Reconstitution of the Lipoprotein Cholesteryl Ester Transfer Process Using Isolated Rat Ovary Plasma Membranes†

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ABSTRACT: Steroidogenic cells are able to utilize lipoprotein-derived cholesteryl esters for steroidogenesis without internalizing intact lipoproteins. In the current report, we provide evidence that an early step in this process may be the selective extraction of cholesteryl esters at the cell (plasma membrane) surface. We have used a highly purified plasma membrane preparation from rat luteinized ovaries for incubation with rat- and human-derived high density (HDL) and low density (LDL) lipoproteins. The lipoproteins were modified with residualizing [^{125}I]apoprotein or [^{3}H]cholesteryl ester markers. Following trypsin treatment to remove intact surface-bound apoprotein particles, the membranes were analyzed for transferred radioactive labels. The results show that all the lipoproteins tested could serve as cholesteryl ester donors. Although far more [^{3}H]cholesteryl ester than [^{125}I]apoprotein radioactivity was transferred to plasma membranes in each case, and varied with the ligand used, the total (net) mass of cholesteryl ester transferred was comparable with the different lipoproteins. These data were confirmed using direct chemical methodology. Transfer was found to be specific for cholesteryl esters or ethers and did not involve other lipoprotein core lipids tested. Endomembranes from the same tissue could not substitute for plasma membranes as the primary cholesteryl ester acceptor. These results provide evidence that a reconstituted lipoprotein–plasma membrane system can simulate the cholesteryl ester extraction process described in situ and suggest uses for this methodology in future experiments designed to understand the transfer process.

Rat steroidogenic tissues have exceptionally high requirements for exogenous cholesterol (Gwynne & Strauss, 1982). Using perfusion techniques for the delivery of lipoproteins to different organs, we have been able to show that the tissue-specific cholesterol requirements are met after perfusion of high and low density lipoproteins (HDL or LDL) and that cholesteryl esters obtained from these lipoproteins are interiorized by the parenchymal cells of the tissues and utilized in hormone production (Reaven et al., 1984, 1986, 1988, 1989; Azhar et al., 1988, 1989; Azhar & Reaven, 1989). Of special interest is the finding that most (i.e., 80–90%) of the internalized cholesteryl esters have entered the steroidogenic cells by a pathway which does not involve internalization of the intact lipoprotein particle (Reaven et al., 1984, 1986, 1988, 1989; Azhar et al., 1988, 1989). It appears that once lipoprotein interaction with the cell surface has occurred, the cells preferentially extract the cholesteryl ester moiety, leaving behind much of the particle which contains the apoproteins. This finding with the luteinized rat ovary (Reaven et al., 1984, 1986, 1988; Azhar et al., 1988) and various rat adrenal models (Azhar et al., 1989; Reaven et al., 1989) has been made also with intact liver (Glass et al., 1983; Stein & Stein, 1985; MacKinnon et al., 1986; Arbeeny et al., 1987) and a variety of cultured cells from these organs (Leitersdorf et al., 1986; Pittman et al., 1987; Bachorik et al., 1987; Despres et al., 1988; Rinninger & Greten, 1990; Azhar et al., 1990). The mechanism by which the selective cholesteryl ester extraction occurs is not known, though we believe it is facilitated (at least in the parenchymal cells of the steroidogenic organs) by the presence of specialized plasma membranes from microvillar regions which trap an extraordinary number of lipoproteins in vivo and during in situ perfusion experiments (Reaven et al., 1988, 1989).

Recently, we have been successful in isolating a highly purified plasma membrane preparation from rat luteinized ovary which has unusual affinity for in vitro binding of cholesterol-rich lipoproteins of diverse apoprotein composition (Reaven et al., 1990). When inspected with the electron microscope, one sees a large proportion of the bound lipoproteins associated with sheets of double membranes which morphologically resemble the specialized microvillar membranes described above. These double membrane sheets account for ~30% of the purified plasma membrane preparation. Lipoprotein binding to the plasma membrane preparation is both saturable and specific (Reaven et al., 1990). High-affinity binding occurs also with the ovary-specific hormone hCG (human chorionic gonadotropin) but not with a variety of other surface active proteins (Reaven et al., 1990). The availability of this unique plasma membrane preparation provides us with the opportunity to further examine the selective extraction of lipoprotein-derived cholesteryl esters referred to above.

In this report, we provide evidence that we have successfully reconstituted the first stage in preferential cholesteryl ester uptake, i.e., the transfer of cholesteryl ester from diverse lipoprotein particles to plasma membranes. Transfer occurs with human- and rat-derived HDL and LDL alike and proceeds with kinetics similar to those observed in situ. The transfer is specific for the cholesteryl ester component of the lipoprotein, does not occur with lipoprotein apoproteins or with other core lipids, and does not proceed efficiently when purified endomembranes of the same cells are used in place of plasma membranes.

Experimental Procedures

Materials. [1α,2α-(n)-^{3}H]Cholesteryl linoleoyl ether (1.5-2.2 TBq/mmol, 40–60 Ci/mmol), [1α,2α-(n)-^{3}H]-

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cholesteryl oleoyl ether (1.5-2.2 TBq/mmol, 40–60 Ci/mmol), [1α,2α-(n)-^{3}H]cholesteryl linoleate (1.5-2.2 TBq/mmol, 40–60 Ci/mmol), and [1α,2α-(n)-^{3}H]cholesteryl oleate (1.5-2.2 TBq/mmol, 40–60 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL. [^{125}I]NaI (sodium iodide) (~629 GBq/mg; ~17 Ci/mg) was purchased from New England Nuclear Research Products, Du Pont Co., Wilmington, DE. All other chemicals used were of analytical grade.

Animals. Superovulated rats were prepared as described previously (Reaven et al., 1984).

Isolation of Plasma Membranes. The plasma membrane fraction from superovulated (luteinized) rat ovaries was prepared by a modification of technique of Bramley and Ryan (1978). In brief, ovaries were homogenized in SET buffer (0.3 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) using an all-glass Dounce-type homogenizer. The homogenate was centrifuged at 800*g* to obtain a nuclear fraction. The sediment (nuclear fraction) was treated with DNAse (100 μg/mL) and RNAase (100 μg/mL), resuspended in SET, layered on a discontinuous sucrose gradient (50, 40, 36, and 30%), and centrifuged at 63000*g* for 90 min. Membranes sedimenting at the SET–30% sucrose interface were collected and used as the plasma membrane fraction. Bovine corpus luteum plasma membranes were prepared as described previously (Azhar & Menon, 1976).

Enzyme Assays. The procedures for the assay of enzyme markers have been described previously (Reaven et al., 1990). [^{125}I]hCG and [^{125}I]lipoprotein binding was carried out as described by Reaven et al. (1990).

Isolation and Iodination of Lipoproteins. Rat (r)HDL, apoE-free human (h)HDL$_3$, and hLDL were isolated by sequential ultracentrifugation (Azhar et al., 1989). Iodination of the lipoproteins was carried out as described previously (Azhar et al., 1989).

Preparation of [^{125}I]DLT–[^{3}H]CLE Lipoprotein. Native plasma lipoproteins (rHDL$_3$, rHDL, or hLDL) were incubated with sonicated dispersions of phosphatidylcholine and [^{3}H]-cholesteryl linoleyl ether ([CLE] or [^{3}H]cholesteryl oleate [CO]) in the presence of partially purified cholesteryl ester transfer protein (Azhar et al., 1989) and diethyl *p*-nitrophenyl phosphate, which suppresses lecithin:cholesterol acyltransferase activity. The [^{3}H]CLE-labeled lipoproteins were then isolated by ultracentrifugation. Doubly labeled [^{125}I]DLT–[^{3}H]CLE lipoprotein was prepared by conjugation of [^{125}I]dilactitol tyramine (DLT) with [^{3}H]CLE–lipoprotein. Average [^{125}I]DLT specific radioactivities of labeled hLDL, hHDL$_3$, and rHDL were 79, 112, and 83 cpm/ng of protein, respectively. Average [^{3}H]CLE specific radioactivities of labeled hLDL, hHDL$_3$, and rHDL were 2600, 2100, and 3500 cpm/μg of protein, respectively. It should be noted that the DLT label was used in these studies (rather than conventionally labeled ^{125}I particles) since particles with the nonreleasable label had previously been employed to demonstrate the selective uptake of cholesteryl esters in situ (Azhar et al., 1989). However, simple ^{125}I-labeled lipoproteins can also be used to demonstrate the selective transfer of cholesteryl esters to plasma membranes.

Preparation of Synthetic [^{125}I]DLT–[^{3}H]CLE–hHDL$_3$ and [^{14}C]Sucrose Octaoleate–HDL$_3$. Synthetic hHDL$_3$ particles containing trace amounts of [^{3}H]CLE and/or [^{14}C]sucrose octaoleate (Rizzi & Taylor, 1978) were prepared according to the procedure of Hirz and Scanu (1970).

Transfer of Cholesteryl Ester from the Lipoproteins to the Plasma Membranes. Donor lipoproteins, (hHDL$_3$, rHDL, and hLDL) containing various lipid core molecules and ^{125}I-labeled apoproteins (to monitor cell surface-associated ligand) were incubated with acceptor plasma membranes (0.05–0.2 mg of protein/mL), 100 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% BSA] in a final volume of 0.5 mL. After incubation at 37 °C for 1 h, transfer was terminated by sedimenting the membranes by centrifugation. The membranes were subsequently treated with trypsin (4 mg/mL, 1 h, 4 °C), in an effort to reduce surface-bound radioactivity. Finally, suitable aliquots of membranes were counted for ^{125}I and ^{3}H radioactivity (after extraction with organic solvents).

In all instances, membrane radioactivity was normalized to micrograms of protein [apoprotein mass (Azhar et al., 1989)] and the amount of [^{3}H]CLE (or ester) transferred to PM was calculated as follows: net cholesteryl ester transferred = total ^{3}H radioactivity (expressed as μg of protein/mg of membrane protein) minus ^{125}I radioactivity (expressed as μg of protein/mg of membrane protein). This calculation is based on two assumptions: (1) that apoprotein ^{125}I will not be transferred to membranes and, as such, can indicate the amount of intact surface-associated protein which remains despite trypsin treatment, and (2) that the surface-associated ^{125}I radioactivity indicates the amount of surface-associated ^{3}H radioactivity (in remaining intact lipoproteins), since both labels are on the same particle (Azhar et al., 1989). Values for [^{3}H]CLE transferred from lipoproteins were subsequently divided by the protein/cholesterol ratio for each donor ligand (i.e., 3.1, 1.8, and 0.67 for hHDL$_3$, rHDL, and hLDL, respectively). Such calculations provide data on the *net mass* of cholesteryl ester which is transferred to plasma membranes (expressed as μg/mg of membrane protein).

Cholesteryl Esterase Treatment of Ovary Plasma Membranes. Aliquots of ovary plasma membranes were incubated with buffer (phosphate-buffered saline, pH 7.4) alone or buffer containing 10 units/mL of cholesteryl esterase (Boehringer Mannheim; 10 units/mg of membrane protein) for 60 min at 30 °C. Following incubation, the membranes were washed extensively and either used immediately in transfer experiments (as described above) or subjected to organic solvent extraction followed by TLC to separate and quantitate cholesteryl esters, cholesterol, and phospholipids.

Miscellaneous Procedures. Cholesterol and cholesteryl ester content of luteal plasma membranes and lipoproteins was determined according to Vahouny et al. (1963). Phospholipid content was measured as previously described by us (Reaven & Azhar, 1981). The protein content of membranes was determined by a modification (Markwell et al., 19816) of the procedure of Lowry et al. (1951). Acyl CoA:cholesterol acyltransferase (ACAT) and cholesteryl esterase activities were measured as described previously (Popplewell & Azhar, 1987).

Results

Membrane Characteristics

The biochemical and morphological characteristics of the plasma membrane used in these studies have been described elsewhere (Reaven et al., 1990). The membranes show all the characteristics associated with a purified plasma membrane fraction, yet the membranes retain the functional characteristics observed in situ. In brief, the activities of specific ovary cell surface markers, such as hCG or adenylate cyclase, are 12–14-fold enriched in the plasma membrane fraction over that found in homogenate from the same tissue (Reaven et al., 1990). In contrast, traditional markers for intracellularly located organelles show no increase in the plasma membrane preparation (Reaven et al., 1990). Acyl CoA:cholesterol

Table I: Quantification of Lipoprotein $hHDL_3$–Cholesteryl Ester ([^{3}H]CLE) Transfer to Plasma Membranes[a]

treatment	radioactivity (μg of protein/mg of membrane protein) [^{125}I]DTT	[^{3}H]CLE	net [^{3}H]CLE transferred (μg of protein/mg of membrane protein)
−trypsin	20.0 ± 2.5	168.8 ± 8.8	
+trypsin	10.1 ± 1.3 (a)	148.8 ± 7.5 (b)	138.7 ± 6.2

[a] Plasma membranes (100 μg of protein) were incubated 1 h, at 37 °C, with [^{125}I]DTT–[^{3}H]CLE–$hHDL_3$ (50 μg of protein), treated with trypsin (4 mg/mL) (or not), and measured for ^{125}I and ^{3}H radioactivity (after extraction with organic solvents). After trypsin treatment, ~50% of the bound ^{125}I radioactivity was released (98% of the intact [^{125}I]DTT as judged by TCA precipitation). Radioactivity was normalized to micrograms of protein (apoprotein mass). Net [^{3}H]CLE transferred is calculated by subtracting *b–a*; i.e., [^{3}H]CLE radioactivity (protein) − [^{125}I]DTT radioactivity (protein) still associated with the plasma membrane fraction after trypsin treatment. The results shown are mean ± SE of four separate experiments.

aceyltransferase (ACAT) was not detected, and no significant activity of cholesteryl esterase was found. The phospholipid:protein and cholesterol:phospholipid ratios of this membrane preparation are 0.47 and 0.54; the ratio of total cholesterol to phospholipid is 0.76.

At the electron microscope level, the fraction contains primarily two components; i.e., small, single-membraned vesicles and sheets of double membranes resembling the specialized microvillar membranes observed in vivo: the double membranes comprise ~30% of the total membrane preparation (Reaven et al., 1990). Various lipoproteins ($hHDL_3$, rHDL, and hLDL) and hHCG show highly specific binding to the plasma membrane preparation and great affinity for the double membrane sheets (Reaven et al., 1990).

Transfer of Lipoprotein-Derived Cholesteryl Esters

(*a*) *Measurements.* Table I demonstrates that incubation of the membranes with [^{125}I]DLT–[^{3}H]CLE–lipoprotein (in this case $hHDL_3$) results in the association of considerably more [^{3}H]CLE than [^{125}I]DLT radioactivity with the membranes: this transferred radioactivity is calculated as micrograms of protein [apoprotein mass (Azhar et al., 1989)] and expressed per milligram of membrane protein. After trypsin treatment, we find that ~50% of the bound [^{125}I]protein is released: 98% of this released radioactivity is found to be TCA-precipitable protein and is assumed to have come from surface-bound, nondegraded apoprotein associated with intact lipoproteins. Since the [^{3}H]CLE label is on the same intact particle, we estimate that an equal amount of [^{3}H]CLE protein is also released. The *remaining* membrane-associated CLE (protein) minus the *remaining* DLT (protein) is calculated as the net amount of cholesteryl ester transferred.

(*b*) *Cholesteryl Ester Transfer from Various Lipoprotein Particles.* Figure 1 compares relative [^{125}I]DLT and [^{3}H]CLE transfer from $hHDL_3$ (panel A), rHDL (panel B), and hLDL (panel C) to plasma membranes. The data indicate that all the particles used are capable of selectively transferred cholesteryl esters (i.e., more [^{3}H]cholesteryl ester than [^{125}I]DLT is transferred when expressed as μg of protein/mg of membrane protein); in the case of $hHDL_3$, this differential is 15-fold, with rHDL it is 5-fold, and with hLDL it is 2-fold. Small differences in [^{125}I]DLT radioactivity are also noticed with the three particles which probably relates to ligand differences in membrane binding capacity.

When the cholesteryl ester transferred by the different lipoproteins is viewed in terms of the starting protein:cholesterol ratios of the particles, one can compare the net mass of the

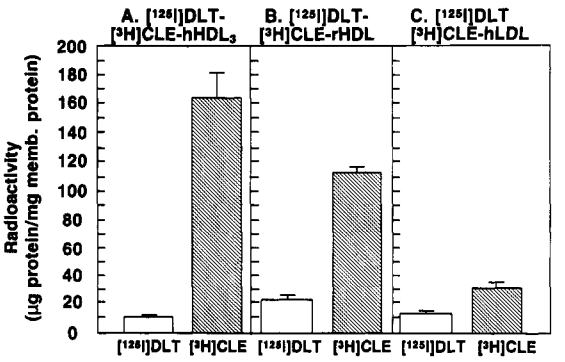

FIGURE 1: Comparison of selective transfer of [^{3}H]CLE to plasma membranes when using $hHDL_3$, rHDL, or hHDL as donor lipoproteins. Aliquots of plasma membranes (0.2 mg/mL) were incubated for 1 h at 37 °C in a medium (final volume, 0.5 mL) containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% BSA, 100 μg of protein/mL of [^{125}I]DLT–[^{3}H]CLE–$hHDL_3$, rHDL, or hLDL ± unlabeled homologous lipoproteins (1000 μg/mL), and 100 μM DTNB. After incubation, transfer was terminated by sedimenting the membranes by centrifugation. The membranes were subsequently treated with trypsin (4 mg/mL; 1 h at 4 °C) in an effort to reduce surface-bound radioactivity. Suitable aliquots of membranes were counted for ^{125}I and ^{3}H radioactivity (after extraction with organic solvents). The results are normalized to micrograms of protein per milligram of membrane protein and show relative differences in uptake of [^{3}H]CLE and [^{125}I]DTT by membranes. The protein to cholesterol ratios for $hHDL_3$, rHDL, and hLDL were 3.1, 1.8, and 0.67, respectively. Results represent mean ±SE of three experiments with each lipoprotein.

cholesteryl ester transferred by $hHDL_3$, rHDL, and hLDL. The relative amounts of CLE transferred from each type of lipoprotein (as shown in Figure 1) can be divided by the protein:cholesteryl ester ratio for each ligand (see Experimental Procedures). When expressed this way, the large [^{3}H]CLE transfer differences obtained with the three ligands are reduced: in fact, $hHDL_3$ and rHDL are shown to be equally effective in the selective transfer of cholesterol ester mass to membranes (i.e., 50 ± 6 and 51 ± 8 μg/mg of membrane protein for $hHDL_3$ and rHDL, respectively) and transfer from hLDL is only 40% less (i.e., 30 ± 4 μg/mg of protein) under identical circumstances.

The selective transfer of [^{3}H]CLE to membranes from each lipoprotein in inhibited 80–85% by the use of excess (500 μg of protein/mL) homologous lipoprotein. Thus each lipoprotein competitively inhibits its own transfer of cholesteryl ester. To a large extent there is also cross competition relating to the transfer process: thus, when [^{125}I]DLT–[^{3}H]CLE–$hHDL_3$ is the ligand, excess homologous ligand competes for 81% of the transfer, excess rHDL competes for 78%, and excess hLDL competes for 70% of the amount of cholesteryl ester transferred.

(*c*) *Compositional Change in Lipoproteins and Membrane Fractions Resulting from Net Transfer of Cholesteryl Esters.* Direct chemical measurements indicate that the net cholesteryl ester content of lipoproteins (hHDL3) decreased about one-half while the cholesteryl ester content of $hHDL_3$-incubated plasma membranes increased ~2-fold during the incubation experiments (Table II). As such, the measured mean (±SE) loss of cholesteryl esters from $hHDL_3$ was 74 ± 13 μg, and the measured gain of cholesteryl esters by the membranes was a comparable 66 ± 9 μg/mg of protein. In the same experiment, the isotopic gain of the membrane fraction was similar; i.e., 59 ± 7 μg of cholesteryl ester was estimated to have been transferred per milligram membrane protein. The fact that the isotopic experiments from these scaled-up studies resulted in values equivalent to those obtained previously added confidence to the use of this methodology.

Table II: Comparison of Different Methods for Quantifying the Mass of Lipoprotein–Cholesteryl Ester Transferred to Plasma Membranes[a]

procedures	$hHDL_3$–cholesterol (μg of total cholesterol/500 μg of $hHDL_3$)	plasma membranes (μg of CE/mg of plasma membrane protein)
(A) chemical[b]		
(a) cholesterol before incubation	161 ± 23	67 ± 9
(b) cholesterol after incubation	87 ± 10	133 ± 19
(c) Δ difference (a–b)	74 ± 13	66 ± 9
(B) isotopic		
calculated mass of CE transfer to plasma membranes		59 ± 7[c]

[a] Incubation conditions for measurement of cholesteryl ester transfer by the chemical procedure were as follows: aliquots of plasma membranes (0.2 mg/mL) were incubated for 1 h at 37 °C in a medium (final volume 5 mL) containing 500 μg of $hHDL_3$ plus a trace amount of [^{125}I]-DLT-[^{3}H]CLE–$hHDL_3$, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% BSA, and 100 μM DTNB. Control incubations contained plasma membranes alone or $hHDL_3$ plus trace amount of [^{125}I]DLT–[^{3}H]CLE. After incubation, transfer was terminated by sedimenting the membranes by centrifugation. The membranes were subsequently treated with trypsin (4 mg/mL, 1 h at 4 °C) to reduce surface-bound lipoprotein. Suitable aliquots of membranes were counted for ^{125}I radioactivity. Also, membranes were extracted with organic solvents, and the mass of cholesteryl ester transferred was measured (Vahouny et al., 1963). Net cholesteryl ester transferred to plasma membranes was calculated by subtracting the amount of lipoprotein cholesterol still associated with plasma membranes after trypsin treatment. Incubation conditions for the isotopic procedure were similar to those described under Table I. The data are expressed as means ± SE of three separate experiments. [b] Results for plasma membrane "free" cholesterol were 96 ± 11, 101 ● 12, and 5 ± 1 for procedures A(a), A(b), and A(c), respectively. [c] Calculations for the transfer of CE mass were as described in the text.

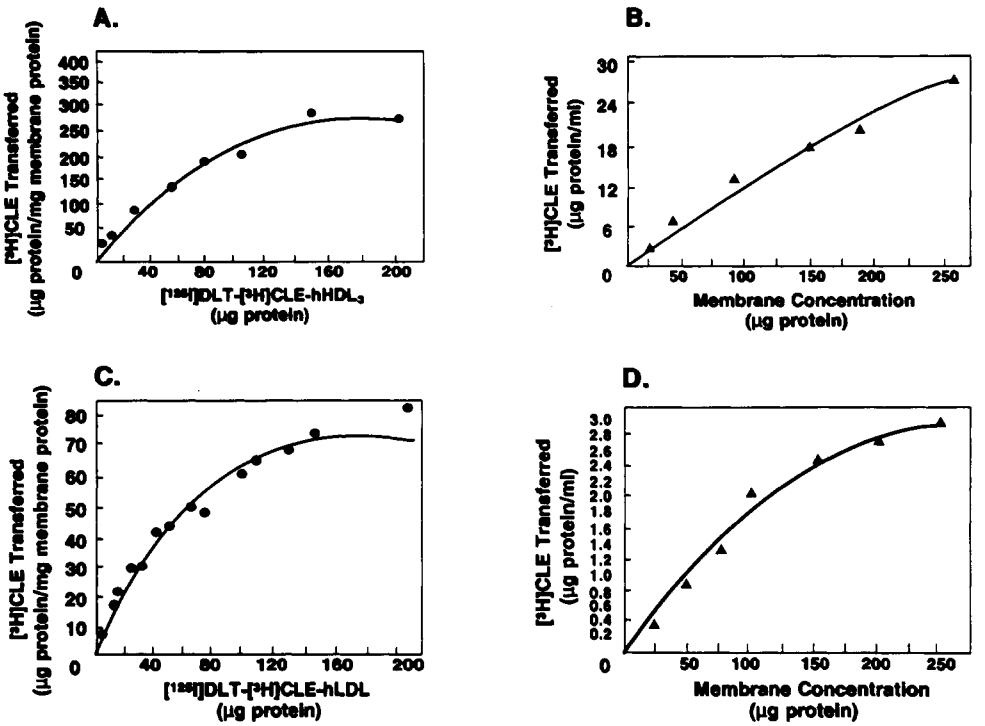

FIGURE 2: Effect of ligand concentration and plasma membrane concentration on the transfer of [^{3}H]CLE from $hHDL_3$ (A, B) and hLDL (C, D). For ligand concentration curves, 100 μg/mL membrane protein was used; for membrane concentration curves, 50 μg/mL ligand protein was used. Lipoprotein concentration, plasma membrane concentration, and incubation time curves were drawn with a Macintosh SE computer using Kaleida graph program (Synergy Software, Reading, PA). Results represent the mean of two separate experiments for each condition.

(d) Kinetics of the Transfer Process and the Various Lipid Controls. Kinetic experiments were carried out with all three lipoproteins, but, for convenience, only data for $hHDL_3$ and hLDL are given. These figures show that the effect of ligand (Figure 2A,C) and membrane concentration (Figure 2B,D) on the process of selective [^{3}H]CLE transfer to membranes is similar, regardless of the ligand used (note that kinetic information for rHDL is also comparable). Thus transfer of [^{3}H]CLE increases linearly to 100 μg with ligand protein and approaches a maximum between 120 and 140 μg of protein; plasma membrane concentration approaches saturation at 250 μg of protein. Transfer begins within 15 min of incubation, is complete in 1 h, and is more efficient at 37 than at 4 °C for both $hHDL_3$ and hLDL (data not shown).

The specificity of transferring [^{3}H]linoleoyl ether to plasma membranes was tested by comparing transfer from HDL_3 particles in which [^{3}H]cholesteryl oleoyl ether was used as a marker. No differences were seen in the ^{3}H radioactivity transferred. Likewise, no differences were seen in the transfer of ^{3}H radioactivity with the use of different biological esters (i.e., cholesteryl linoleate, and cholesteryl oleate). All these particles transferred between 140 and 150 μg of protein/mg of membrane protein under the conditions of the experiments, suggesting that the use of the CLE component was a reasonable particle for tracking cholesteryl esters. In addition, it was determined that if lipoproteins were labeled in the fatty acid position (rather than the cholesterol moiety), no differences in the rate of transfer occurred (data not shown).

In Figure 3, we examine the question of whether lipoprotein cholesteryl esters (or ethers) are specifically transferred in our system or could an irrelevant lipoprotein–core lipid component be transferred as well? For this purpose, we reconstituted $hHDL_3$ with a [^{3}H]CLE core (panel B) similar to that used in the standard procedure (panel A). We compared [^{3}H]CLE

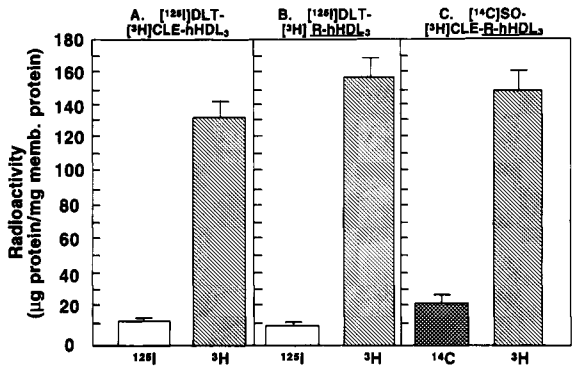

FIGURE 3: Comparison of selective [^{3}H]cholesterol transfer from $hHDL_3$ or from synthetic (reconstituted) $hHDL_3$ or from reconstituted particles colabeled with [^{14}C]sucrose octaoleate (SO). In a given incubation, the ratio of lipoprotein cholesteryl ester to plasma membrane cholesteryl ester for various $hHDL_3$ preparations was in the range of 3.0–3.2. Results are mean ± SE of three experiments. *R* = synthetic (reconstituted) $hHDL_3$. [^{125}I] = [^{125}I]DLT; [^{3}H] = [^{3}H]CLE; [^{14}C] = [^{14}C]SO.

Table III: Effect of Cholesteryl Esterase Treatment of Plasma Membranes on Transfer of $hHDL_3$–[^{3}H]CLE[a]

treatment	cholesteryl ester [μmol/(mg of membrane protein)]	[^{3}H]CLE transferred [μg of protein/(mg of membrane protein)]
control	0.127 ± 0.007 (100.0)	180.8 ± 19.7 (100.0)
cholesteryl esterase	0.038 ± 0.004 (29.8)	180.0 ± 23.8 (99.5)

[a] Incubation conditions were the same as described under Experimental Procedures. The results are mean ± SE of eight separate experiments. The number in parentheses represents the percent change after cholesteryl esterase treatment.

transfer from this particle to that achieved with a reconstituted particle containing both [^{3}H]CLE and an irrelevant core lipid ester [^{14}C]sucrose octaoleate (SO) (panel C). Although the results show that [^{3}H]CLE is transferred equally well from "native" and "reconstituted" HDL particles (panels A, B, and C), only minimal transfer occurs from the sucrose octaoleate component (panel C).

To ascertain whether the movement of [^{3}H]CLE from the lipoprotein particle to plasma membranes is a function of the endogenous pool of membrane cholesteryl ester (acting to facilitate "exchange" of radioactivity), we carried out experiments in which the plasma membrane fraction was treated with cholesteryl esterase prior to incubation with lipoproteins ($hHDL_3$). Table III shows that cholesteryl esterase was effective in reducing the cholesteryl ester content of the membrane fraction to a very low level but that treatment in no way affected the transfer of [^{3}H]CLE radioactivity.

(*e*) *Membrane Controls for Cholesteryl Ester Transfer.* Cholesteryl ester transfer from [^{125}I]DLT–[^{3}H]CLE–$hHDL_3$ particles to plasma membranes was also compared to [^{3}H]CLE transfer to potential non-plasma membrane acceptor membranes prepared from the same tissue source. When equal concentrations of the different membranes are used, 3 times more cholesteryl ester is transferred to plasma membranes than to either SER or RER (data not shown).

Finally, identical CE transfer experiments were carried out using [^{125}I]–[^{3}H]CLE–$hHDL_3$ particles and isolated bovine corpus luteum plasma membranes. This fraction was not as pure as the rat plasma membranes used and contained relatively fewer images of the double membranes believed to trap the lipoproteins prior to CE transfer (Reaven et al., 1990). CE transfer was observed with the bovine membranes but represented only 50% (when expressed per milligram of membrane protein) of that seen with the rat plasma membranes (data not shown).

Discussion

These experiments show that various intact cholesterol-rich lipoproteins [doubly labeled with radioactive nonreleasable apoprotein and cholesteryl ester (or ether) markers] rapidly and selectively transfer the cholesteryl ester moiety of lipoproteins to purified ovary plasma membranes. The characteristics of the system show dependence on ligand and membrane concentration, time, and temperature and suggest that it is a true physiological process and not simply an artifact of an in vitro reconstitution system. The transfer is specific for the cholesteryl ester (or ether) portion of the lipoprotein and does not involve the transfer of lipoprotein apoproteins or other core lipid esters (e.g., octaoleate) of the lipoproteins. Endomembranes (e.g., SER and RER) from the same tissue source cannot substitute for plasma membranes as the primary cholesteryl ester acceptor.

Human and rat HDL and human LDL can serve as cholesteryl ester donors under the conditions used in the studies. Although large differences exist in the relative amount of cholesteryl esters which can be transferred from each lipoprotein type, the calculated mass of transferred lipid is quite similar when lipid to protein ratios of the donor particles are taken into consideration. That the calculated mass of transferred radioactive lipid is, in fact, a reliable value can be seen from control experiments in which direct chemical measurement of $hHDL_3$–cholesteryl ester transfer to membrane was entirely comparable to the reported [^{3}H]CLE transfer. Additional experiments show that the loss of lipoprotein–cholesterol ester equals the cholesteryl ester gain of the plasma membranes during the incubation period. Finally, transfer is prevented in the presence of excess unlabeled lipoprotein of the same class but is also largely prevented in cross-competition experiments where excess unlabeled lipoproteins (e.g., rHDL or hLDL) are incubated with a different labeled lipoprotein such as $hHDL_3$.

In considering these data, several questions come to mind regarding the mechanism of the transfer process. Is this true transfer of cholesteryl ester from lipoproteins to membranes or something else which may be better termed "lipid fusion" or "lipid exchange"? Although it is not always easy to make these distinctions, several experiments were done which unequivocally favor the transfer theory. In one experiment, lipoproteins were reconstituted with a large core lipid (sucrose octaoleate) which cannot be transferred to membranes. If the mechanism we are studying merely involved stripping away of particle apoprotein and subsequent fusion of the core lipid with plasma membranes, then one would expect that the labeled sucrose octaoleate molecule (like the labeled CLE molecule on the same particle) would have become associated with plasma membranes. Since only the CLE moiety was membrane associated after the incubation period, we believe this rules against the idea that lipid "fusion" is responsible for our findings.

Likewise were cholesteryl ester "exchange" the explanation of data, one would not expect to find an absolute loss of cholesteryl ester mass from the incubated lipoproteins or an absolute gain of cholesteryl ester mass in the membrane fraction. Nor would one expect to see the process dependent on ligand or membrane concentration, or time, or temperature of the incubation. Finally, for cholesteryl ester exchange to occur, there must be an endogenous membrane cholesteryl ester pool to exchange with. Most plasma membranes studied seem to have little cholesteryl ester (Rouset et al., 1968; Steck & Wallach, 1970; Seltzman, 1975), and our data suggest that the ovarian plasma membranes used in this study are also not

especially rich in this molecule. Furthermore, if one treats the membranes with cholesteryl esterase to hydrolyze those cholesteryl esters which exist (i.e., bring the endogenous pool to even lower levels) and then use the membranes in the incubation system, [^{3}H]CLE is accepted by the membranes to the same extent as before the hydrolysis (Table III). Together, these experiment suggest that cholesteryl ester "exchange" is not the explanation for our findings.

Thus we are left with the notion of direct lipoprotein cholesteryl ester transfer to the ovarian plasma membranes. The idea that plasma membranes can directly "extract" cholesteryl esters from lipoproteins supports the notion advanced from earlier in situ perfusion (Reaven et al., 1984, 1986, 1988, 1989; Azhar et al., 1988, 1989; Azhar & Reaven, 1989) studies that intact lipoproteins can contribute their cholesteryl esters to the surface of steroidogenic cells. Indeed, we have hypothesized that the specialized microvillar plasma membranes of the ovary and adrenal provide a trapping mechanism for various lipoproteins so that this process of cholesteryl ester transfer can proceed efficiently (Reaven et al., 1988). The problem now is to understand how so much cholesteryl ester can exist in plasma membranes given the low solubility of this molecule in biological membranes (Grover et al., 1979; Valic et al., 1979; Gorrissen et al., 1981; Hamilton & Small, 1982; Hamilton et al., 1983; Spooner et al., 1986). Conceptually, this issue is less important when considering intact cells since a great deal of cholesteryl ester could be moved "through" the plasma membrane domain if intracellular acceptors were available. In the reconstituted system of this study, no such intracellular acceptors were available, and we have to account for more cholesteryl ester uptake than one might predict. Several possibilities exist. For one, cholesteryl ester uptake is a direct function of the phospholipid to protein (PL/P) ratio of membranes (Hamilton & Small, 1982; Hashimoto & Fogelman, 1990; Lange & Steck, 1985), and this ratio changes with the cell type (Seltzman et al., 1975; Eberhagen & Jossiphov, 1970; Molitoris & Simon, 19185; Meier et al., 1984) and even specific plasma membrane domains within the same cell (Molitoris & Simon, 1985; Meier et al., 1984). Many membranes PL/P ratios are in the 0.2–0.7 range (Eberhagen & Jossiphov, 1970; Molitoris & Simon, 1985; Meier et al., 1984); some PL/P ratios, such as bovine adrenal plasma membranes, are much higher and are in the 1.2 range (Seltzman et al., 1975). Our measurements show that ovarian plasma membrane PL/P is ~0.5. It is possible, therefore, that these membranes can accept a substantial amount of cholesterol esters in soluble form, though it is not yet clear precisely how much. Studies with liver sinusoidal membranes, in fact, show that in vivo ethinyl estradiol treatment results in a 14-fold increase in membrane cholesteryl ester content (Rosario, 1988). On the other hand, cholesteryl esters could exist in membranes in a different physical (i.e., nonsoluble) state (Janiak et al., 1974, 1979). As such, lipids, as in artificial membranes, could partition in layers or be dispersed in microdroplets before being hydrolyzed, or bound to transfer proteins, or transferred directly to intracellular membranes. We do not yet know which of these mechanisms is operating in the lipoprotein cholesteryl ester–membrane transfer system we've described, but the idea that the membranes are capable of accepting cholesteryl esters is consistent with these possibilities.

This brings us to the final point. The findings in this report demonstrate that an in vitro reconstituted system is capable of reproducing what we believe is an early stage of a complex in vivo process. In utilizing this system, we have hope of unraveling mechanistic and regulatory details of the transfer process which would be difficult to do, if not impossible, in intact systems.

Registry No. CLE, 73505-32-5.

References

Arbeeny, C. M., Rifici, V. A., & Eder, H. A. (1987) *Biochim. Biophys. Acta 917*, 9–17.

Azhar, S., & Menon, K. M. J. (1976) *J. Biol. Chem. 251*, 7398–7404.

Azhar, S., & Reaven, E. (1989) *Biochim. Biophys. Acta 1004*, 61–66.

Azhar, S., Cooper, A., Tsai, L., Maffe, W., & Reaven, E. (1988) *J. Lipid Res. 29*, 869–882.

Azhar, S., Stewart, D., & Reaven, E. (1989) *J. Lipid Res. 30*, 1799–1810.

Azhar, S., Tsai, L., & Reaven, E. (1990) *Biochim. Biophys. Acta 1047*, 148–160.

Bachorik, P. S., Virgil, D. G., & Kwiterovich, P. O., Jr. (1987) *J. Biol. Chem. 262*, 13636–13645.

Bramley, T. A., & Ryan, R. J. (1978) *Endocrinology 103*, 778–795.

Despres, J.-P., Fong, B. S., Jimenez, J., Julien, P., & Angel, A. (1988) *Am. J. Physiol. 254*, E667–E675.

Eberhagen, D., & Jossiphov, J. (1970) *J. Physiol. Chem. 351*, 399–374.

Glass, C. G., Pittman, R. C., Weinstein, D. B., & Steinberg, D. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5435–5439.

Gorrissen, H., MacKay, A. L., Wassall, S. R., Valic, M. I., Tulloch, A. P., & Cushley, R. J. (1981) *Biochim. Biophys. Acta 644*, 266–272.

Grover, A. K., Forrest, B. J., Buchinski, R. K., & Cushley, R. J. (1979) *Biochim. Biophys. Acta 550*, 212–221.

Gwynne, J. T., & Strauss, J. F., III (1982) *Endocr. Rev. 3*, 299–329.

Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem. 257*, 7318–7321.

Hamilton, J. A., Miller, K. W., & Small, D. M. (1983) *J. Biol. Chem. 258*, 12821–12826.

Hashimoto, S., & Fogelman, A. M. (1980) *J. Biol. Chem. 255*, 8678–8684.

Hirz, R., & Scanu, A. M. (1970) *Biochim. Biophys. Acta 207*, 364–366.

Janiak, M. J., Loomis, C. R., Shipley, G. G., & Small, D. M. (1974) *J. Mol. Biol. 86*, 325–339.

Janiak, M. J., Small, D. M., & Shipley, G. G. (1979) *J. Lipid Res. 20*, 183–199.

Lange, Y., & Steck, T. L. (1985) *J. Biol. Chem. 260*, 15592–15597.

Leitersdorf, E., Israeli, A., Stein, O., Eisenberg, S., & Stein, Y. (1986) *Biochim. Biophys. Acta 878*, 320–329.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

MacKinnon, M., Savage, J., Wishart, R., & Barter, P. (1986) *J. Biol. Chem. 261*, 2648–2552.

Markwell, M. A. K., Hass, S. M., Tolbert, N. E., & Bieber, L. L. (1981) *Methods Enzymol. 72*, 296–303.

Meier, P. J., Sztul, E. S., Reuben, A., & Boyer, J. L. (1984) *J. Cell Biol. 98*, 991–1000.

Molitoris, G., & Simon, F. R. (1985) *J. Membr. Biol. 83*, 207–215.

Pittman, R. C., Knecht, T. P., Rosenbaum, M. S., & Taylor, C. A., Jr. (1987) *J. Biol. Chem. 262*, 2443–2450.

Popplewell, P. Y., & Azhar, S. (1987) *Endocrinology 121*, 64–73.

Reaven, E., & Azhar, S. (1981) *J. Cell Biol. 89*, 300–308.

Reaven, E., Chen, Y.-D. I., Spicher, M., & Azhar, S. (1984) *J. Clin. Invest. 74*, 1384-1397.
Reaven, E., Chen, Y.-D. I., Spicher, M., Hwang, S.-F., Mondon, C. E., & Azhar, S. (1986) *J. Clin. Invest. 77*, 1971-1984.
Reaven, E., Boyles, J., Spicher, M., & Azhar, S. (1988) *Arteriosclerosis 8*, 298-309.
Reaven, E., Spicher, M., & Azhar, S. (1989) *J. Lipid Res. 30*, 1551-1560.
Reaven, E., Shi, X.-Y., & Azhar, S. (1990) *J. Biol. Chem. 265*, 19100-19111.
Rinninger, F., & Greten, H. (1990) *Biochim. Biophys. Acta 1043*, 318-326.
Rizzi, G. P., & Taylor, H. M. (1978) *J. Am. Oil Chem. Soc. 55*, 398-401.
Rosario, J., Sutherland, E., Zaccaro, L., & Simon, F. R. (1988) *Biochemistry 27*, 3939-3946.
Rouser, G., Nelson, G. J., Fleischer, S., & Simon, G. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 5-69, Academic Press, New York.
Seltzman, T. P., Finn, F. M., Widnell, C. C., & Hofmann, K. (1975) *J. Biol. Chem. 250*, 1193-1196.
Spooner, P. J. R., Hamilton, J. A., Gantz, D. L., & Small, D. M. (1986) *Biochim. Biophys. Acta 860*, 345-353.
Steck, T. L., & Wallach, D. F. H. (1970) *Methods Cancer Res. 5*, 93-153.
Stein, Y., & Stein, O. (1985) *Adv. Exp. Med. Biol. 183*, 37-46.
Vahouny, G. V., Borja, C. R., & Weersing, S. (1963) *Anal. Biochem. 6*, 555-559.
Valic, M. I., Gorrissen, H., Cushley, R. J., & Bloom, M. (1979) *Biochemistry 18*, 854-859.

Folate Binding Protein from Kidney Brush Border Membranes Contains Components Characteristic of a Glycoinositol Phospholipid Anchor†

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ABSTRACT: A number of cell surface proteins have been shown to be anchored to the plasma membrane by a covalently attached glycoinositol phospholipid (GPL) in amide linkage to the C-terminus of the mature protein. We applied several criteria to establish that folate binding protein (FBP) in brush border membranes of rat kidney contains a GPL anchor. Brush border membranes were isolated and labeled with [^{3}H]folate, and the complex of FBP and [^{3}H]folate was shown to be released to the supernatant by incubation with purified bacterial phosphatidylinositol-specific phospholipase C (PIPLC) but not by incubation with a purified bacterial phosphatidylcholine-specific phospholipase C. The FBP-[^{3}H]folate complex both in crude extracts and after FBP purification by ligand-directed affinity chromatography interacted with Triton X-114 micelles, and prior incubation with PIPLC prevented this detergent interaction. Individual residues characteristic of GPL anchors were found to be covalently associated with FBP following polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These included glucosamine and ethanolamine, which were radiolabeled by reductive methylation and identified by chromatography on an amino acid analyzer, and inositol phosphate, which was inferred by Western blotting with an anti-CRD antisera. This antisera gave positive immunostaining only after FBP had been cleaved by PIPLC, a reliable diagnostic of a GPL anchor. The relationship between GPL-anchored FBP in biological membranes and soluble FBP in biological fluids also is discussed.

Integral membrane proteins generally maintain intimate contact with the hydrophobic phase of phospholipid bilayer membranes through hydrophobic amino acid residues in one or more transmembrane segments (Sabatini et al., 1982). These proteins require detergents for solubilization and bind detergents in extracts. However, it has become apparent that the membrane association of several intracellular proteins is mediated at least in part by covalently attached lipid groups. The attachments can involve acylation of protein residues by the fatty acids myristate or palmitate (Sefton & Buss, 1987) or the isoprenylation of cysteine residues by farnesyl or geranylgeranyl groups (Maltese, 1990). Recently, a new class of more than 50 extracellular membrane proteins has emerged that are anchored, not by a transmembrane peptide segment, but solely by a glycoinositol phospholipid (GPL) linked covalently to the protein C-terminus. Many features of proteins with GPL anchors are covered in excellent review articles by Ferguson and Williams (1988) and Cross (1990).

The most widely applied criterion for the identification of GPL-anchored proteins is their selective release from intact cells or plasma membrane preparations by highly purified

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