2007

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Selective Cholesterol Dynamics between Lipoproteins and Caveolae/Lipid Rafts†

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Received April 12, 2007; Revised Manuscript Received October 1, 2007

ABSTRACT: Although low-density lipoprotein (LDL) receptor-mediated cholesterol uptake through clathrin-coated pits is now well understood, the molecular details and organizing principles for selective cholesterol uptake/efflux (reverse cholesterol transport, RCT) from peripheral cells remain to be resolved. It is not yet completely clear whether RCT between serum lipoproteins and the plasma membrane occurs primarily through lipid rafts/caveolae or from non-raft domains. To begin to address these issues, lipid raft/caveolae-, caveolae-, and non-raft-enriched fractions were resolved from purified plasma membranes isolated from L-cell fibroblasts and MDCK cells by detergent-free affinity chromatography and compared with detergent-resistant membranes isolated from the same cells. Fluorescent sterol exchange assays between lipoproteins (VLDL, LDL, HDL, apoA1) and these enriched domains provided new insights into supporting the role of lipid rafts/caveolae and caveolae in plasma membrane/lipoprotein cholesterol dynamics: (i) lipids known to be translocated through caveolae were detected (cholesteryl ester, triacylglycerol) and/or enriched (cholesterol, phospholipid) in lipid raft/caveolae fractions; (ii) lipoprotein-mediated sterol uptake/efflux from lipid rafts/caveolae and caveolae was rapid and lipoprotein specific, whereas that from non-rafts was very slow and independent of lipoprotein class; and (iii) the rate and lipoprotein specificity of sterol efflux from lipid rafts/caveolae or caveolae to lipoprotein acceptors *in vitro* was slower and differed in specificity from that in intact cells—consistent with intracellular factors contributing significantly to cholesterol dynamics between the plasma membrane and lipoproteins.

Cellular cholesterol homeostasis involves a balance of cholesterol uptake (dietary and *de novo* synthesized), intracellular storage, and efflux. Although cholesterol is an essential component of membranes and a precursor of steroids, excess cholesterol is associated with diabetic complications, atherosclerosis, cardiovascular disease, and other diseases. Since intracellular storage of cholesterol in esterified form is limited in peripheral tissues, mammals have evolved a complex mechanism termed reverse cholesterol transport (RCT)[1] for removal (efflux) of excess cellular cholesterol from peripheral cells and transport via serum lipoproteins to liver hepatocytes, followed by uptake, oxidation to bile acids, and excretion into bile (reviewed in refs *1*−*4*). Cholesterol efflux from peripheral cells involves three distinct classes of proteins:

First, intracellular cholesterol must be transported from intracellular sites to the plasma membrane. This involves both protein-mediated and vesicular processes (reviewed in refs *3* and *4*). In protein-mediated molecular cholesterol transfer, cholesterol synthesized in endoplasmic reticulum translocates to the plasma membrane as a complex with one of two proteins in the cytosol: (i) sterol carrier protein-2 (SCP-2), which directly interacts with caveolin-1 localized in the cytofacial leaflet of the plasma membrane (*5*, *6*); or (ii) caveolin-1/chaperones (reviewed in refs *3*, *4*, *6*, and *7*). In the vesicular pathway, cholesterol traffics to the plasma membrane from Golgi as caveolar vesicles, a process inhibited by SCP-2 overexpression (reviewed in ref *3*, *4*, and *6*). SCP-2's inhibition of vesicular trafficking is consistent with its high affinity for long-chain fatty acyl CoAs (*8*) and phosphatidylinositol (*9*), both of which regulate vesicular fusion and budding from the Golgi (reviewed in refs *10*−*12*). The protein-mediated molecular cholesterol transport and vesicular transport differ in that the former is not energy driven.

Second, cholesterol arriving at the plasma membrane is translocated across the plasma membrane for efflux to lipoproteins by processes that are as yet not clearly resolved. The transbilayer distribution of cholesterol in the plasma membrane from peripheral cells favors enrichment in the cytofacial leaflet as indicated by selective quenching of fluorescent sterols (*13*−*17*) and photo-cross-linking (*18*). Although spontaneous transbilayer migration of cholesterol in model membranes is relatively slow (reviewed in ref *19*),

† This work was supported in part by the USPHS, National Institutes of Health Grants GM31651 (F.S. and A.B.K.), GM63236 (J.M.B.), and DK70965 (B.P.A.), and a Mentored Quantitative Research Career Development Award (K25) DK62812 (A.M.G.).

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[1] Abbreviations: Caveolae/rafts, non-detergent affinity-purified lipid rafts; non-rafts, non-detergent affinity-purified non-rafts; DRM, detergent-resistant membranes; DSM, detergent-soluble membranes; ConA, concanavalin A; SCP-2, sterol carrier protein-2; DHE, dehydroergosterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo A1, apolipoprotein A1; VLDL, very low density lipoprotein; BHT, butylated hydroxytoluene; BLT-1, 2-hexyl-1-cyclopentanone thiosemicarbazone; PBS, phosphate-buffered saline; RTC, reverse cholesterol transport.

10.1021/bi700690s CCC: $37.00
Published on Web 11/09/2007

that in plasma membranes is fast (seconds to minutes) (*16*, *17*, *19*). Several candidate proteins have been postulated to facilitate cholesterol transbilayer migration from the cytofacial to the exofacial leaflet for subsequent efflux: (i) Caveolin-1 is localized at the cytofacial leaflet of the plasma membrane, directly binds cholesterol *in vitro* (*20*, *21*), and is cross-linked by photoactivatable sterol in membranes (*22*, *23*). However, there is as yet no direct evidence that this interaction is energy-driven and/or directly facilitates cholesterol translocation/flipping across the plasma membrane for efflux (reviewed in refs *1*, *3*, and *4*). (ii) Although there is no evidence demonstrating direct binding of cholesterol by P-glycoprotein (P-gp, also known as MDR, multidrug resistance protein), P-gp has been reported to enhance cholesterol transbilayer migration to the exofacial leaflet for efflux (reviewed in refs *24* and *25*). Other reports indicate that P-gp also enhances cholesterol desorption from the plasma membrane for transport to the endoplasmic reticulum and esterification (reviewed in ref *26*) and that P-gp-mediated lipid translocation is energy driven (reviewed in refs *2* and *27*). Thus, P-gp may mediate bidirectional energy-driven cholesterol transport at the plasma membrane. (iii) ABCA1, an energy-driven ATP-binding cassette transporter, has also been suggested to elicit unidirectional cholesterol transport to the exofacial leaflet of the plasma membrane of extrahepatic tissues (reviewed in refs *2*, *27*, and *28*). However, photo-cross-linking studies indicate that ABCA1 does not directly interact with cholesterol (*29*), and it has been suggested that ABCA1 acts indirectly through enhancing phospholipid desorption, which in turn facilitates transbilayer cholesterol transport/desorption (reviewed in refs *2* and *28*). Taken together, these data indicate that transbilayer cholesterol transport mediated by P-gp and ABCA1 is energy-driven (reviewed in refs *2* and *27*).

Third, two distinct lipoprotein pathways serve as acceptors of cholesterol desorbed from the cell surface. First, apolipoprotein A1 (apoA1) serves as the acceptor of ABCA1-mediated phosphatidylcholine efflux, which facilitates cholesterol desorption to form precursor HDL in serum (reviewed in refs *2* and *28*). Second, serum HDL tethered to scavenger receptor class B1 (SR-B1) facilitates bidirectional cholesterol transfer from the plasma membrane (*2*, *30*).

Whereas the organizing principle of low-density lipoprotein (LDL)-mediated cholesterol uptake through clathrin-coated pits is now well understood, a similar concept for HDL-mediated cholesterol efflux is only recently emerging. There is extensive biochemical evidence from cell fractionation studies suggesting the presence of cholesterol-rich domains ("lipid rafts") in plasma membranes, yet only recently has the existence of sterol-rich domains been demonstrated in plasma membranes of living cells (reviewed in ref *3*). Real-time multiphoton imaging of dehydroergosterol (DHE), a naturally occurring fluorescent sterol, demonstrated for the first time that in plasma membranes of living cells sterol is not randomly distributed but instead is localized in sterol-rich and -poor regions of the plasma membrane (*3*, *31*−*33*). Thus, it would appear that cholesterol-rich rafts do exist in living cells and are not just an artifact induced by subcellular fractionation protocols or nonphysiological probe molecules. Most important, biochemical fractionation studies of cells and tissues to isolate these sterol-rich/lipid rafts show that proteins involved in RCT are localized in lipid rafts/caveolae, i.e., SR-B1 and caveolin-1 (reviewed in refs *1*−*4*, *30*, *34*, and *35*). ABCA1 (*36*−*40*) and P-gp (*25*, *41*−*48*) are also found in lipid rafts/caveolae or possibly in domains intermediate between lipid rafts/caveolae and non-rafts. The above findings suggest that cholesterol-rich lipid rafts/caveolae may provide a nexus for mediating transfer of cholesterol between the plasma membrane and ABCA1-bound apoA1 or SR-B1-bound HDL. However, to date there have been no biochemical studies examining sterol dynamics between serum lipoproteins (apoA1, HDL, LDL, VLDL) and purified lipid rafts/caveolae versus non-rafts. To address this question, lipid rafts/caveolae, caveolae, and non-rafts were isolated from peripheral cell types (fibroblasts, MDCK cells) according to two classic methods: First, detergent-resistant membranes (DRM) and detergent-soluble membranes (DSM) were isolated to allow comparison of the results with the earlier biochemical characterizations of lipid rafts/caveolae. Second, caveolae-enriched rafts and non-rafts were isolated by an affinity chromatography method not utilizing detergents or high-pH carbonate buffer (*34*, *49*−*52*). These fractions were used to determine if (i) caveolae-enriched domains were better donors of cholesterol to lipoproteins (HDL, VLDL, LDL, apoA1) than non-rafts and (ii) sterol dynamics between lipoproteins (HDL, VLDL, LDL, apoA1) and caveolae-enriched domains or non-rafts was directional, i.e., reflecting that observed with intact cells.

EXPERIMENTAL PROCEDURES

Materials. High-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein A1 (apoA1), and very low-density lipoprotein (VLDL) were purchased from EMD Biosciences (San Diego, CA). BLT-1 was purchased from Chem Bridge (San Diego, CA). Probucol was purchased from VWR International (West Chester, PA). The following antibodies were purchased from the indicated commercial sources: rabbit polyclonal anti-sera to caveolin-1 and flotillin-1 (BD Transduction Laboratories, Palo Alto, CA); SR-B1 and ABCA1 (Novus Biologicals, Littleton, CO); cholera toxin B (Sigma, St. Louis, MO); P-gp (Santa Cruz Biotech, Inc., Santa Cruz, CA); calnexin (Stressgen Biotech, Victoria, BC, Canada); tubulin (Upstate, Lake Placid, NY); mouse monoclonal antibodies to Na^+,K^+-ATPase (Affinity BioReagents, Inc., Golden, CO); protein disulfide isomerase (PDI) (MBL International Corp., Woburn, MA); clathrin (heavy chain, BD Transduction Laboratories). Cholesterol (99+% pure) and egg PC (99+% pure) were purchased from Steraloids (Wilmington, NH). EDTA, tris-base, sucrose, phosphate-buffered saline (PBS), PMS-F, cholera toxin B, and Percoll were obtained from Sigma Chemical (St. Louis, MO). All solutions in which water was used contained Milli-Q/deionized water.

Dehydroergosterol Synthesis. Dehydroergosterol (DHE) occurs naturally in yeast and Red Sea sponge. However, DHE utilized herein was chemically synthesized from ergosterol (99+% pure) by use of a method developed in our laboratory (*31*, *53*). This method yielded DHE with a high degree of purity (99+%), as ascertained by high-performance liquid chromatography (*34*, *49*).

Cell Culture. Murine L-cells were grown to confluency at 37 °C and 5% CO_2 in Higuchi medium (*54*), supplemented

with fetal bovine serum (10%, Hyclone, Logan, UT), as described earlier (*34*). Madin Darby Canine Kidney (MDCK; American Type Culture Collection) cells were grown as described earlier (*52*) in high-glucose (4.5 g/L) Dulbecco's Modification of Eagle's medium (D-MEM) (Cellgrow/Mediatech, Herndon, VA) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), sodium bicarbonate (43.9 mM), fetal bovine serum (5%), Serum Supreme (5%), penicillin (100 units/L), streptomycin (100 μg/L), and Fungizone (μg/L). Cells stocks, maintained in 175 cm^2 flasks (Sarstedt, Newton, NC), were expanded into 500 cm^2 trays (Corning, NY) and grown to 85% confluency for membrane isolations.

Isolation of Plasma Membrane Microdomains. Plasma membranes were isolated from L-cell fibroblasts and resolved into lipid raft/caveolae- and non-raft-enriched fractions by a detergent-free, high-pH carbonate buffer-free, concanavalin-1 (ConA) affinity chromatography method as described earlier (*34*). Detergent-resistant membranes (DRM) and detergent-soluble membranes (DSM) were isolated from MDCK cells as described previously (*52*). Plasma membranes (PM) from MDCK cells, isolated without the use of detergents, were resolved into caveolae- and non-raft-enriched membrane fractions according to a modified concanavalin A affinity chromatography method exactly as described in ref *52*.

Western Blot Analysis of Lipid Rafts/Caveolae and Non-Raft Fractions. To determine the relative purity of the lipid rafts/caveolae-, caveolae-, and non-raft-enriched fractions eluted from the ConA affinity columns, aliquots of each fraction derived from L-cell fibroblasts or MDCK cells were separated by SDS-PAGE and analyzed by Western blotting to detect relative expression levels of caveolin-1, SR-B1, ABCA1, P-gp, flotillin, clathrin, Na^+,K^+-ATPase, calnexin, and PDI. Briefly, aliquots of each fraction were separated by SDS-PAGE and analyzed as follows: Samples (10 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, 0.05% Tween-20) for 1 h at room temperature and then washed two times with TBST. The blots were incubated overnight at room temperature with the respective primary antibodies in 1% gelatin in TBST. After several washes with TBST, the blots were incubated for 2 h at room temperature with the appropriate secondary antibody (alkaline phosphatase conjugates) in 1% gelatin TBST. The blot was washed three times with TBST, and bands of interest were visualized by development with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets according to the manufacturer's protocol. Images of each blot were acquired using a single-chip charge-coupled device (CCD) video camera and a computer workstation (IS-500 system from Alpha Innotech, San Leandro, CA). Densitometric analysis of image files was then performed (mean 8-bit gray scale density) using Scion Image (Scion Corp., Frederick, MD) to obtain relative levels of respective protein detected in each fraction expressed as integrated density values. The fold increase was calculated on the basis of expression levels in the homogenate fraction.

Lipid Extraction and Analysis. Lipids from L-cell lipid rafts/caveolae and non-raft fractions were extracted, resolved into individual classes (cholesterol, phospholipid, cholesteryl ester, triacylglycerol, fatty acid), and quantitated as described earlier (*34*). Ganglioside GM1 levels were determined by a dot-blot technique as described elsewhere (*34*). Protein concentration was determined according to the method of Bradford from the dried protein extract residue digested overnight in 0.2 M KOH (*55*). Amounts of individual lipid classes were expressed as nanomoles of lipid per milligram of protein. All glassware used for lipid analyses was washed with sulfuric acid−chromate before use.

Labeling of Lipid Rafts/Caveolae, Caveolae, Non-Rafts, and Lipoproteins with DHE. DHE-labeled donor and unlabeled acceptor membrane fractions were prepared from MDCK or L-cell fibroblasts exactly as described in refs *13* and *52*. DHE-labeled donor lipoproteins (VLDL, LDL, HDL, apoA1) were prepared as described by Yeagle et al. (*56*) and later modified by Smutzer (*57*). With regard to the latter, the DHE, cholesterol, and egg PC at a molar ratio of 1:2:5 were mixed in chloroform. The solution was dried under N_2 and subsequently under vacuum, at room temperature, to remove any residual solvent. Next, a buffer containing 150 mM NaCl, 10 nM histidine, and 1 mM EDTA at pH 7.4 was added to a final concentration of 5 mg of phospholipid/mL, and multilammelar liposomes were formed by vortexing at room temperature. The liposomes were then incubated with the lipoproteins at a ratio of 24 mg of lipoprotein to 5 mg of liposome lipid (per milliliter of solution) and incubated in the dark for 1 h at room temperature under N_2 and gentle stirring. The samples were then centrifuged for 20 min at 40 000 rpm in an SW41Ti rotor in a Beckman XL90 Ultracentrifuge (Beckman Instruments, Fullerton, CA) to remove multilamellar vesicles as described (*57*).

Measurement of DHE Steady-State Fluorescence Polarization. All measurements of steady-state fluorescence polarization were made with a PC1 spectrofluorometer with photon-counting electronics (ISS Instruments, Inc., Champaign, IL) exactly as described earlier (*49*). Small amounts of residual light scatter contributing to polarization measurements were corrected by converting polarization to anisotropy according to the relationship $r = 2P/(3 - P)$ and subtracting the residual fluorescence anisotropy from all experimental data. To avoid potential inner filter artifacts, absorbance of sample solutions at the excitation wavelengths were kept below 0.15. Polarization/anisotropy measurements comprise both static (limiting anisotropy) and dynamic (rotational rate) parameters. Earlier studies showed that agents which fluidize membranes (ethanol, anesthetics) primarily alter the static (i.e., limiting anisotropy), but not dynamic (rotational), components of polarization/anisotropy measurements (*58*−*63*). Since limiting anisotropy represents a measure of the cone angle of fluorescence probe rotation in lipid bilayers, limiting anisotropy and, consequently, polarization of DHE measured at very low, non-self-quenching DHE concentrations represent a useful relative measure of sterol mobility in the purified membrane domain or lipoprotein.

Measurement of Sterol Exchange: DHE Release from Self-Quenching Fluorescence Polarization Assay. Sterol exchange between the isolated membrane fractions and lipoproteins was determined by using a fluorescent sterol (DHE) exchange assay previously developed by our laboratory (*34*, *49*, *52*, *64*). DHE was used as a probe for cholesterol dynamics because it (i) is a natural product, (ii) is fluorescent, (iii) closely resembles cholesterol in structure, (iv) has exchange

kinetics very similar to those of cholesterol in model membranes and biological membranes, (v) is taken up by cultured cells such that >80% of endogenous sterol is replaced by DHE without altering membrane lipid composition or sterol-sensitive enzymes, (vi) co-distributes with cholesterol in model and biological membranes, (vii) does not alter the activity of sterol-sensitive membrane proteins, (viii) is nontoxic to cultured cells or animals, and (ix) is readily incorporated into lipoproteins *in vivo* and *in vitro* (reviewed in refs *13*, *19*, *31*, and *64*−*73*). The molecular basis for the fluorescent sterol (DHE) exchange assay, described in detail in an earlier publication (*64*), was recently reviewed (*74*). Briefly, the underlying basis of the DHE exchange assay is that fluorescence self-quenching of DHE occurs in the donor membrane or lipoprotein, which contain high levels of DHE, thereby resulting in low DHE fluorescence polarization values. Upon addition of a 10-fold excess (by sterol) of acceptor membrane or lipoprotein, the donor exchanges DHE one-for-one with cholesterol in the acceptor, thereby resulting in release from self-quenching of DHE and increased DHE polarization. To obtain baseline DHE fluorescence polarization of the donor (1.5 μg of protein/mL in 2 mL of 10 mM PIPES buffer), polarization was measured for 20 min to ensure a stable signal baseline and to obtain an initial value of DHE polarization. This was followed by addition of a 10-fold excess of acceptor without DHE (15 μg/mL in the 2 mL sample). The DHE polarization was subsequently recorded during 20 s intervals for 3−4 h to monitor sterol transfer between membranes and lipoproteins.

Inhibitors of ABCA1- and SR-B1-Mediated Sterol Dynamics between ApoA1 and MDCK Caveolae. To resolve the relative contributions of ABCA1 and SR-B1 to sterol dynamics between caveolae and apoA1, DHE exchanges between MDCK caveolae donors and apoA1 acceptor were repeated in the presence of BLT-1 to inhibit SR-B1-mediated sterol efflux (*75*), Probucol to inhibit ABCA1-mediated sterol efflux (*75*), and anti-ABCA1 to inhibit ABCA1-mediated sterol efflux with anti-tubulin as a control. In brief, before each exchange stock solutions of 10 mM Probucol or BLT-1 were made in DMSO. Prior to initiating exchange, the MDCK donor caveolae were incubated with Probucol (20 μM), BLT-1 (10 μM), anti-ABCA1 (20 μM), or anti-tubulin (20 μM) in the dark for 30 min as described earlier (*75*). A baseline polarization of the donor with these treatments was recorded for 30 min, followed by addition of the acceptor apoA1. Data were recorded for 4 h. For each treatment, a total of $n = 2-3$ runs was used for calculations.

Standard Curves for the Sterol Exchange Assay. Standard curves for sterol exchange were prepared basically as described earlier by our laboratory (*34*, *49*, *52*, *64*). The standard curve that calculates the fraction of DHE remaining in the donor during an exchange is a polynomial equation involving polarization P of the exchange in the form of

$$P = \Sigma\, b_n X_d^{\,n} \tag{1}$$

where X_d is the mole fraction of DHE left in the donor. For sterol exchange between donor and acceptor, a polynomial with two terms yielded a fit with $r^2 = 0.9999$, that is, eq 2

$$P(x) = b_0 + b_1 X_d + b_2 X_d^{\,2} \tag{2}$$

where $b_0 = 0.3261$, $b_1 = 0.016$, and $b_2 = -0.144$.

Calculation of the Initial Rate of Sterol Transfer. The initial rate of DHE exchange between donor and acceptor was estimated from the first 10 min of exchange data by using the standard curve described above in eq 2 (*76*). In essence, eq 2 is the definitive relationship that describes the exchange between donor and acceptor. Taking the time derivative of eq 2 yields

$$(dP/dt) = b_1(dX_d/dt) + 2b_2X_d(dX_d/dt) \tag{3}$$

As $t \rightarrow 0$, $X_d \rightarrow 1$ (i.e., initial rate criteria), and by rearranging eq 3, the following expression is obtained:

$$[1/(b_1 + 2b_2)](dP/dt)_{t\rightarrow 0} = (dX_d/dt)_{t\rightarrow 0} \tag{4}$$

To obtain the molar transfer rate of DHE (d[DHE]/dt) from donor to acceptor, dX_d/dt was transformed into d[DHE]/dt by factoring in the initial donor protein concentration (1.5 μg of protein/mL), the total sterol/protein concentration in the donor (1011.02 pmol of total sterol/μg of protein), and the values of b_1 (0.016) and b_2 (−0.144). Combining this information with eq 4 yielded eq 5:

$$(d[DHE]/dt)_{t\rightarrow 0} = -2253 \text{ pmol} \times (dP/dt)_{t\rightarrow 0} \tag{5}$$

The initial rate of DHE transfer was directly estimated by substituting the initial measured rate of fluorescence polarization change per unit time (i.e., minutes) for $(dP/dt)_{t\rightarrow 0}$.

Calculation of the Kinetic Parameters of Sterol Exchange. The kinetic parameters of exchange between donor/acceptor pairs were determined by use of the standard curve equation, i.e., eq 2, and the equation for a one-exponential exchange

$$X = f_1 \exp(-kt) + f_2 \tag{6}$$

where f_1 and f_2 are the exchangeable and nonexchangeable fractions, respectively, of the sterol in the exchange assay and k is the rate constant of the exchange. The expression for X in eq 6 was substituted into eq 2 to obtain the expression describing the exchange:

$$P(x) = b_0 + b_1[f_1 \exp(-kt) + f_2] + b_2[f_1 \exp(-kt) + f_2]^2 \tag{7}$$

The exchange curves were fit to eq 8 with r^2 values varying from 0.97 to 0.99. The halftime, $t_{1/2}$, of the exchanges was defined by the following equation:

$$t_{1/2} = (\ln 2)/k \tag{8}$$

Data and Statistical Analyses. All curve-fitting and data analyses herein were performed by use of SigmaPlot (Jandel Scientific, San Rafael, CA) scientific data analysis and graphing software.

RESULTS

Lipid Raft/Caveolae, Caveolae, and Non-Raft Distribution of Proteins and Lipids Transported by Lipoproteins Interacting with the RCT Pathway. As indicated under Experimental Procedures, lipid raft/caveolae- and non-raft-enriched fractions were simultaneously isolated by ConA affinity chromatography of purified plasma membranes from L-cell fibroblasts (*34*), whereas caveolae- and non-raft-enriched

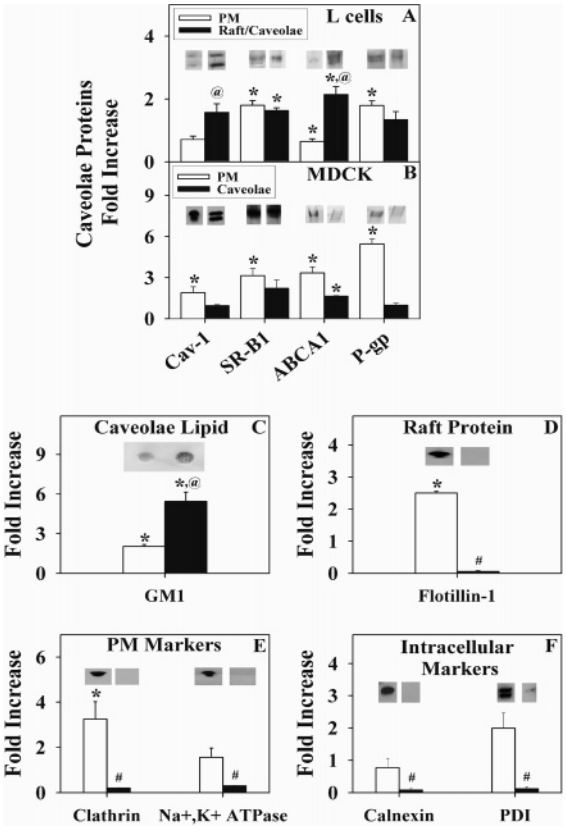

FIGURE 1: Quantitative analysis of specific lipid raft and non-raft markers in membranes isolated from L-cell fibroblasts and MDCK cells. Caveolae protein markers [caveolin-1 (cav-1), SR-B1, ABCA1, and P-gp] in membranes isolated from L cells (panel A) and MDCK cells (panel B) were quantified as described under Experimental Procedures. Fold increases of caveolae lipid marker GM1 (panel C), non-caveolar raft protein marker flotillin-1 (panel D), non-raft PM markers clathrin and Na^+,K^+-ATPase (panel E), and intracellular markers calnexin and PDI (panel F) were determined for MDCK-derived membranes. Open bar indicates plasma membrane (PM); solid bar indicates caveolae-enriched fractions. Western blots are shown as insets. Values represent the mean ± SE ($n = 3-7$). ∗ indicates $P < 0.05$ fold increase vs homogenate; # indicates $P < 0.05$ fold decrease vs homogenate; @ indicates $P < 0.05$ fold increase vs PM.

plasma membrane fractions were isolated by concanavalin A affinity chromatography modified as described in ref *52*. To confirm the purity of the ConA adherent and non-adherent fractions, Western blot and lipid analyses were performed to determine the presence of select protein and lipid markers.

The ConA adherent fraction isolated from L-cell plasma membranes was enriched in the caveolae markers caveolin-1 (Figure 1A) and SR-B1 (Figure 1A), the lipid raft marker flotillin (not shown), and other proteins detected in lipid rafts/caveolae including ABCA1 and to a lesser extent P-gp (Figure 1A). Concomitantly, the ConA adherent fraction was deficient in the non-raft marker to Na^+,K^+-ATPase, which was enriched in the ConA non-adherent fraction (not shown). These data confirmed earlier findings indicating that the ConA adherent fraction isolated from L-cells was enriched in lipid rafts/caveolae (*34*). To further confirm that the ConA non-adherent and adherent fractions from L-cell plasma membranes represented non-raft- and lipid raft-enriched fractions, lipid analyses for marker lipids was performed as described under Experimental Procedures. Lipid rafts from peripheral cells are known to be enriched in total lipids, cholesterol, and phospholipids (reviewed in ref *52*). Lipid extraction and analysis revealed that the lipid raft/caveolae fraction was enriched in total lipid, cholesterol, and phospholipid as compared to non-rafts—3.5-, 3-, and 6.3-fold, respectively (Table 1). In addition, the lipid rafts/caveolae contained small amounts of cholesteryl ester and triacylglycerol (Table 1). Triacylglycerol was enriched 2.1-fold in lipid rafts/caveolae as compared to non-rafts, whereas the opposite was observed for cholesteryl ester (Table 1). Cholesteryl ester and triacylglycerol represented only 0.4 and 1.5% of total lipid, respectively, in lipid rafts/caveolae, but significantly more (4.7 and 2.5% of total lipid, respectively) in non-rafts (Table 1). Although cholesteryl ester and triacylglycerol are known to be taken up through lipid rafts/caveolae from SR-B1-bound lipoproteins, neither apoB nor apoA1 was detected in the lipid rafts/caveolae or non-raft fractions (not shown), indicating that the presence of small amounts of cholesteryl ester and triacylglycerol in lipid rafts/caveolae or non-rafts was not due to contaminant adherent lipoproteins. Thus, lipid rafts/caveolae were lipid rich, cholesterol-rich, and phospholipid-rich and contained small amounts of lipids (cholesteryl ester, triacylglycerol).

Table 1: Lipid Distribution in L-Cell Lipid Rafts/Caveolae and Non-Rafts[a]

lipid	non-rafts (nmol/mg of protein)	lipid rafts/caveolae (nmol/mg of protein)
cholesterol	345 ± 86	1016 ± 75*
cholesteryl ester	43 ± 13	13 ± 3*
triacylglycerol	23 ± 6	48 ± 5*
phospholipid	266 ± 24	1681 ± 152*
total lipid	904 ± 104	3117 ± 152*

[a] Lipids were extracted, resolved into individual classes by thin layer chromatography, and quantitated as described under Experimental Procedures. Total lipid represents the sum of cholesterol, cholesteryl ester, triacylglycerol, phospholipid, and small amounts of other lipids (e.g., fatty acids, sphingolipids, etc.) not shown. Values represent the mean ± SD ($n = 3-7$). * indicates $P < 0.05$ vs non-rafts.

The ConA adherent fraction isolated by the detergent-free method from MDCK cell plasma membranes was also rich in a caveolae protein marker [SR-B1 (Figure 1B)] and other proteins such as ABCA1 (Figure 1B) and less so P-gp (Figure 1B)—confirming earlier findings (*52*). The 3-fold enrichment of GM1 (Figure 1C), a caveolae lipid marker, between the PM and caveolae fraction closely resembles the approximately 4-fold PM to caveolae enrichment observed with detergent-free cryoEM methodologies (*77*). However, the ConA adherent fraction isolated from MDCK cells was essentially deficient in the lipid raft marker protein flotillin-1 (Figure 1D). Flotillin-1 is a known lipid raft, non-caveolar marker (*78*). In contrast, the non-raft protein markers clathrin and Na^+,K^+-ATPase were absent from the ConA adherent fraction (Figure 1E) but enriched in the non-adherent fraction (not shown). The ConA adherent fraction was also devoid of the intracellular markers calnexin and PDI (Figure 1F). These data together with earlier findings (*52*) indicated that the ConA adherent fraction isolated from MDCK cell plasma membranes was highly enriched in caveolae, but not lipid rafts.

Thus, on the basis of protein as well as lipid markers and consistent with earlier findings (*34*, *52*), in subsequent sections the ConA adherent fraction isolated from L-cells

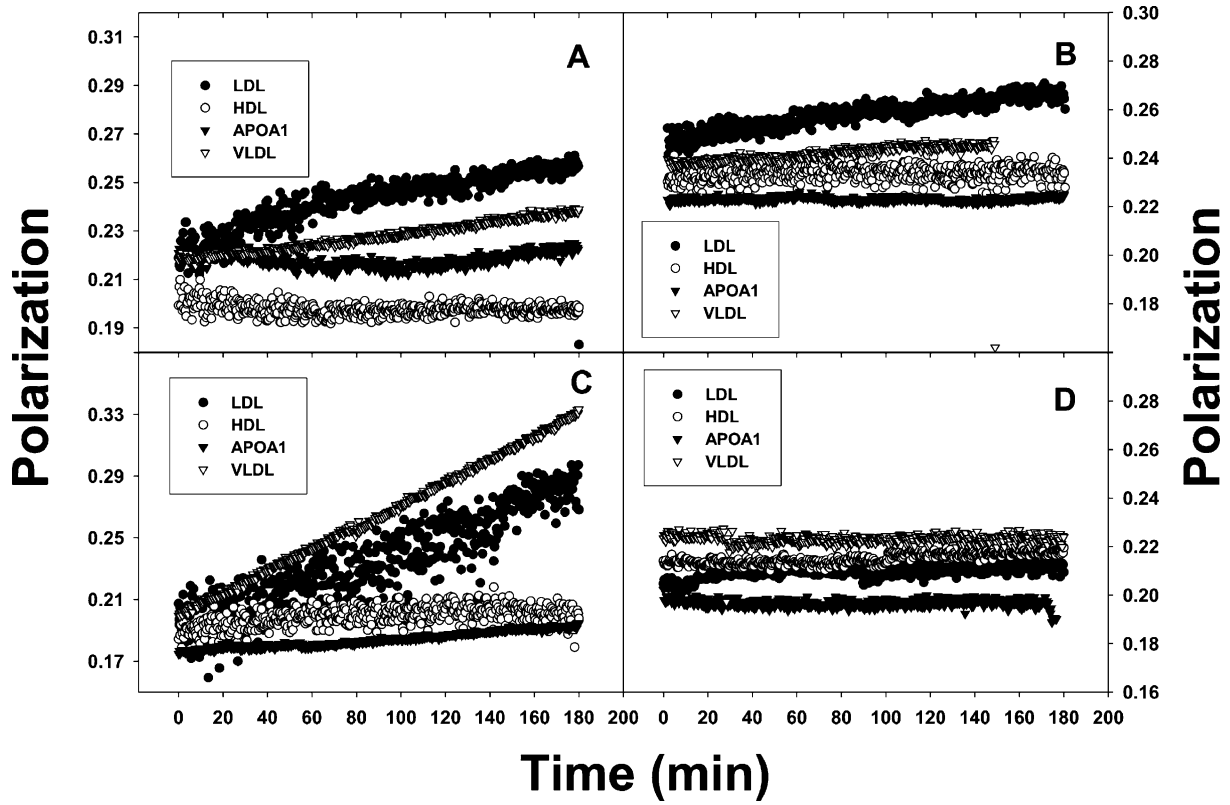

FIGURE 2: Sterol exchange between L-cell and MDCK-derived plasma membrane (caveolae-enriched and non-raft) donors to lipoprotein acceptors. DHE exchanges between L-cell lipid rafts/caveolae donors (panel A), L-cell non-raft donors (panel B), MDCK caveolae donors (panel C), and MDCK non-raft donors (panel D) to lipoprotein acceptors were measured by monitoring DHE polarization as described under Experimental Procedures: ●, exchange to LDL; ○, sterol exchange to HDL; ▼, exchange to apoA1; ▽, sterol exchange to VLDL. All curves show sterol exchange after addition of 10-fold excess (by sterol) acceptor lipoprotein.

will be referred to as lipid rafts/caveolae, whereas that isolated from MDCK cells will be referred to as caveolae.

Sterol Exchange between L-Cell Plasma Membrane Lipid Raft/Caveolae or Non-Raft Donors and Lipoprotein Acceptors. The molecular basis for the polarization sterol exchange assays is release of DHE from self-quenching. In donor membrane fractions (labeled with DHE) the DHE content is high, resulting in self-quenching and low polarization. In contrast, as the donor DHE is exchanged one-for-one with cholesterol in the acceptor (present at 10-fold excess to minimize back-transfer of DHE and not labeled with DHE) the DHE is released from self-quenching, and polarization increases as the exchange process proceeds. Examination of representative sterol exchange curves indicated that in membrane fractions isolated from L-cell fibroblasts sterol transfer from lipid raft/caveolae donors to lipoprotein acceptors was relatively slow and dependent on the type of lipoprotein acceptor (Figure 2A). These visually apparent trends were confirmed by quantitative analysis of multiple sterol exchange curves (Table 2). The initial rates of molecular sterol exchange between caveolae/raft DHE donors and lipoprotein acceptors differed nearly 9-fold, depending on the lipoprotein, and were in the order LDL > VLDL > HDL, apoA1 (Table 2). Multiexponential analysis of multiple exchange curves revealed that they best fit two components: (i) a relatively rapidly exchangeable sterol pool with half-times near 1−4 h comprised about 12−20% of total sterol; (ii) a very slowly exchangeable sterol pool (half-time of days) represented the majority (>80%) of sterol (Table 2). The half-times of molecular sterol transfer from the rapidly exchangeable sterol pool in lipid rafts/caveolae donors to lipoprotein acceptors were shortest in the order LDL > VLDL > HDL, apoA1 (Table 2). However, the fraction of sterol in both the rapidly exchangeable and very slowly exchangeable sterol pool in the donor lipid rafts/caveolae was independent of the lipoprotein acceptor.

In contrast, representative exchange curves indicated that sterol exchange between non-raft donors and lipoprotein acceptors appeared to be slower (Figure 2B) than that in the reverse direction (Figure 2A). This was confirmed by examination of initial rates of molecular sterol exchange between non-raft donors and lipoprotein acceptors, which were all very slow and similar for all lipoproteins except for exchange with apoA1, which was the slowest of any transfer examined (Table 2). Finally, non-rafts had significantly less or no rapidly exchangeable sterol pool (Table 2). Thus, sterol exchange between lipid rafts/caveolae donors and lipoprotein acceptors proceeded most rapidly to LDL and VLDL. However, none of the lipoproteins examined altered the proportion of rapidly and slowly/nonexchangeable sterol pools in the donor lipid rafts/caveolae. In contrast, non-rafts were very poor sterol donors to lipoprotein acceptors and exhibited a very large or exclusively very slowly exchangeable sterol pool.

Sterol Exchange between MDCK Plasma Membrane Caveolae or Non-Raft Donors and Lipoprotein Acceptors. Representative sterol exchange curves indicated that sterol transfer from MDCK caveolae to lipoprotein acceptors was rapid (Figure 2C). The initial rates of molecular sterol exchange between MDCK caveolae donors and lipoprotein acceptors were in the order VLDL > LDL > HDL, apoA1 (Table 2). Multiexponential analysis showed that the ex-

Table 2: Sterol Exchange between Lipid Rafts/Caveolae, Non-Raft Fractions, and Lipoprotein Acceptors[a]

donor	acceptor	initial rate (pmol/min)	$t_{1/2}$ (min)	f_1	f_2
		L-Cell Fibroblasts			
lipid rafts/caveolae	LDL	0.693± 0.034	41 ± 6	0.171 ± 0.016	0.829 ± 0.046
	HDL	0.107± 0.021$^+$	205 ± 28$^+$	0.131 ± 0.013	0.869 ± 0.023
	ApoA1	0.080± 0.015$^+$	260 ± 21$^+$	0.124 ± 0.024	0.876 ± 0.029
	VLDL	0.232± 0.040$^+$	138 ± 18$^+$	0.192 ± 0.022	0.808 ± 0.035
non-rafts	LDL	0.129± 0.026*	144 ± 20*	0.112 ± 0.021*	0.888 ± 0.031
	HDL	0.062± 0.018	−*	−*	1.000*
	ApoA1	0.036± 0.009*	−*	−	1.000*
	VLDL	0.066± 0.028*	178 ± 27	0.069 ± 0.019*	0.931 ± 0.027*
		MDCK Cells			
caveolae	LDL	0.389 ± 0.037	52 ± 9	0.127 ± 0.011	0.873 ± 0.039
	HDL	0.139 ± 0.015$^+$	109 ± 21$^+$	0.096 ± 0.028	0.904 ± 0.035
	ApoA1	0.103 ± 0.018$^+$	131 ± 15$^+$	0.079 ± 0.017$^+$	0.921 ± 0.021
	VLDL	0.528 ± 0.022$^+$	140 ± 31$^+$	0.221 ± 0.017$^+$	0.779 ± 0.024
non-rafts	LDL	0.031 ± 0.009*	−*	−*	1.000*
	HDL	0.039 ± 0.006*	−*	−*	1.000*
	ApoA1	0.036 ± 0.007*	−*	−*	1.000*
	VLDL	0.029 ± 0.009*	−*	−*	1.000*

[a] DHE was incorporated at low, non-self-quenching levels into the isolated L-cell lipid rafts/caveolae and MDCK caveolae domains as described under Experimental Procedures. Polarization values represent the mean ± SD ($n = 7$). * indicates $P < 0.05$ vs caveolae-enriched domains; $^+$ indicates $P < 0.05$ vs LDL.

change curves again best fit to two components: a relatively rapidly exchangeable sterol pool with half-times near 1−2 h comprised about 10−20% of total sterol, and a very slowly exchangeable sterol pool (half-time of days) represented the majority (>80%) of sterol (Table 2). The half-times of molecular sterol transfer from the rapidly exchangeable sterol pool in caveolae to lipoproteins were shortest in the order LDL > VLDL, HDL, apoA1 (Table 2). Again, the fraction of rapidly exchangeable and very slowly exchangeable sterol pool in the donor caveolae was largely independent of the lipoprotein acceptor, although small differences were seen with apoA1 and VLDL (Table 2).

In contrast, sterol exchange between MDCK non-raft donors and lipoprotein acceptors was extremely slow as compared to exchange between caveolae donors and lipoprotein acceptors (Figure 2D). Initial rates of molecular sterol exchange between MDCK non-raft donors and lipoprotein acceptors were as much as 18-fold slower between non-raft donors and lipoprotein acceptors than between caveolae donors and lipoprotein acceptors (Table 2). Furthermore, there were no significant differences in initial rates of sterol exchange from non-raft donors to lipoprotein acceptors regardless of lipoprotein type (Table 2). Finally, MDCK non-rafts had no rapidly exchangeable sterol pool, regardless of the type of lipoprotein acceptor (Table 2). Thus, sterol transfer from MDCK non-raft donors to lipoprotein acceptors was slower than that from caveolae, unresponsive to lipoproteins, and exhibited only a very slowly exchangeable sterol pool.

Sterol Exchange between MDCK DRM or DSM Donors and Lipoprotein Acceptors. To determine if MDCK sterol exchange dynamics between caveolae (purified by affinity-chromatography without the use of detergents) and lipoprotein acceptors was also reflected by caveolae isolated with the use of detergents, DRM and DSM were isolated as described under Experimental Procedures. Representative sterol exchange curves between MDCK DRM donors and lipoprotein acceptors indicated that exchange to LDL and VLDL was more rapid than that to HDL or apoA1 acceptors (Figure 3A). This was confirmed by comparison quantitative analysis of multiple exchanges which indicated that the initial rates of sterol transfer from DRM donors to lipoprotein acceptors were in the relative order VLDL > LDL, HDL, apoA1 (Table 3). Multiexponential analysis of exchange curves from DRM donors to lipoprotein acceptors best fit to two components: a relatively rapidly exchangeable sterol pool with half-times near 1−2 h comprised about 10−20% of total sterol, and a very slowly exchangeable component (half-time of days) represented the majority (>80%) of sterol (Table 3). The half-times of molecular sterol exchange between the rapidly exchangeable sterol pool in DRM donors to lipoprotein acceptors were shortest in the order VLDL > LDL, HDL, apoA1 (Table 3). The fraction of rapidly exchangeable and very slowly exchangeable sterol pool in the donor DRM was largely independent of the lipoprotein acceptor, although small differences were seen between apoA1 and VLDL (Table 3). In contrast, sterol exchange between MDCK DSM donors and lipoprotein acceptors was 6−20 times slower than that from DRM, unresponsive to the type of lipoprotein acceptor, and exhibited no rapidly exchangeable sterol pool (Table 3). Thus, the kinetics and relative order of initial rates of molecular sterol exchange between DRM donors and lipoprotein acceptors also indicated that VLDL and LDL were the best sterol acceptors. Thus, DSM were poor sterol donors to any of the lipoproteins examined and exhibited only a single very slowly exchangeable sterol pool.

Sterol Exchange between Lipoprotein Donors and L-Cell Plasma Membrane Lipid Rafts/Caveolae or Non-Raft Acceptors. Since the above studies indicated that LDL and VLDL were better sterol acceptors from lipid rafts/caveolae and DRM, it was important to determine if this was also the case in the reverse direction, i.e., if LDL and VLDL were good sterol donors to lipid rafts/caveolae acceptors. Therefore, DHE was incorporated into the different lipoproteins (HDL, LDL, VLDL, apoA1) by using a method initially prescribed by Yeagle et al. (*56*) and later modified by Smutzer (*57*). To characterize the spectral parameters of DHE

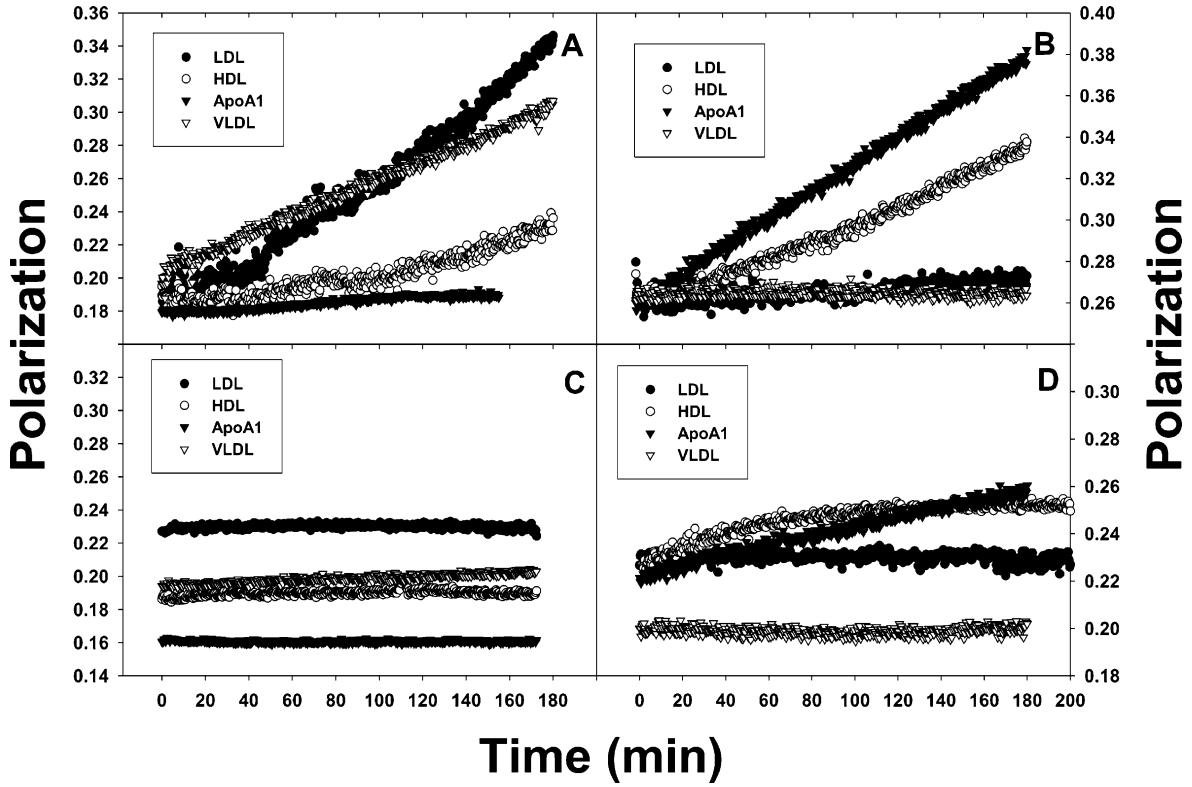

FIGURE 3: MDCK and L-cell membrane donor/acceptor sterol exchanges to lipoproteins. DHE exchanges between MDCK detergent-resistant membrane (DRM) donors and lipoprotein acceptors (panel A), lipoprotein donors and L cell lipid rafts/caveolae acceptors (panel B), lipoprotein donors and L cell non-raft acceptors (panel C), and lipoprotein donors and MDCK caveolae acceptors were measured by monitoring DHE polarization as described under Experimental Procedures: ●, exchange to LDL; ○, sterol exchange to HDL; ▼, exchange to apoA1; ▽, sterol exchange to VLDL.

Table 3: Sterol Exchange between MDCK Donors (DRM, DSM) and Lipoprotein Acceptors[a]

donor	acceptor	initial rate (pmol/min)	$t_{1/2}$ (min)	f_1	f_2
DRM	LDL	0.185 ± 0.023	127 ± 32	0.143 ± 0.041	0.857 ± 0.032
	HDL	0.115 ± 0.019$^+$	145 ± 28	0.106 ± 0.019	0.894 ± 0.028
	ApoA1	0.101 ± 0.024$^+$	169 ± 18	0.092 ± 0.016	0.908 ± 0.027
	VLDL	0.699 ±0.018$^+$	46 ± 14$^+$	0.198 ± 0.038	0.802 ± 0.036
DSM	LDL	0.034 ± 0.013*	−*	−*	1.000*
	HDL	0.036 ± 0.012*	−*	−*	1.000*
	ApoA1	0.041 ± 0.009*	−*	−*	1.000*
	VLDL	0.033 ± 0.010*	−*	−*	1.000*

[a] DHE was incorporated at low, non-self-quenching levels into the isolated detergent resistant membranes (DRM) and detergent soluble membranes (DSM) as described under Experimental Procedures. Values represent the mean ± SD ($n = 7$). * indicates $P < 0.05$ vs DRMs; $^+$ indicates $P < 0.05$ vs LDL.

in HDL, LDL VLDL, and apoA1, the polarization and fluorescence emission maxima were determined for each lipoprotein−DHE complex (Table 4). DHE polarization values did not significantly differ between lipoproteins, ranging from 0.1786 ± 0.0072 (apoA1−DHE) to 0.1904 ± 0.0083 (VLDL−DHE). DHE emission maxima were also similar in all DHE−lipoprotein complexes, which characteristically exhibited emission maxima at 357 ± 3, 374 ± 2, and 388 ± 4 nm (Table 4). Comparison of these spectra and emission maxima with those of crystalline DHE (*31*) showed the absence of crystalline DHE in the lipoprotein−DHE complexes. These spectral properties of DHE confirmed those previously reported for DHE in VLDL, LDL, and HDL (*56*, *57*, *71*, *72*, *79*, *80*). Finally, since the purified lipoprotein fractions (especially VLDL and LDL) lack the sequestering activity of lecitihin:cholesterol acyltransferase, all of the DHE incorporated into the lipoproteins was unesterified DHE,

Table 4: Spectral Parameters of DHE Incorporated into Different Lipoproteins[a]

		emission maxima (nm)		
lipoprotein	polarization	peak 1	peak 2	peak 3
HDL	0.1833 ± 0.0099	357	374	387
ApoA1	0.1786 ± 0.0072	358	373	388
LDL	0.1892 ± 0.0078	355	374	392
VLDL	0.1904 ± 0.0083	358	375	385

[a] Dehydroergosterol (DHE) was incorporated at low, non-self-quenching levels into HDL, ApoA1, LDL, and VLDL as described under Experimental Procedures. The fluorescence polarization values represent the mean ± SE ($n = 30$). All values are ±SE. Emission maxima of the three peaks of DHE correspond with those observed previously for membranes.

confirming earlier studies (*72*, *79*, *80*). Thus, all exchange curves between lipoproteins and purified plasma membrane

Table 5: Sterol Exchange between Lipoprotein Donors and Plasma Membrane Domain (Caveolae-Enriched, Non-Rafts) Acceptors[a]

donor	acceptor	initial rate (pmol/min)	$t_{1/2}$ (min)	f_1	f_2
		L-Cell Fibroblasts			
LDL	lipid rafts/caveolae	0.083 ± 0.016	167 ± 21	0.083 ± 0.006	0.917 ± 0.024
HDL		$0.269 \pm 0.029^{+}$	113 ± 17	$0.188 \pm 0.023^{+}$	$0.812 \pm 0.026^{+}$
ApoA1		$0.435 \pm 0.031^{+}$	$85 \pm 16^{+}$	$0.229 \pm 0.027^{+}$	$0.721 \pm 0.034^{+}$
VLDL		0.056 ± 0.011	−	−	1.000
LDL	non-rafts	0.037 ± 0.009*	−*	−*	1.000*
HDL		0.051 ± 0.018*	−*	−*	1.000*
ApoA1		0.045 ± 0.026*	−*	−*	1.000*
VLDL		0.042 ± 0.019	−	−	1.000*
		MDCK Cells			
LDL	lipid rafts/caveolae	0.112 ± 0.013	159 ± 18	0.106 ± 0.009	0.894 ± 0.011
HDL		$0.370 \pm 0.032^{+}$	109 ± 14	$0.243 \pm 0.022^{+}$	$0.757 \pm 0.034^{+}$
ApoA1		$0.296 \pm 0.024^{+}$	175 ± 29	0.129 ± 0.011	0.871 ± 0.043
VLDL		$0.043 \pm 0.010^{+}$	$-^{+}$	$-^{+}$	$-^{+}$
LDL	non-rafts	0.039 ± 0.010*	−*	−*	1.000*
HDL		0.040 ± 0.012*	−*	−*	1.000*
ApoA1		0.039 ± 0.019*	−*	−*	1.000*
VLDL		0.044 ± 0.015	−*	−*	1.000*

[a] DHE was incorporated at low, non-self-quenching levels into HDL, ApoA1, LDL, and VLDL as described under Experimental Procedurs. Values represent the mean ± SD ($n = 7$). * indicates $P < 0.05$ vs lipid rafts/caveolae; + indicates $P < 0.05$ vs LDL.

fractions represent exchange (not net transfer) of unesterified sterol, in the absence of sequestering actions of esterification enzymes.

Examination of representative sterol exchange curves between lipoprotein donors and lipid rafts/caveolae isolated from L-cell plasma membranes indicated that sterol exchange was most marked from apoA1 (Figure 3B, ▼) and HDL (Figure 3B, ○) donors. In contrast, exchange from LDL (Figure 3B, ●) and VLDL (Figure 3B, ▽) donors to lipid rafts/caveolae acceptors was very slow. These trends were confirmed by quantitative analysis of initial rates from multiple exchanges (Table 5). The initial rate of sterol exchange between lipoprotein donors and L-cell lipid rafts/caveolae acceptors was most rapid in the order apoA1 > HDL > LDL, VLDL (Table 5). Furthermore, initial rates of sterol exchange between apoA1 and HDL donors and L-cell lipid rafts/caveolae acceptors (Table 5) were 5.4- and 2.5-fold, respectively, faster than in the reverse direction (Table 3). In contrast, initial rates of sterol exchange between LDL or VLDL donors and L-cell caveolae/lipid raft acceptors (Table 5) were 8.3- and 4.1-fold, respectively, slower than in the reverse direction (Table 3). Multiexponential analysis of the sterol exchange curves from lipoprotein donors to L-cell fibroblast lipid rafts/caveolae acceptors best fit to two components for apoA1, HDL, and LDL donors, whereas that from VLDL donors was too slow to resolve (Table 5). The half-times of exchange of the most rapidly exchangeable component from lipoprotein donors to the lipid rafts/caveolae acceptors were in the order of the most rapid to the least rapid: apoA1, HDL > LDL ≫ VLDL (Table 5). Lipoprotein donors with shortest half-times and fastest initial rates had the largest rapidly exchangeable sterol fraction—apoA1 and HDL (Table 5). In contrast, sterol exchange between the respective lipoprotein donors and non-raft acceptors isolated from L-cell plasma membranes was almost nondetectable (Figure 3C). There were no statistically significant differences in initial rates of sterol exchange between the various lipoprotein donors and non-raft acceptors. Initial rates of sterol exchange between apoA1, HDL, LDL, or VLDL donors and non-raft acceptors were 9.7-, 5.3-, 2.2-, and 1.3-fold, respectively, slower than to lipid rafts/caveolae acceptors (Table 5). In summary, as shown by fast initial rates and small half-times of sterol exchange, apoA1 and HDL were the best sterol donors to L-cell fibroblast lipid rafts/caveolae acceptors. Furthermore, apoA1 and HDL donors exhibited a larger exchangeable sterol fraction as compared to LDL and VLDL. Non-rafts were poor sterol acceptors, regardless of the type of lipoprotein donor, and all of the lipoprotein donors exhibited only a single very slowly exchangeable sterol pool.

Sterol Transfer from Lipoprotein Donors to MDCK Plasma Membrane Caveolae and Non-Raft Acceptors. Representative sterol exchange curves between lipoprotein donors and MDCK cell caveolae acceptors also appeared most rapid for apoA1 (Figure 3D, ▼) and HDL (Figure 3D, ○) donors. Initial rates of sterol exchange between lipoprotein donors and caveolae acceptors were in the order HDL, apoA1 > LDL > VLDL (Table 5). Initial rates of sterol exchange between apoA1 and HDL donors and MDCK caveolae acceptors (Table 5) were 3.6- and 2.7-fold, respectively, faster than in the reverse direction (Table 3). In contrast, initial rates of sterol exchange between LDL or VLDL donors and MDCK cell caveolae acceptors (Table 5) were 3.4- and 12.2-fold, respectively, slower than in the reverse direction (Table 3).

Exchange curves between lipoprotein donors and MDCK caveolae acceptors best fit to two components for the more rapid exchanges (apoA1, HDL, LDL donors), but not from VLDL donors, which was too slow to resolve (Table 5). The HDL and less so apoA1 donors appeared to have the largest rapidly exchangeable sterol fraction, whereas that of LDL and VLDL was smaller or nonexistent (Table 5). Lipoprotein donors exhibiting the shortest half-times and fastest initial rates generally had the largest rapidly exchangeable sterol fraction—apoA1 and HDL (Table 5). The half-times of exchange of the rapidly exchangeable sterol pool between the various lipoprotein donors and the MDCK caveolae acceptors were in the approximate order from the fastest (i.e., shortest half-time) to the slowest HDL, apoA1, LDL ≫ VLDL (Table 5).

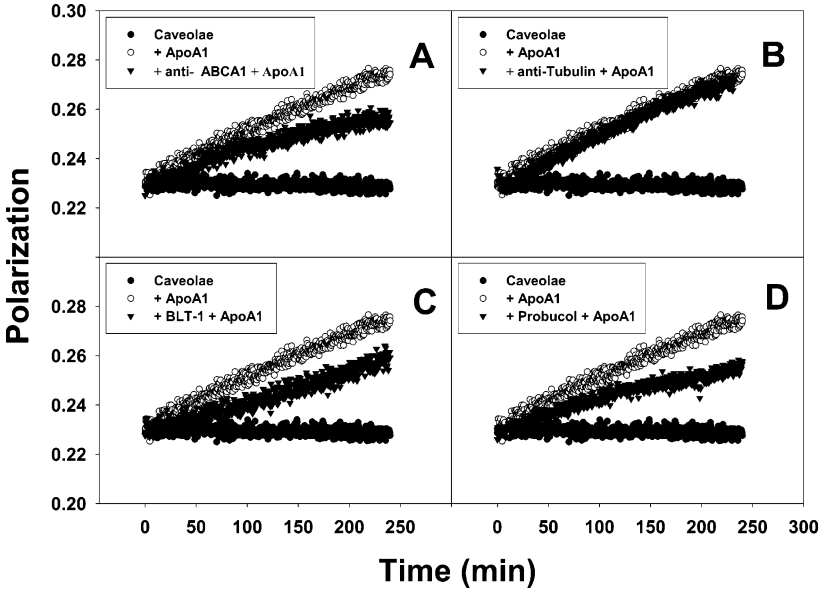

FIGURE 4: Sterol transfer from MDCK caveolae donors to apolipoprotein A1 acceptor in the presence of different inhibitors. DHE exchange between MDCK caveolae donors and apoA1 was measured by monitoring DHE polarization as described under Experimental Procedures. The effect of several inhibitors including anti-ABCA1 (panel A), BLT-1 (panel C), Probucol (panel D), and a control for nonspecific adsorption of antibodies (anti-tubulin, panel B) on sterol exchange was determined: ●, MDCK caveolae donor only; ○, sterol exchange to the apoA1 without inhibitors present; ▼, exchange to the respective inhibitors (anti-ABCA1, BLT-1, Probucol) or control (anti-tubulin). All curves show sterol exchange from donor MDCK caveolae after addition of 10-fold excess (by sterol) acceptor apoA1.

In contrast, there were no differences in initial rates of sterol exchange between the various lipoprotein donors and MDCK cell non-raft acceptors. Furthermore, initial rates of sterol exchange between apoA1, HDL, LDL, or VLDL donors and MDCK non-raft acceptors were 7.6-, 9.3-, 2.9-, and 1.0-fold, respectively, slower than to MDCK caveolae/lipid rafts (Table 5). In summary, apoA1 and HDL were the best sterol donors to MDCK cell caveolae acceptors. Furthermore, apoA1 and HDL tended to exhibit a larger rapidly exchangeable sterol fraction than LDL or VLDL. MDCK cell non-rafts were poor sterol acceptors from lipoproteins, regardless of the type of lipoprotein, and all of the lipoprotein donors exhibited only a single very slowly exchangeable sterol pool with non-raft acceptors.

Relative Contributions of ABCA1 and SR-B1 to Sterol Exchange between MDCK Caveolae Donors and ApoA1 Acceptors: Effect of Inhibitors. Sterol exchange between MDCK caveolae donors and apoA1 acceptor was examined in the presence of several inhibitors [BLT-1 to inhibit SR-B1 (*75*), Probucol to inhibit ABCA1 (*75*), and anti-ABCA1 to inhibit ABCA1] and one non-inhibitor (anti-tubulin) to apoA1 sterol exchange. As shown by representative exchange curves, anti-ABCA1 reduced sterol exchange between MDCK caveolae donors and apoA1 (Figure 4A, ▼) as compared to control in the absence of anti-ABCA1 (Figure 4A, ○) to reduce the initial rate of sterol exchange by 27 ± 8% (n = 3). This inhibition was not due to nonspecific antibody adsorption as treatment with anti-tubulin, a non-inhibitor of apoA1, did not affect sterol exchange (Figure 4B) or initial rate. These findings were confirmed with the ABCA1-inhibiting drug Probucol, which inhibited sterol exchange (Figure 4C, ▼) as compared to control in the absence of Probucol (Figure 4C, ○) to reduce the initial rate of sterol exchange by 35 ± 9% (n = 3). Finally, the SR-B1 inhibitor BLT-1 reduced sterol exchange (Figure 4C, ▼) as compared to control in the absence of BLT-1 (Figure 4C, ○) to reduce the initial rate of sterol exchange by 45 ± 16% (n = 3). In summary, inhibitors of ABCA1 (i.e., anti-ABCA1, Probucol) and SR-B1 (BLT-1) were observed to decrease sterol exchange between MDCK caveolae donors and apoA1. Taken together, these data suggest that 72−80% of sterol exchange between MDCK caveolae donors and apoA1 is associated with ABCA1 and SR-B1 in nearly equal proportion. Furthermore, finding that a significant proportion (20−28%) of cholesterol flux was not due to either ABCA1 or SR-B1 was consistent with findings in other peripheral cell types (*75*).

DISCUSSION

Although the organizing principle of LDL-mediated cholesterol uptake (i.e., LDL receptor-mediated uptake through clathrin coated pits) is now well understood, much remains to be determined regarding the molecular details and organizing principles for selective cholesterol uptake/efflux. As indicated in the introduction, it is not yet completely clear whether selective cholesterol uptake/efflux in peripheral cells can be attributed to plasma membrane microdomains termed lipid rafts/caveolae. Furthermore, two rather than one distinct lipoprotein pathways serve selective cholesterol efflux/uptake (also called reverse cholesterol transport or RCT) from the cell surface of peripheral cells: (i) Apolipoprotein A1 (apoA1) interacts with ABCA1 and is the acceptor of phosphatidylcholine efflux, which together with cholesterol desorption results in the formation of HDL precursors in serum (reviewed in ref *2* and *28*). This unidirectional pathway is energy-requiring, and other lipoproteins do not bind and/or serve as effective acceptors (reviewed in ref *2*). Interestingly, cross-linking studies of aortic endothelial cells after HDL incubation have established direct interactions between ABCA1 and both HDL and caveolin-1, but not HDL and caveolin-1. This suggests that ABCA1 may act as a structural platform between HDL and caveolin-1 on the cell surface during cholesterol transfer to HDL (*81*). (ii) Serum HDL tethered to SR-B1 facilitates cholesterol transfer from the

plasma membrane in an energy-independent, bidirectional process (*2*, *30*). This pathway differs markedly from the ABCA1 pathway in that SR-B1-mediated cholesterol transfer is energy-independent, bidirectional, and less specific for only one class of lipoprotein, i.e., HDLs (*2*, *30*, *82*−*88*). Whereas binding of lipoproteins to the SR-B1 receptor appears to be required for efficient cholesterol efflux, high-affinity binding of the lipoprotein to the receptor is insufficient to account for the observed relative order of cholesterol efflux (reviewed in ref *2*). For example, SR-B1 binds lipoproteins with the following order of affinities: apoA1 > VLDL > HDL > LDL (*83*, *88*). In contrast, effectiveness in SR-B1-mediated efflux of radiolabeled cholesterol from peripheral cells is in the order HDL > VLDL, LDL (reviewed in refs *2*, *83*, and *88*). The same approximate order of effectiveness was noted in SR-B1-mediated uptake of fluorescent sterol measured by real-time confocal imaging in living cells: HDL > LDL > VLDL (*89*). Interestingly, however, regardless of the relative affinities of SR-B1 for the respective lipoprotein classes or their individual activities in mediating sterol efflux/uptake, other lipoproteins do not effectively compete with HDL for mediating RCT from intact peripheral cells (reviewed in ref *2*, *83*, *88*, and *89*). To further investigate the molecular determinants of RCT from peripheral cells, studies were designed to probe the effectiveness of the various lipoprotein classes to act as acceptors (efflux) and donors (uptake) from/to two different types of lipid rafts/caveolae and non-raft preparations isolated from peripheral cell types (L-cell fibroblasts, MDCK cells): (i) affinity chromatography (no detergent, no high-pH carbonate buffer) was used to resolve lipid rafts/caveolae and non-rafts from purified plasma membranes; (ii) DRM and DSM were purified from cells through the use of Triton X-100.

Caveolae represent only a subfraction of lipid rafts (*90*−*92*). Consistent with this, the amount of protein found in L-cell lipid rafts/caveolae and MDCK caveolae enriched fractions represents a small percentage of the total protein in plasma membrane, 10 and 2%, respectively, in keeping with the results of others (*93*). Although ultrastructural studies show that flask-shaped caveolae typically occupy <10% of the cell surface in most cells, caveolin is found not only in flask-shaped caveolae but also at or near the PM in linear membrane patches or clustered invaginations (grapes on a string) (*90*, *94*). In addition, caveolin has been localized at intracellular sites including intracellular transport/endocytic vesicles and caveosomes (*90*, *94*), endoplasmic reticulum and trans Golgi network (*94*), and lipid droplets (*95*) and isolated from the cytosol as soluble chaperone complexes (*21*, *96*). The lack of >2-fold enrichment of caveolin-1 from homogenate levels in the ConA-isolated L-cell lipid raft/caveolae and MDCK caveolae fractions presented herein was thus in keeping with the known presence of caveolin in locations other than plasma membrane caveolae—thereby accounting for the smaller enrichment of caveolin in ConA-purified membranes. Furthermore, other known plasma membrane lipid raft/caveolae markers (SR-B1 and GM1) were enriched in the lipid rafts/caveolae and caveolae isolated by the nondetergent ConA methodology. It is important to note that even though caveolin is distributed to plasma membrane locations other than plasma membrane caveolae, only the cholesterol dynamics of lipid rafts/caveolae and caveolae isolated by nondetergent methods were responsive to lipoproteins (present work) and intracellular cholesterol transport proteins such as SCP-2 (*34*, *49*, *52*). Finally, the structure of lipid rafts/caveolae and caveolae isolated by nondetergent methods differed markedly from that of the bulk plasma membrane and non-rafts (*34*, *49*, *52*). Taken together, these findings suggested that not only the presence of caveolin but also structural properties of lipid rafts/caveolae and caveolae contribute to a cholesterol membrane environment responsive to extracellular lipoproteins and intracellular cholesterol binding proteins. Thus, findings derived from these studies contribute the following new insights:

First, lipid rafts/caveolae were enriched in lipids trafficked through the RCT pathway. For example, the data presented herein with lipid rafts/caveolae isolated by nondetergent, non-high-pH carbonate buffer, ConA affinity chromatography were enriched in both cholesterol and phospholipid, confirming earlier studies with lipid rafts/caveolae isolated by ConA affinity chromatography (*34*, *50*, *51*) or by other nondetergent, non-high-pH carbonate buffer-based methods (*97*−*99*). However, because of preferential interaction of cholesterol with sphingolipids and phospholipids with saturated acyl chains, the caveolae/lipid rafts, caveolae, and lipid rafts are selectively enriched in these raft-promoting lipids (i.e., SM, GM1, phospholipids with saturated acyl chains) (*10*, *34*, *50*−*52*, *74*, *75*, *97*−*105*). In contrast, DRM exhibit much lower phospholipid content arising from detergent-induced selective loss of phospholipids, especially phosphatidylcholine, which is the major plasma membrane phospholipid (*97*, *99*, *106*, *107*). Thus, it is unlikely that DRM equate with lipid rafts/caveolae isolated by nondetergent methods (*92*, *107*). Finally, lipid rafts/caveolae also contained low amounts of cholesteryl ester and triacylglycerol. It is important to note that detection of these lipids in lipid rafts/caveolae was not due to contamination by adherent lipoproteins, which are especially rich in cholesteryl ester and triacylglycerol. Western blotting did not detect either apoB or apoA (protein components of HDL, LDL, and VLDL) in lipid rafts/caveolae. Further support for the presence of these lipids in lipid rafts/caveolae comes from the finding that SR-B1 mediates selective uptake not only of cholesterol but also of other lipids present in lipoproteins including phospholipid, cholesteryl ester, and triacylglycerol (*2*, *30*, *82*−*88*, *108*). Thus, the lipid distribution within lipid rafts/caveolae was consistent with the transient tethering of bound lipoproteins to SR-B1, which may facilitate the transmembrane flux of these lipids (reviewed in refs *2* and *109*).

Second, it was shown for the first time that, despite being more fluid than lipid rafts/caveolae and DRM (*34*, *49*, *52*), the sterol exchange assays showed that non-rafts and DSM were very poor sterol donors or acceptors to/from lipoproteins, regardless of lipoprotein class (HDL, apoA1, LDL, VLDL) involved. This lack of responsiveness to extracellular cholesterol transfer proteins (HDL, VLDL, LDL, apoA1) was also observed with intracellular cholesterol transfer proteins such as sterol carrier protein-2 (SCP-2) (*34*, *52*). Sterol exchange between non-rafts or DSM and lipoproteins was likely poor/very slow because (i) lipoprotein receptors mediating RCT (i.e., ABCA1, SR-B1, caveolin-1, P-gp) were not enriched in non-rafts as shown herein and earlier (reviewed in refs *1*−*4*, *30*, and *34*−*42*). Likewise, DSM were also not enriched in RCT proteins (reviewed in refs *3*, *25*,

44−*48*, and *52*). (ii) Neither caveolin-1, with which the intracellular cholesterol transport protein SCP-2 directly interacts at the cytofacial leaflet (*5*), nor SCP-2 (*34*) is localized in non-rafts.

Third, sterol exchange assays indicated that lipid rafts/caveolae and caveolae isolated from purified plasma membranes and DSM isolated from peripheral cell types (L-cell fibroblasts, MDCK cells) were excellent sterol donors to lipoproteins. This was consistent with lipid rafts/caveoale and caveolae being enriched in RCT proteins (caveolin-1, SR-B1, ABCA1, P-gp, ABCG1) known to induce the formation of more aqueous-exposed, cholesterol oxidase-accessible cholesterol in plasma membranes and/or in cholesterol-rich microdomains (*24*, *83*, *110*−*115*). For example, SRB1 increases the size of the membrane cholesterol pool available to cyclodextrin acceptors (*116*). Caveolin-1 and SR-B1, but not ABCA1 (*117*, *118*), directly bind cholesterol and/or are cross-linked by photoactivatable cholesterol (*4*, *20*, *21*, *29*, *117*−*120*). However, it is important to note that intracellular factors must also contribute to cholesterol dynamics in lipid rafts/caveolae. For example, the half-times of sterol exchange between lipid rafts/caveolae donors and HDL acceptors shown herein were 2−3 h, and the half-times of sterol exchange in the reverse direction, i.e., uptake from HDL donors to purified lipid rafts/caveolae acceptors, were even shorter, <2 h. However, these dynamics were significantly slower than those observed with intact cells exposed to lipoproteins that bind to SR-B1, i.e., on the order of a few minutes (reviewed in refs *2* and *89*). These data suggest that translocation of sterol across the lipid rafts/caveolae bilayer and efflux/uptake to bound HDL (or other lipoproteins) may require participation of other processes or additional intracellular factors present only in intact cell which may include the ATP requiring translocases ABCA1 and P-gp. ABCA1 has been shown to be directly associated with caveolin-1 (*81*). Incubation with anti-ABCA1 antibody or the ABCA1 inhibitor BLT-1 both inhibited ABCA1-mediated sterol flux to apoA1 (Figure 4). Although these data suggest that ABCA1 induced a more rapidly exchangeable sterol pool even in the absence of ATP, nevertheless this exchange was still slower than observed with intact cells. Alternately, vesicular and/or protein-mediated cholesterol transfer from intracellular sites to/from lipid rafts/caveolae may affect sterol exchange since SCP-2 overexpression inhibits HDL-mediated sterol efflux in fibroblasts (*6*).

Fourth, the lipoprotein specificity for sterol exchange with lipid rafts/caveolae and/or DRM *in vitro* suggested that LDL and VLDL were better sterol acceptors, whereas apoA1 and HDL were better sterol donors. Earlier studies also showed that HDL depleted cholesterol from DRM, but comparative studies with other lipoproteins were not reported (*40*). It should be noted that the lipoprotein specificity presented herein showing LDL and VLDL as the best sterol acceptors from highly purified lipid rafts/caveolae *in vitro* was opposite from that observed in intact living cells, where HDL and apoA1 are the best cholesterol acceptors from intact peripheral cell donors (reviewed in refs *2*, *83*, and *88*). These specificities *in vitro* versus in intact cells were not correlated with differences in respective binding affinities of SR-B1 for these lipoprotein classes (*83*, *88*) or with differences in cholesterol packing density among the different lipoprotein acceptors (*121*, *122*). Taken together, these results indicate that the specificity of cholesterol transfer to lipoproteins in the serum is likely influenced by additional factors: (i) Lipoproteins in serum compete for binding to the SR-B1 receptor. Surprisingly, despite the fact that SR-B1 binds LDL and VLDL with similar or somewhat weaker affinities than HDL, the lipoproteins do not compete with HDL for binding/mediating RCT from intact peripheral cells (reviewed in refs *2*, *83*, *88*, and *89*). (ii) The energy requirement of the ABCA1 and P-gp transporters *in vivo* and in intact cells correlates with highest specificity of ABCA1-mediated sterol efflux for apoA1 and pre-HDL (reviewed in ref *2*). Since purified lipid rafts/caveolae do not contain the energy production machinery of the cell, the lack of ATP in these studies performed with purified lipid rafts/caveolae *in vitro* likely contributes significantly to the reduced effectiveness of apoA1 and possibly HDL as well. (iii) Intracellular factors present only in intact cells may contribute to determining sterol efflux patterns. Sterol transfer from intracellular sites is mediated by both vesicular (caveolar vesicles, non-caveolar vesicles) and protein-mediated (caveolin-1, SCP-2) pathways that take different paths favoring the ABCA1 or the SR-B1 pathway (reviewed in refs *2*, *3*, *7*, and *123*). For example, SCP-2 facilitates SR-B1-mediated molecular sterol efflux to HDL, but concomitantly inhibits vesicular-mediated sterol efflux to HDL in SCP-2 overexpressing fibroblasts—the latter likely due to SCP-2's affinity for ligands that regulate vesicle budding from the Golgi (*6*).

Fifth, lipoproteins were excellent donors to lipid rafts/caveolae *in vitro*, in the order apoA1, HDL > VLDL, LDL—consistent with the cholesterol packing densities in lipoproteins such as HDL and LDL, i.e., weaker in HDL and tighter in LDL, that make HDL a better cholesterol donor (*121*, *122*).

Finally, these studies with biochemically resolved distinct plasma membrane fractions—cholesterol-poor non-rafts and cholesterol-rich caveolae/lipid rafts—were not due to artifactual segregation of the plasma membrane into cholesterol-rich and -poor regions during the isolation procedure (*110*, *124*). Real-time multiphoton imaging of DHE in L-cell fibroblasts established the existence of sterol-rich and sterol-poor domains in plasma membranes of living cells (*31*, *32*, *125*). Video imaging of DHE in HepG2 cells also confirmed a nonrandom distribution of DHE in the plasma membrane as evidenced by DHE highly clustered in microvilli (*126*). Microvilli are sterol-rich microdomains or rafts, are present in peripheral cell types as well as polarized cells, are highly enriched in sterol (*127*, *128*), and are enriched in ABC transporters and SR-B1, both of which mediate cholesterol transport (*126*, *129*−*131*). In living cells, the size range of sterol-rich domains, 200−565 nm (*32*), is generally smaller than microvilli, 500−1000 nm (*132*−*135*). These findings were confirmed by electron microscopy and immunofluorescence of fixed cells wherein cholesterol-rich domains were detected with the noncytolytic perfringolysin O within microvilli/filopodia (*136*). When cholesterol-rich lipid rafts are disrupted, filipin indiscriminately labels all cholesterol in the plasma membrane (*136*, *137*). Thus, these data indicate that cholesterol-rich rafts do exist in living cells and are not a potential artifact of biochemical isolation procedures.

In summary, the data presented herein provided new insights supporting the role of lipid rafts/caveolae in RCT: (i) lipids known to be translocated through lipid rafts/caveolae are enriched (cholesterol, phospholipid) and, in the

case of lipids poorly soluble in bilayers (i.e., cholesteryl ester, triacylglycerol), detectable in small amounts in lipid rafts/caveolae; (ii) lipoprotein-mediated sterol uptake/efflux occurs via lipid rafts/caveolae rather than non-rafts; and (iii) the rate of lipoprotein specificity of sterol efflux from lipid rafts/caveolae to lipoprotein acceptors *in vitro* is slower and differs in specificity from that in intact cells, where HDL and apoA1, rather than LDL and VLDL, preferentially enhance sterol efflux from intact peripheral cells (reviewed in refs *1*, *2*, *4*, *6*, *28*, *35*, *37*, *38*, *109*, *124*, and *138*). These findings suggest that additional factors (e.g., energy-driven cholesterol transbilayer translocation, intracellular vesicular transport, intracellular protein-mediated molecular cholesterol transport, etc.) contribute significantly to regulating the direction of sterol transfer between plasma membrane lipid rafts/caveolae and serum lipoproteins/apoproteins.

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BI700690S