterol utilization is high and SCP-2 expression greatest (rev. in (7,27). The observation that SCP-2 expression stimulated cholesterol uptake in transfected cells is also consistent with SCP-2 affecting the balance of influx/efflux in favor of influx/retention of cholesterol (rev. in (7,32). The ability of SCP-2 cells to retain cholesterol, along with longer half-times and changing pool size contributed to the overall effect of inhibition of HDL-mediated cholesterol efflux. Interestingly, the data suggest that the SCP-2 protein may have an opposite effect on cellular cholesterol balance as compared to caveolin, a cholesterol binding protein also enriched in tissues where cholesterol utilization is high, i.e. liver and steroidogenic tissues (rev. in (8). Thus, the opposite effects of caveolin and SCP-2 on HDL-mediated cholesterol efflux suggest that these two proteins may participate in fine-tuning cellular cholesterol efflux versus uptake.
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Table 1. Kinetic analysis of HDL-mediated NBD-cholesterol efflux from intact cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Half-times (min⁻¹)</th>
<th>Pool Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_{1/2}^b$</td>
<td>$t_{1/2}^d$</td>
</tr>
<tr>
<td>Control</td>
<td>1.2±0.13</td>
<td>13.1±1.4</td>
</tr>
<tr>
<td>SCP-2</td>
<td>1.1±0.20</td>
<td>26.9±2.2*</td>
</tr>
</tbody>
</table>

(*) Parameters were derived from the following multi-parameter exponential decay equation: when $t < T_0$, $y = (Ae^{bt} + Ce^{dt})$; when $t \geq T_0$, $y = (Ae^{bT_0} + Ce^{dT_0})e^{-(t-T_0)}$ where A and C represent the fraction of available NBD-cholesterol in each rate differentiated pool; b, d, and h are the apparent rate constants; t is time in minutes; and $T_0$ (derived from the fitted curve) is the time at which depletion of the pools increased to completion (18.2±0.6 and 16.8±0.9 min for the control and SCP-2 expressor, respectively). (*) indicates p<0.0025 as compared to control, n=11-14.
Table 2. Effect of SCP-2 expression on HDL-mediated NBD-cholesterol efflux kinetics from cytoplasm and lipid droplets.

<table>
<thead>
<tr>
<th></th>
<th>Half-times (min⁻¹)</th>
<th>Pool Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t₁/₂</td>
<td>t₂/₂</td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9±0.1</td>
<td>14.7±0.8</td>
</tr>
<tr>
<td>SCP-2</td>
<td>1.4±0.1*</td>
<td>31.4±2.0*</td>
</tr>
<tr>
<td><strong>Lipid droplet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.66±0.06</td>
<td>8.4±0.30</td>
</tr>
<tr>
<td>SCP-2</td>
<td>0.56±0.20</td>
<td>18.1±0.76*</td>
</tr>
</tbody>
</table>

( a ) Parameters were derived from the following multi-parameter exponential decay equation: when t < T₀, \( y = (Ae^{-bt} + Ce^{-dt}) \); when t ≥ T₀, \( y = (Ae^{-bT₀} + Ce^{-dT₀})e^{-h(t-T₀)} \) as described in “Methods.” ( b ) Parameters were derived from the biexponential decay equation: \( y = Ae^{-bt} + Ce^{-dt} \). (*) indicates significance P<0.005 as compared to the control, n=6-9 (cytoplasm) and n=11-14 (lipid droplets).
FIGURE LEGENDS

Fig. 1. **Efflux of [³H]cholesterol from L-cell fibroblasts expressing SCP-2.** Mock transfected control and transfected L-cells expressing SCP-2 were labeled with [³H]-cholesterol before incubation with HDL (98 µg protein/ml) to start the efflux process. Aliquots were removed at timed intervals and counted to determine the fraction of [³H]-cholesterol remaining in the cells. Values represent means ± S.E.M. from 3-5 cell wells. If not clearly seen, error bars were obscured by the markers. Symbols indicate the following: solid circles, medium without HDL; solid triangles, mock transfected cells + HDL; open triangles, SCP-2 expressing cells + HDL.

Fig. 2. **[³H]cholesterol efflux dependence on HDL concentrations.** Mock transfected control cells and SCP-2 expressors were labeled with [³H]-cholesterol to show the extent of efflux (panel A) and initial rates (panel B) after 4 hours incubation at various concentrations of HDL (54-250 µg protein/ml). Values are means ± S.E.M. from 3-5 samples and represent the percentage of [³H]-cholesterol released to the medium. (*) indicates significance, P<0.047. (**) indicates significance, P<0.017 as compared to the control. Open and solid bars refer to mock transfected control and SCP-2 expressing cells, respectively.

Fig. 3. **Efflux of NBD-cholesterol from L-cell fibroblasts expressing SCP-2.** Single cell efflux of NBD-cholesterol was examined from mock transfected control (closed triangles) and SCP-2 expressing cells (open triangles). Efflux was initiated by addition of HDL (10 µg protein/ml) to cells prelabeled with NBD-cholesterol. Values from cells in medium only were subtracted to correct for minimal losses due to photobleaching effects. Values represent means ± S.E.M. from 11-14 cells.

Fig. 4. **NBD-cholesterol efflux dependence on HDL concentrations.** The extent of efflux (panel A) and initial rates (panel B) are shown after 10 minutes incubation at several concentrations of
HDL (5-30 µg protein/ml) in control (open bars) and SCP-2 expressing cells (solid bars) prelabeled with NBD cholesterol. Values are means±S.E.M. of cells from several dishes and represent the percentage of NBD-cholesterol released to the medium. (*) indicates significance, P<0.006, as compared to the control.

Fig. 5. Inhibition of efflux from SCP-2 overexpressing cells loaded with [3H]-cholesterol and NBD-cholesterol. The extent of inhibition with cells loaded with [3H]-cholesterol (Open bars) and NBD-cholesterol (Closed bars) was compared. The inhibition was comparable at similar ratios of HDL/cell number over the range of HDL concentrations examined (1.0-2.0 x10^{-4} HDL µg protein/ #cells).

Fig. 6. NBD-cholesterol efflux by HDL in SCP-2 overexpressing cells. Transfected L-cells expressing the SCP-2 protein were labeled with NBD-cholesterol (0.35 µM) and incubated with HDL (10 µg protein/ml). Cells at 2 min (panel A), 28 min (panel B), and 58 min (panel C) during the efflux process were examined with the Bio-Rad MRC-1024 confocal system as described in Methods. Arrows indicate high retention lipid droplets during the time course. Objective 63x.

Fig. 7. Intracellular distribution of NBD-cholesterol and nile red in transfected L-cells. Co-localization patterns of NBD-cholesterol and nile red were shown using pseudo-coloring derived from confocal image acquisition from red- and green-specific PMT channels. A 24-bit RGB image was created from the red plus green plus blue (null) channels. Red and green are additive in RGB color space yielding yellow-orange. L-cells expressing the SCP-2 protein stained with NBD-cholesterol and nile red were combined to yield yellow-orange (panel A) where co-localization occurred. Superimposition of the probes was graphically demonstrated in a pixel fluorogram (panel B) where co-localization of NBD-cholesterol (green) and nile red (red) resulted in yellow to orange points falling along the diagonal line in
the fluorogram. The correlation coefficients corresponding to red and green were proportional to the
degree of fluorescence probe co-localizing in each component of the image relative to the total
fluorescence and indicated the extent of overlap between the probes. The cells were examined using the
Bio-Rad MRC-1024 confocal system. Objective 63x.

Fig. 8. **Double-label immunofluorescence with ADRP and nile red or NBD-cholesterol in
transfected L-cells expressing SCP-2.** Cells were simultaneously labeled for nile red (panel A) and
ADRP with FITC-conjugated goat anti-rabbit IgG (panel B) or ADRP with Texas Red-conjugated goat
anti-rabbit IgG (panel D) and NBD-cholesterol (panel E). Co-localization (panels C and F) were shown
using pseudo-coloring resulting in yellow to orange where superimposition occurred. Inset figures were
lipid droplets located within the larger frame.

Fig. 9. **Western blot analysis of SCP-2 expression clones showing expression levels of
ADRP, caveolin, and SRB-1.** Samples from control and SCP-2 expressing cells were run on 12%
tricine gels. Panel A: lane 1, untransfected L-cell homogenate; lane 2, lipid droplet fraction isolated from
L-cells; lane 3, SCP-2 cell homogenate; and lane 4, lipid droplet fraction isolated from SCP-2 expressing
cells. Panel B: lane 1, untransfected L-cell homogenate; and lane 2, SCP-2 cell homogenate. Panel C:
lane 1, untransfected L-cell homogenate; and lane 2, SCP-2 cell homogenate. The blots were probed
with polyclonal rabbit antibodies against ADRP (Panel A), caveolin (Panel B), or SRB-1 (Panel C) as
described in Methods.

Fig. 10. **NBD-cholesterol efflux from the cytoplasmic and lipid droplet compartments.**
Single cell efflux of NBD-cholesterol from the lipid droplet and the cytoplasmic compartments of the
cell was shown plotted against time. Cells were loaded with 0.35 µM NBD-cholesterol and incubated
with 10 µg protein/ml of medium to start the efflux process. Cells were imaged using the Bio-Rad MRC-
1024 confocal system and analyzed by Metamorph software as described in Methods. Symbols refer to mock transfected control (solid triangles) and SCP-2 expressing L-cells (open triangles).
Fig. 4
Figure 5

![Graph showing % Inhibition vs. μg HDL / # of cells (x 10^-4)]

- Open bars represent [³H]cholesterol
- Black bars represent NBDcholesterol

% Inhibition

μg HDL / # of cells (x 10^-4)