The rate of transfer of unesterified cholesterol from rat erythrocytes to emulsions modeling nascent triglyceride-rich lipoproteins and chylomicrons depends on the degree of fluidity of the surface

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We have measured the rate of transfer of unesterified cholesterol from rat erythrocyte to triolein emulsions modeling nascent triglyceride-rich lipoproteins. Emulsions (mean diameter ~130 nm) were prepared with low cholesterol content (less than 2%) and various phosphatidylcholines that resulted in fluid (egg yolk phosphatidylcholine, dimyristoyl phosphatidylcholine) at transition (dipalmitoyl phosphatidylcholine) and solid (distearoyl phosphatidylcholine) surfaces at 37° C. Emulsions were incubated for 0, 20, 60, and 180 min with rat erythrocytes. Incubation mixtures initially contained approximately equal masses of phospholipid in the emulsion surfaces and the outer layer of plasma membrane of rat erythrocytes. There was a gradual and significant increase ($P < 0.05$) in the percent mass of unesterified cholesterol and consequently unesterified cholesterol to phospholipid molar ratio in the surface phase of three reisolated emulsions with time: dimyristoyl phosphatidylcholine-low cholesterol- >> egg yolk phosphatidylcholine-low cholesterol- > dipalmitoyl phosphatidylcholine-low cholesterol-triolein. There was no significant change in the composition of the surface phase of distearoyl phosphatidylcholine-low cholesterol-triolein emulsions. Therefore, transfer of unesterified cholesterol to the surface phase of emulsions during incubation with intact rat erythrocytes at 37° C in the absence of transfer proteins and plasma proteins is attributable to the degree of surface fluidity of emulsions. (J. Nutr. Biochem. 4:630–634, 1993.)

Keywords: Surface fluidity; triolein emulsions; phospholipids; rat erythrocytes; unesterified cholesterol

Introduction

Phospholipid-stabilized triglyceride-rich protein-free emulsions, such as Intralipid (Kabivitrum, Stockholm, Sweden), have been employed extensively in human parenteral nutrition[1-4] and in metabolic studies as models for plasma and lymph chylomicrons and very low density lipoproteins and their remnants.[5-12]

In a series of in vivo studies in rats it was demonstrated that the metabolic fate of model emulsions depends on the phospholipid composition and the cholesterol content of the surface monolayer.[13-15] Furthermore, the physical state of lipid molecules at the lipid-water interface and the surface pressure of the surface monolayer play important roles in the binding of apolipoproteins to the surface[11,15] and in the lipolysis of emulsions by hepatic or lipoprotein lipase.[16,17]

The asymmetric distribution of lipids in cell mem-

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branes has been well documented.[18] Earlier studies have demonstrated that the surface components of erythrocytes, mainly unesterified cholesterol, exchange and transfer to circulating plasma lipoproteins.[19–22]

In this investigation, well-characterized protein-free triolein emulsions containing low unesterified cholesterol concentrations and various phosphatidylcholines resulting in fluid (egg yolk phosphatidylcholines, EYPC; dimyristoyl phosphatidylcholine, DMPC) at chain melting transition (dipalmitoyl phosphatidylcholine, DPPC) and solid (distearoyl phosphatidylcholine, DSPC) surfaces at 37° C[7–9,11,14,15,17,23] were incubated for up to 3 hr at 37° C with intact rat erythrocytes and the change in total lipid and phase (surface and oil) compositions of the reisolated emulsions was determined.

Methods and materials

Materials

EYPC was from Avanti Polar Lipids (Birmingham, AL USA). DMPC, DPPC, DSPC, and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO USA) and triolein was from Nu Chek Prep (Elysian, MN USA). All lipids were >99% pure by thin layer chromatography and were used without further purification. Glycerol tri[9,10(n)-^{3}H]oleate (TO, triolein) was purchased from Amersham Corp. (Arlington Heights, IL USA) and its purity (>99%) was confirmed by thin layer chromatography and β-liquid scintillation spectrometry (Rackbeta 1217, Pharmacia LKB Nuclear, Inc., Gaithersburg, MD USA).

Preparation of rat erythrocytes

Male Sprague-Dawley rats (325–375 g) were purchased from Taconic Farms (Germantown, NY USA) and were maintained on Purina Rat Chow (Ralston Purina, St. Louis, MO USA) with free access to drinking water. Blood was collected from fed rats under ether anesthesia with citrate-dextrose solution as anticoagulant. Erythrocytes were sedimented by centrifugation at 3000 rpm for 15 min in a Beckman J6 centrifuge (Beckman Instruments, Inc., Palo Alto, CA USA) at 10° C. Plasma and buffy coat were removed. Packed erythrocytes were resuspended and washed three times with 10 volumes of 150 mM NaCl. The lipid composition of rat erythrocytes was: unesterified cholesterol (UC) 1.05 ± 0.03 mg/mL and phospholipids (PL) 2.77 ± 0.08 mg/mL (mean ± SEM; $n=16$). Therefore, the UC/PL molar ratio was 0.76 ± 0.02, assuming a PL molecular weight of 775.

Preparation and characterization of emulsions

Emulsions were prepared by sonication above their respective phosphatidylcholine acyl chain melting temperatures.[11,13,14,23] Briefly, glycerol tri[9,10(n)-^{3}H]oleate (40 mg; specific activity 0.5 μCi/μmol TO), cholesterol (0.75 mg), and one of the phospholipids (either EYPC, DMPC, DSPC, or DPPC; 9.25 mg) dissolved in chloroform were mixed in glass vials. Chloroform was evaporated under a stream of N_2 and lipid films were further dried down in a vacuum dessicator at 4° C for about 18 hr. Ten mL of 150 mM aqueous NaCl (pH 7.2) was added to the vials at appropriate temperatures (EYPC 0° C; DMPC 25–30° C; DPPC 45–50° C; DSPC 60–65° C) to rehydrate the dry lipid films. The lipid mixtures were sonicated at the above temperatures for 11 min at ~30% of maximum power (Branson Sonifier Model, W-350, Branson Sonic Power Co., Danbury, CT USA; 1 cm probe tip). The sonicated lipid mixtures were transferred into polyallomer ultracentrifuge tubes, overlayered with 1.5–2.0 mL double distilled water, and floated by ultracentrifugation in a SW41 rotor (Beckman Instruments) for 11 min at 23,000 rpm and 25° C. The floated emulsions were collected by tube slicing and were used within 1 hr after preparation. A 10 μL aliquot of each emulsion was fixed with 2% OsO_4 and negatively stained with 2% phosphotungstate.[24] Particle size was determined from electron micrographs of negatively stained emulsions. Specific activity of ^{3}H triolein was determined on aliquots of nonextracted emulsions. Mass of PL as phosphatidylcholine was quickly determined by a modified phospholipase D assay[25] to estimate the mass of emulsion needed per tube in incubation experiments. Initially, equal amounts of PL were present in surfaces of emulsions and in the outer layer of lipid bilayers of rat erythrocytes. It was assumed that 60% of PC is in the outer lipid layer of rat erythrocytes.[26]

Incubation of emulsions and rat erythrocytes

In each experiment all four sets of emulsions were incubated with 25% rat erythrocytes and 75% (vol/vol) Krebs-Henseleit bicarbonate buffer (KHB) for 0, 20, 60, and 180 min at 37° C. KHB (pH 7.4) contained 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 8.8 mM D-glucose.

Racks containing siliconized glass tubes in duplicate for each time point were placed in a shaking water bath at 37° C. First, 1000 μL containing 25% red blood cells (~400 μg PC) and 75% KHB (vol/vol) were added to each tube. Next, a model emulsion containing 300–400 μg PC (2.3–3.0 mg total lipid) was added to each tube in 50–160 μL, and the tubes were covered with Parafilm (VWR Scientific, Boston, MA). The specific activity of ^{3}H triolein in the emulsions was 490, 690, and 910 cpm/μg per assay tube in the three experiments, respectively. Recovery of ^{3}H was between 96–108%.

Aliquots of the original emulsions (0 time) and the incubation tubes from 20, 60, and 180 min time points were placed on ice and extracted for lipid analysis.[27] Blank tubes contained 25% erythrocytes and 75% (vol/vol) KHB and were incubated simultaneously with tubes containing emulsions.

At each time point emulsions were refloated by centrifugation at 3,000 rpm for 15 min at 10° C. Emulsions were transferred into glass tubes and were extracted with methanol/chloroform (2:1, vol/vol).[27]

Chemical analyses

Analytical assays were performed on lipids extracted into the chloroform layer.[28] PL was quantitated either by the method of Bartlett[29] or a phospholipase D assay.[25] Triglycerides (TG) were measured by a semienzymatic method (Sigma Chemical Co., Kit #320-UV). Total and unesterified cholesterol were measured by a modified cholesterol oxidase method.[30] Erythrocyte lipids were extracted by the method of Rose and Oklander.[31]

Morphology of emulsions

Particle size (diameter) was measured from electron micrographs of negatively stained emulsions.[28]

Phase analysis of emulsions

Lipid compositions of the surface and the oil phases of the emulsions were calculated from phase diagrams of emul-

sions[7-9] using a computer program ("Lipoprotein Phase Analysis Program") developed by Miller and Small.[22,32]

Statistical analysis

Data are shown (*Table 1* and *Figure 1*) as means of three experiments ± SEM unless otherwise indicated. Statistical significance was determined by one-way analysis of covariance (ANOCOVA ONEWAY) that combined one-way analysis of variance with linear regression on a single dependent variable. Significance was set at $P < 0.05$. Best-fit lines and ANOCOVA ONEWAY were determined by the RS1 computer program (BBN Software Products, Cambridge, MA USA).

Results and discussion

The lipid compositions (weight percent) of the original emulsions (0 min) are shown in *Table 1*. All four types of emulsions were constructed to have a low cholesterol content (less than 10%) on the particle surfaces, which are similar to that of nascent TG-rich particles like chylomicrons.[9,22] The initial UC to PL molar ratios of the surface phases of the four emulsions were calculated from "Lipoprotein Phase Diagram Analysis" computer program[22] and were similar between 0.16 and 0.22. Emulsion particles appeared spherical by negative staining electron microscopy. The mean diameters of emulsions (±S.D.) from negatively stained preparations in one experiment were, in nm, EYPC-LoC: 104 ±43 ($n=390$); DMPC-LoC: 79 ±41 ($n=398$); DPPC-LoC: 78 ±32 ($n=423$); and DSPC-LoC: 106 ±35 ($n=341$).

There was a linear and significant ($P < 0.05$) increase in the percent mass of UC of emulsions made with DMPC (from 1.2 to 3.2), EYPC (from 1.1 to 1.8), and DPPC (from 1.3 to 1.9) from direct chemical measurements (*Table 1*). At 37° C, DMPC and EYPC emulsions have fluid surfaces while the DPPC emulsion is at the transition temperature between solid and fluid. However, the total composition of DSPC-LoC-triolein emulsions with solid surface at 37° C due to saturated acyl chains remained statistically unaltered during the 3-hr incubation with intact rat erythrocytes (*Table 1*). To further investigate the changes in the composition of the oil and surface phases of the reisolated emulsions the percent lipid compositions were plotted on triangular coordinates (not shown)[22] and phase compositions were also analyzed by a computer program[22] (*Table 1*). The distribution coeffi-

Table 1 Total and phase composition (weight percent) of triolein emulsions before and after incubations with intact rat erythrocytes

Emulsions Incubation time	Total composition (%)			Oil phase (%)		Surface phase (%)		
	TG	UC	PL	TG	UC	TG	UC	PL
DMPC-LoC								
0 min	88.3 ± 1.2	1.0 ± 0.2	10.8 ± 1.0	99.3 ± 0.1	0.30 ± 0.04	2.8 ± 0.02	6.6 ± 0.8	90.6 ± 0.7
20 min	88.7 ± 1.1	1.2 ± 0.2	10.1 ± 0.9	99.6 ± 0.05	0.40 ± 0.05	2.7 ± 0.03	8.7 ± 1.2	88.6 ± 1.1
60 min[a]	87.8 ± 1.5	1.8 ± 0.5	10.4 ± 1.1	99.5 ± 0.1	0.53 ± 0.10	2.6 ± 0.07	11.8 ± 2.3	85.5 ± 2.2
180 min	84.9 ± 1.9	3.2 ± 0.8	12.0 ± 1.1	99.2 ± 0.1	0.80 ± 0.11	2.5 ± 0.07	17.6 ± 2.3	79.9 ± 2.3
EYPC-LoC								
0 min	89.3 ± 1.2	1.1 ± 0.2	9.6 ± 0.9	99.8 ± 0.1	0.37 ± 0.06	2.8 ± 0.04	8.1 ± 1.3	89.2 ± 1.2
20 min	87.6 ± 2.3	1.3 ± 0.2	11.1 ± 2.1	99.6 ± 0.03	0.40 ± 0.03	2.7 ± 0.02	8.8 ± 0.8	88.4 ± 0.8
60 min	89.1 ± 1.4	1.4 ± 0.2	9.6 ± 1.2	99.6 ± 0.04	0.45 ± 0.04	2.7 ± 0.03	10.0 ± 1.0	87.4 ± 0.9
180 min	89.1 ± 1.1	1.8 ± 0.3	9.1 ± 1.0	99.4 ± 0.08	0.61 ± 0.08	2.6 ± 0.05	13.4 ± 1.8	84.0 ± 1.8
DPPC-LoC								
0 min	86.7 ± 0.3	1.3 ± 0.1	12.1 ± 0.3	99.6 ± 0.01	0.36 ± 0.02	2.8 ± 0.01	7.9 ± 0.3	89.3 ± 0.3
20 min	86.6 ± 0.6	1.4 ± 0.2	12.0 ± 0.5	99.7 ± 0.03	0.39 ± 0.04	2.7 ± 0.03	8.6 ± 0.9	88.6 ± 0.9
60 min[a]	85.8 ± 0.7	1.6 ± 0.2	12.6 ± 0.7	99.6 ± 0.04	0.43 ± 0.04	2.7 ± 0.03	9.5 ± 0.8	87.8 ± 0.8
180 min[a]	86.2 ± 0.8	1.9 ± 0.4	11.9 ± 0.5	99.5 ± 0.1	0.53 ± 0.09	2.6 ± 0.1	11.6 ± 1.8	85.8 ± 1.8
DSPC-LoC								
0 min	86.7 ± 1.2	1.3 ± 0.3	12.0 ± 0.8	99.6 ± 0.04	0.40 ± 0.04	2.7 ± 0.03	8.8 ± 0.9	88.5 ± 0.9
20 min	85.9 ± 0.8	1.4 ± 0.3	12.6 ± 0.6	99.6 ± 0.06	0.39 ± 0.06	2.7 ± 0.04	8.6 ± 1.4	88.6 ± 1.4
60 min[a]	85.8 ± 0.6	1.4 ± 0.3	12.7 ± 0.4	99.6 ± 0.08	0.39 ± 0.08	2.7 ± 0.06	8.5 ± 1.8	88.8 ± 1.8
180 min	86.0 ± 0.7	1.6 ± 0.3	12.4 ± 0.5	99.6 ± 0.06	0.45 ± 0.06	2.7 ± 0.04	9.8 ± 1.3	87.5 ± 1.2
Comparison of slopes of lines:								
DMPC versus EYPC	N.S.	$P < 0.05$	N.S.	N.S.	N.S.	$P < 0.05$	$P = 0.053$	$P = 0.053$
DMPC versus DPPC	N.S.	$P < 0.05$	N.S.	N.S.	$P < 0.05$	$P < 0.05$	$P = 0.010$	$P = 0.010$
DMPC versus DSPC	N.S.	$P < 0.01$	N.S.	$P < 0.05$	$P < 0.05$	$P < 0.01$	$P = 0.002$	$P = 0.002$

The four sets of [^{3}H] triolein emulsions EYPC-LoC, DMPC-LoC, DPPC-LoC, and DSPC-LoC were prepared as described in Methods and materials. To each assay tube 1000 μL of 25% intact rat erythrocytes (~700 μg PL and 250 μg UC) and 75% (vol/vol) KHB (pH 7.4) and 1 model emulsion (containing ~300–400 μg PL) were added. Tubes were incubated at 37° C in a shaking water bath. At designated time points tubes were removed and erythrocytes were pelleted. Emulsions were reisolated by centrifugation and lipids were extracted with methanol/chloroform (2:1, vol/vol). TG, PL, and UC in chloroform layers were quantified as described. Total compositions (weight percent) are from direct chemical measurements. Phase (oil and surface) compositions were calculated by a computer program "Lipoprotein Phase Diagram Analysis",[22,32] Data are the means of three experiments ± SEM unless indicated otherwise.

[a]Mean of two experiments ± SEM. Statistical analysis was by ANOCOVA ONEWAY that compared slopes of lines.

N.S., not significant.

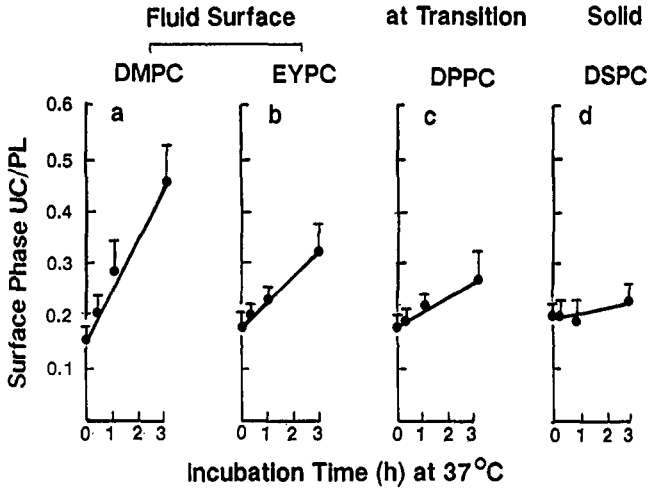

Figure 1 The changes in UC to PL molar ratio in the surface phase of reisolated emulsions following incubations with intact rat erythrocytes [25%] and KHB [75%, vol/vol] for 0, 20, 60, and 180 min at 37° C. The data points drawn are the means of three separate experiments + one SEM. Data points were calculated from *Table 1* by the computer program "Lipoprotein Phase Diagram Analysis".[22,23] The best-fit line was drawn by RS1 computer program. Comparison of the slopes of lines by ANOCOVA ONEWAY are: DMPC versus EYPC, $P < 0.050$; DMPC versus DPPC, $P < 0.010$; DMPC versus DSPC, $P < 0.002$.

cient of cholesterol between surface and core $K_{s/c}$ used here was $K_{s/c} = 22.0$. This value was derived from data on EYPC-low cholesterol-triolein emulsions.[7,22] Distribution coefficients for other emulsions were assumed to be similar.

There was a linear and significant increase in the percent mass of UC in the surface phase of three reisolated emulsions with time: DMPC-LoC from 6.6 to 17.6 ($P < 0.001$), EYPC-LoC from 8.1 to 13.4 ($P < 0.005$), and DPPC-LoC from 7.5 to 11.6 ($P < 0.05$) (*Table 1*). Consequently there was a parallel decrease (percent mass) in PL content of the surface phase of these emulsions (*Table 1*). These changes resulted in concomitant significant increases in the UC/PL molar ratio of the surface phase of the three reisolated emulsions with time: DMPC-LoC ($P < 0.001$), EYPC-LoC ($P < 0.01$), and DPPC-LoC ($P < 0.05$) (*Figure 1a-c*). However, there was no significant change in lipid composition of the surface phase of the reisolated DSPC-LoC emulsions (*Table 1 and Figure 1d*).

In conclusion, there was a gradual, significant increase in the percent UC of the surface phase of protein free phospholipid-triolein emulsions with low cholesterol content of similar size (diameter <130 nm) and overall composition. However, the rate of transfer of UC from erythrocytes depends on the phospholipid acyl chains and their consequent fluidity. The rate of transfer was fastest and most significant to the fluid DMPC surface, followed by the fluid EYPC surface, slower to DPPC (at its melting transition), and slowest (or perhaps absent) to the solid DSPC surface.

Earlier studies from this laboratory[13,14] identified the liver as the central organ of uptake of these emulsions with the various phospholipid surfaces in the intact rat. Our studies continue (unpublished results) to dissect the metabolic fate and the consequent cellular distribution of those emulsions in the perfused rat liver system. Because we employ rat erythrocytes as oxygen carriers,[28] this in vitro incubation study with the emulsions and red cells will enable us to evaluate the possible contribution of red blood cell lipids to compositional changes of emulsion during the course of liver perfusions up to 3 hr.

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