Free Fatty Acids Modulate Intermembrane Trafficking of Cholesterol by Increasing Lipid Mobilities: Novel ^{13}C NMR Analyses of Free Cholesterol Partitioning†

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ABSTRACT: Cholesterol and free fatty acids in membranes modulate major biological processes, and their cellular metabolism and actions are often coordinately regulated. However, effects of free fatty acid on cholesterol−membrane interactions have proven difficult to monitor in real time in intact systems. We developed a novel ^{13}C NMR method to assess effects of free fatty acids on molecular interactions of cholesterol within—and transfer between—model membranes. An important advantage of this method is the ability to acquire kinetic data without separation of donor and acceptor membranes. Large unilamellar phospholipid vesicles (LUV) with phosphatidylcholine/cholesterol ratios of 4:1 served as cholesterol donors. Small unilamellar vesicles (SUV) made with phosphatidylcholine were acceptors. The $^{13}C_4$-cholesterol peak is narrow in SUV, but very broad in LUV, spectra; the increase in intensity of this peak over time monitored transfer. Oleic acid and other long chain free fatty acids [saturated (C12−18) and unsaturated (C18)] dose-dependently increased mobilities of lipids in LUV (phospholipid and cholesterol) and cholesterol transfer rates, whereas short (C8−10) and very long (C24) chain free fatty acids did not. Decreasing pH from 7.4 to 6.5 (±oleic acid) had no effect on cholesterol transfer, and 5 mol % fatty acyl-CoAs increased transfer rates, demonstrating greater importance of the fatty-acyl tail over the headgroup. In LUV containing sphingomyelin, transfer rates decreased, but the presence of oleic acid increased transfer 1.3-fold. These results demonstrate free fatty acid-facilitated cholesterol movement within and between membranes, which may contribute to their multiple biological effects.

Both cholesterol and free fatty acids (FFA)[1] regulate the structure and function of cellular membranes and many pathways of lipid metabolism (*1*−*3*). Maintenance of physiologic membrane cholesterol levels is necessary for normal cell function. The planar, hydrophobic structure of cholesterol leads to lipid ordering, which alters membrane fluidity, permeability, and protein function (*4*). Likewise, alterations in fatty acid levels in membranes (free and incorporated into phospholipids) change fluidity, resulting in altered function of membrane-associated proteins including transporters, receptors, enzymes, and signaling molecules (*5*, *6*).

Cholesterol is solubilized by membrane phospholipids, including phosphatidylcholine (PC) and sphingomyelin (SM). SM has high affinity for cholesterol, and by sequestering cholesterol, forms microdomains termed rafts (*7*). Cholesterol and rafts are important in cell signaling, endocytic pathways, cell migration, and in specific adverse pathological conditions such as Alzheimer's disease, prion disease, and cancer (*7*−*11*). How FFA may affect raft function has yet to be established, but preliminary research shows that the partitioning of FFA is only weakly dependent on membrane phase structure and may therefore affect raft and nonraft domains similarly (*12*).

Plasma membrane is the major reservoir of cholesterol, possessing as much as 90% of total cellular cholesterol, with only relatively small amounts present in intracellular membranes (*13*, *14*). Membrane protein and lipid content (e.g., SM to total phospholipid ratios), selective vesicular transport by proteins (e.g., Niemann Pick C protein), and kinetic barriers to cholesterol release from the plasma membrane (e.g., sequestration by rafts) may contribute to this heterogeneous distribution of cholesterol between cellular membranes (*4*). Our previous work suggested that FFA promote redistribution of intracellular cholesterol, specifically from the plasma membrane into intracellular regulatory pools (*2*, *15*). This role of FFA in cholesterol redistribution led us to question if FFA have direct physical effects on cholesterol within membranes and upon its transport.

Despite recent progress in our knowledge of cholesterol trafficking, for example, in the regulation of proteins such

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[1] Abbreviations: $^{13}C_4$-cholesterol, cholesterol labeled with ^{13}C at the C_4 position; EM, electron microscopy; FA, fatty acid; FFA, free (unesterified) fatty acid; HDL, high-density lipoprotein; LUV, large, unilamellar vesicle; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; SM, sphingomyelin; SREBP, sterol regulatory element-binding protein; SUV, small, unilamellar vesicle.

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as SREBP (*16*, *17*), there have been few advances in methods for tracking cholesterol movement and defining related biophysical mechanisms for this movement. Cholesterol transfer has been studied for many years using model systems, yet we were unable to find a method that did not require separation of donor and acceptor membranes, which could perturb cholesterol distribution. Accordingly, we developed a ^{13}C NMR method to allow real time measurement of cholesterol transfer, without alteration of experimental samples. We applied this method to study effects of FFA on cholesterol transfer between model membranes. Our results support a model whereby FFA increase mobility of both cholesterol and PC within membranes, thus enhancing transfer of cholesterol between membranes. These alterations in physical interactions of cholesterol with surrounding lipids could contribute to a number of the modulating effects of FFA on cell biology.

EXPERIMENTAL PROCEDURES

Materials. Egg yolk L-α-lecithin PC, beef brain SM, and HEPES buffer were purchased from Avanti Polar Lipids. Unlabeled cholesterol and FFA were purchased from NU-CHEK-Prep. and cholesterol specifically labeled with ^{13}C at the C_4 position from Cambridge Isotope Laboratories. 2-Methyl-2-propanol, chloroform, and other common reagents were purchased from Sigma Chemical Co.

Methods. Vesicles. SUV and LUV were made by drying PC (±cholesterol, ±SM) under nitrogen, followed by 1 h lyophilization. Samples were resolubilized in 2-methyl-2-propanol, frozen by immersion in liquid N_2, and lyophilized for 24 h. SUV were resuspended to 60 mM PC in 25 mM HEPES (10% D_2O) buffer and hydrated under nitrogen for 1 h at room temperature. SUV were formed by sonication at an intermediate power level in the pulsed mode for 1 h under nitrogen while the sample was continuously immersed in an ice bath. Low-speed centrifugation for 20 min was then used to remove metal fragments released from the sonicator tip. With this sonication procedure, the levels of FFA and lysophosphatidylcholine are <1% (*18*). SUV containing SM were resuspended at 1:1 (PC/SM, 30 mM).

For LUV, dried samples were resuspended to 60 mM PC and 30 mM $^{13}C_4$-cholesterol. After hydration under nitrogen, samples were put through five consecutive freeze−thaw cycles (alternate liquid nitrogen, warm water immersions) (*19*). To create unilamellar structures, samples were extruded 10 times at room temperature through two stacked 100 nm polycarbonate filters using a 10 mL Thermobarrel Extruder of Lipex Biomembranes, Inc. Some cholesterol precipitates in polycarbonate filters during extrusion; therefore, final PC/cholesterol ratios were determined by chemical analysis and were 4:1. For LUV containing SM, lipids were resuspended at a PC/SM/cholesterol ratio of 1:1:1 (30 mM). The final PC/SM/cholesterol ratio was determined by chemical analysis to be 2:2:1. SUV and LUV were maintained under nitrogen.

FFA. FFA were solubilized by adding crystalline K^+FA^- to 80 mM in water with an excess molar ratio of KOH (1.2:1). FFA were added to vesicles, allowed to equilibrate for 30 min, and adjusted to pH 7.4. Because of the insolubility of lignoceric acid (24:0) and to ensure distribution in both bilayers of the fatty acyl-coAs, these additives were co-dried with lipids for vesicle preparation.

Electron Microscopy (EM). Vesicles were examined by negative staining transmission EM with a Philips CM12 Transmission Electron Microscope. SUV/LUV were unilamellar and uniform in size within each population (Peng, S., and Hamilton, J. A., unpublished results).

Dynamic Light Scattering (DLS) Measurements. A Beckman Coulter N4 Plus instrument was used for vesicle size determination. LUV and SUV were measured alone or mixed in triplicate at a 1:1 ratio with aliquots removed at consecutive 2 h time points. Aliquots were diluted 200-fold, and vesicle size measurements were acquired for 5 min using a 90° angle.

^{13}C NMR Spectroscopy. ^{13}C NMR measurements were made at room temperature using a 10 mm broadband probe in a Bruker 300 MHz AMX NMR. Spectra of LUV or SUV alone were obtained after 1:1 (v/v) dilution in H_2O (10% D_2O for lock and shim signal) and being placed under nitrogen. Individual spectra of LUV with cholesterol and SUV without cholesterol were used to assess vesicle integrity and electronically summed to create the zero time point. For transfer experiments, LUV and SUV were mixed (1:1 ratio), placed under nitrogen, and consecutive spectra were obtained continuously for at least 40 h. No significant spectral changes were observed over the entire acquisition periods, nor were there any visual changes in the lipids (e.g., yellowing), which indicates no significant alterations in chemical composition (e.g., lipid degradation), in agreement with lipid oxidation studies in vesicle systems (*20*). For experiments testing the total amount of cholesterol transferred to SUV, the LUV/SUV mixtures were sonicated (after equilibrium was reached) in the same manner used to generate SUV. Spectra of the newly formed SUV were taken, and the difference between pre- and post-sonication peak areas (C_4-cholesterol/choline) were determined.

Kinetic Analysis. Intensities of C_4-cholesterol peaks were measured by peak height and total area. To obtain adequate signal-to-noise ratios of the C_4 peak, each NMR spectrum required almost 1 h (53 min), and the recorded cholesterol intensity represented an average over this time. Sequential spectra (time points) were fit using Origin 6.0. Cholesterol flip-flop occurs on a time scale of seconds or faster (*21*−*23*) and thus would not affect the kinetics observed in our experiments, which were first order. Similar relative rates of transfer (k^{FFA}/k^{noFFA}) were derived from peak heights and areas.

PC and Cholesterol Analysis. PC (Bartlett Method) (*24*) and cholesterol (horseradish peroxidase colorimetric enzyme assay) (*25*) concentrations of vesicles were measured to ensure consistency between experiments.

Statistics. Time curves were generated from peak heights or areas, first-order kinetic fits performed using Origin 6.0, and transfer rates obtained. For almost all values, three or more separate kinetic experiments were performed. Means, standard deviations (std), and t-tests were performed using Excel2000.

RESULTS

Monitoring Cholesterol Transfer by NMR. To monitor transfer by NMR, we exploited the differences in line widths of cholesterol in SUV or LUV. As shown in Figure 1, the ^{13}C NMR spectrum of SUV made with PC but without

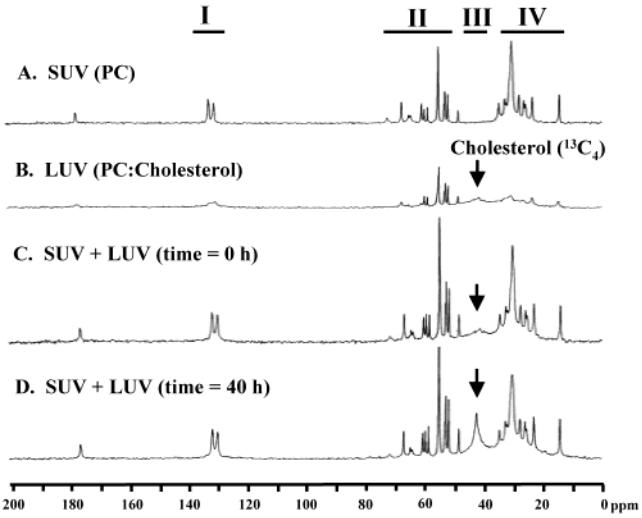

FIGURE 1: ^{13}C NMR spectra of vesicles. (A) SUV (60 mM PC) after 2048 scans with a pulse interval of 1.5 s. (B) LUV (60 mM PC and 15 mM cholesterol). (C) SUV + LUV at $t = 0$, generated by computer addition of SUV and LUV spectra shown in panels A and B, respectively. (D) Spectrum of the SUV + LUV mixture at 40 h, after transfer equilibrium. Increase of the $^{13}C_4$-cholesterol peak (41.9 ppm) over time monitored cholesterol transfer. General regions of the spectra are labeled at the top. Region I includes the olefinic carbons of PC. Region II includes various carbons of choline within PC and HEPES buffer. Region III (arrow) represents the ^{13}C-cholesterol (C_4 position). Note the ^{13}C-cholesterol peak is large relative to PC peaks because 100% of cholesterol present is labeled with ^{13}C, whereas only naturally abundant ^{13}C is being measured for PC. Region IV includes various fatty acyl carbons from PC.

cholesterol is characterized by narrow resonances (Figure 1A). For SUV made with PC and cholesterol (4:1, 60:30 mM), similarly narrow phospholipid line widths were observed. By contrast, the spectrum of LUV containing PC and $^{13}C_4$-cholesterol (4:1 PC/cholesterol, Figure 1B) is characterized by broad resonances, including a very broad C4 peak. The larger diameter of LUV as compared to SUV results in slower rotation of vesicles and broader line widths (*26*). Thus, when SUV are added to cholesterol containing LUV, only cholesterol that has transferred to SUV will be detected as a narrow peak. Note that the $^{13}C_4$-cholesterol peak is intense relative to all other peaks in LUV or SUV spectra. This is because all cholesterol present is labeled at the C_4 position with ^{13}C, whereas the ^{13}C peaks in PC represent only what is naturally abundant (approximately 1.1% of all carbon atoms).

The individual spectra of SUV and LUV were added together to provide a spectrum equivalent to time zero (Figure 1C). Figure 1D shows a spectrum after equilibrium peak intensity was attained. Increase in the $^{13}C_4$-cholesterol peak in SUV was measured at sequential time points and was used to monitor cholesterol transfer at room temperature. All data for cholesterol transfer fit first-order kinetics (Figure 2A); therefore, first-order rate constants were determined.

Total Amount of Cholesterol Transferred from LUV to SUV. Because it is difficult to separate a mixture of LUV and SUV, we developed the following approach to determine the total amount of cholesterol transferred from LUV to SUV. First, we sonicated LUV/SUV mixtures that had reached transfer equilibrium (after at least 40 h) to convert the entire sample to SUV and then obtained new NMR spectra. Since LUV and SUV were mixed at equal amounts of PC for

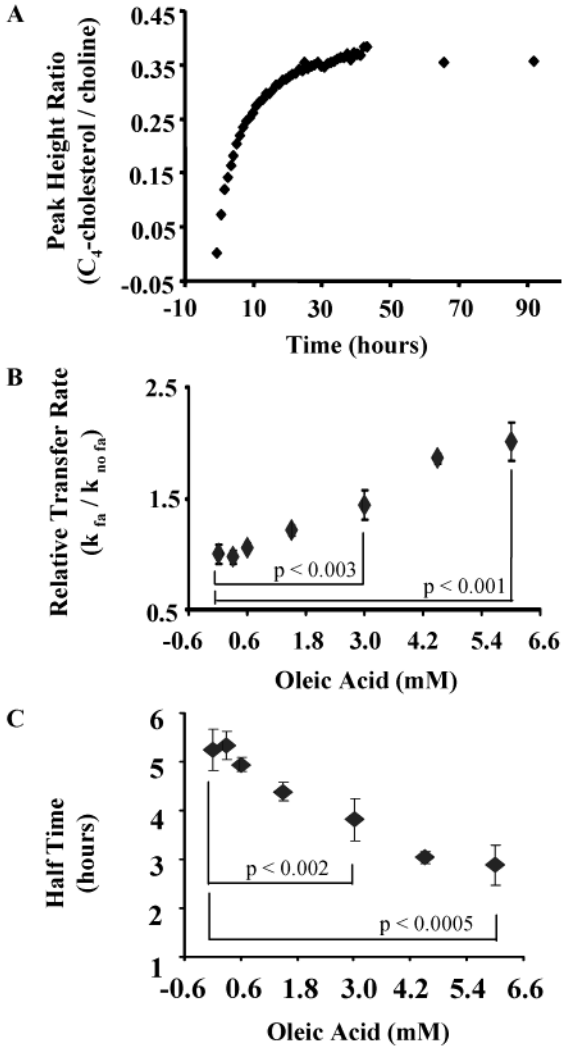

FIGURE 2: OA dose-dependently increases the rate of cholesterol transfer from LUV to SUV. (A) A representative kinetic curve generated by measuring cholesterol uptake within SUV. Each point represents an individual ^{13}C NMR spectrum. Curves were fit to first-order kinetics, and rates and half-times of transfer were calculated. (B) Cholesterol transfer rates dose-dependently increased with increasing OA addition. (C) Cholesterol transfer half-times dose-dependently decreased with OA addition.

transfer experiments, and initial LUV contained PC/cholesterol at 4:1, sonication of this mixture produced SUV with a PC/cholesterol ratio of 8:1. Thus, the spectra of these new SUV are equivalent to equilibrium spectra in which 50% of the cholesterol in LUV had transferred to SUV. The area of the equilibrium cholesterol peak in the LUV/SUV mixture was 79.3 ± 1.7% of the new cholesterol peak in the freshly sonicated mixture (average of three separate experiments, ±SD). This is equivalent to 39.7 ± 0.9% (0.793 × 50% = 39.7%) of the cholesterol in LUV transferring to SUV at equilibrium.

Oleic Acid Effects on Cholesterol Transfer. As a new application of this method, we investigated if FFA modulate transfer of cholesterol between membranes and focused on oleic acid (OA), the most abundant FA in human plasma (*27*). Separate transfer experiments were performed with increasing OA concentrations. The sequential time points of all experiments fit first-order kinetics, and first-order rate constants were determined. In Figure 2B, rate constants were plotted against OA concentration, and a linear increase in

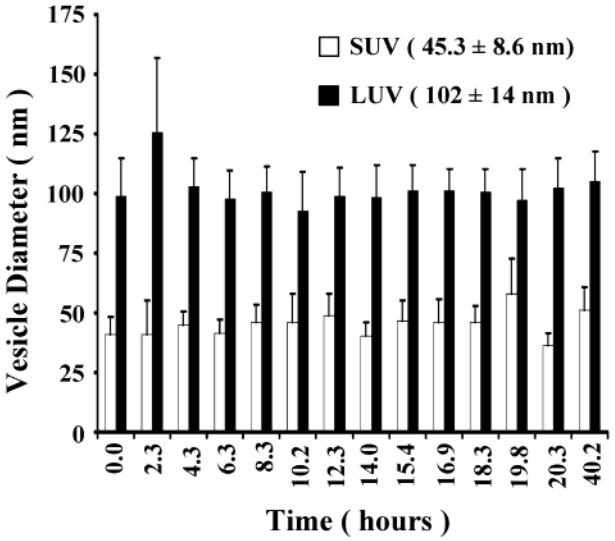

FIGURE 3: Vesicle fusion does not occur during the time course of cholesterol transfer. Dynamic light scattering was used to determine vesicle size during cholesterol transfer. LUV and SUV (+6 mM OA) were mixed in triplicate at a 1:1 molar ratio (PC in LUV/PC in SUV), aliquots were removed at consecutive time points, diluted 200-fold, and vesicle size measurements were acquired for 5 min using a 90° angle. Data shown is an average of the triplicates at each time point.

cholesterol transfer rates ($n = 18$, $r^2 = 0.9$) was observed with OA addition (0−10 mol % relative to PC, or 0−6 mM). Transfer rates increased 2-fold with 6 mM OA as compared to experiments without. Half-times (Figure 2C) showed a continuous decrease with increasing OA, from 4.7 to 0.4 h without FFA to 2.4−0.4 h with 6 mM OA. Similar dose-dependent increases in transfer rate were observed with palmitate (C16:0) at 5 and 10 mol % (data not shown).

Vesicle Characterization. Preliminary data using the above NMR method indicated that increases in donor vesicle size (decreases in curvature) decrease cholesterol transfer rates (Hamilton, J.A., Johnson, R.A., and Peng, S., unpublished data). It is also conceivable that transfer could occur via vesicle fusion, rather than by the unimolecular mechanism of desorption from donor vesicles, followed by aqueous transfer and then adsorption to acceptor vesicles. To rule out the possibility of vesicle fusion or aggregation, we used three approaches to determine vesicle integrity. First, we observed that no significant spectral changes occurred during spectra acquisition except for the increasing $^{13}C_4$-cholesterol peak. Fusion or increase in vesicle size would have resulted in overall broadening of spectra, whereas a decrease in size would have increased and narrowed spectral peaks. Second, SUV/LUV mixtures were examined by electron microscopy at 0 and 24 h after mixing, and no detectable fusion or change in particle size was observed. Last, dynamic light scattering was used to monitor vesicle sizes of SUV/LUV mixtures at 2 h intervals over 40 h in the presence of 6 mM oleate, the highest FFA concentration used in our experiments (Figure 3). The average vesicle diameters were 102 ± 14 nm for LUV, and 45.3 ± 8.6 nm for SUV. No significant change in the number or size of vesicles was observed over the 40 h time period. As cholesterol transfer equilibrium was reached well before 40 h, changes in vesicle size or fusion could not account for cholesterol transfer from LUV to SUV.

Oleic Acid and Lipid Order. We analyzed individual LUV and SUV spectra and measured changes in peak height with

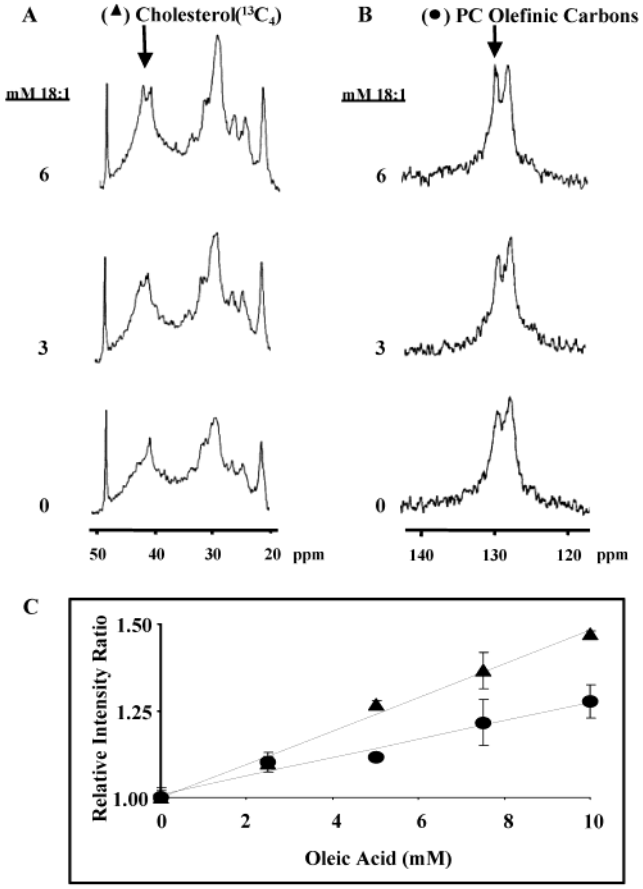

FIGURE 4: Oleic acid dose-dependently increases cholesterol and PC mobilities. (A) The $^{13}C_4$-cholesterol region of the LUV spectra after sequential OA additions, final [OA] = 6 mM. (B) PC olefinic carbon region of LUV spectra after sequential OA additions, final [OA] = 6 mM. (C) A plot of data from experiments shown in panels A and B, where OA dose-dependently increases the mobility (peak sharpness or peak height) of cholesterol and PC within LUV. ▲, cholesterol; ●, olefinic carbons of PC.

increasing amounts of OA (Figure 4). For LUV at a fixed cholesterol concentration (4:1 PC/cholesterol) the C4 peak is asymmetric, with a maximal intensity at 40.7 ppm and a shoulder at 41.9 ppm. OA dose-dependently increased the peak height (decreased the line width) of this C_4-cholesterol peak centered at 41.9 ppm (Figure 4A). Second, increasing FFA was associated with concentration-dependent narrowing of PC peaks within regions outlined in Figure 1; region I (129.9 ppm peak) (enlarged in Figure 4B), region II (66.2 ppm peak), and region IV (25.7−30.5 ppm peaks) increased approximately 33%. These dose-dependent increases in peak height correspond to decreasing line width and increasing mobility of both cholesterol and PC within LUV after OA addition (Figure 4C). No differences in cholesterol or PC mobilities were observed with OA addition in SUV.

Effects of Different FFA. After establishing OA effects, we questioned if other FFA induced similar alterations. C18 fatty acids with increasing degrees of unsaturation (C18:0, C18:1, C18:2, and C18:3) increased transfer rates at a 3 mM concentration, with no significant differences ($t_{1/2} = 3.4 \pm 0.2$ h) between them (Table 1). We also examined saturated FFA with differing chain lengths (C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, and C24:0) (Table 2). No increases were observed in cholesterol transfer rates with C8:0, C10:0, and C24:0. Approximately 1.4-fold increased transfer

Table 1: Effects of Saturated and Unsaturated FFA on Cholesterol Transfer from LUV to SUV[a]

free fatty acid (3 mM)	relative first-order transfer rate (k^{FFA}/k^{none})
none	1.00 ± 0.09
18:0	1.47 ± 0.25
18:1	1.44 ± 0.19
18:2	1.32 ± 0.05
18:3	1.29 ± 0.08

[a] LUV were prepared at the molar ratio of 4:1 PC/cholesterol and mixed with PC SUV, ±FFA at 3 mM (5 mol %), and rates of cholesterol transfer were measured. The experimental mean (±SD) without FFA was set at 1.00 ($k = 0.15 \pm 0.01$ h^{-1}), and all other experiments (±SD) are represented relative to this value.

Table 2: Effects of Fatty Acid Chain Length on Cholesterol Transfer from LUV to SUV[a]

A. free fatty acid (3 mM)	relative first-order transfer rate (k^{FFA}/k^{none})
none	1.00 ± 0.09
8:0	1.12 ± 0.01
10:0	0.98 ± 0.46
12:0	1.53 ± 0.29
14:0	1.49 ± 0.07
18:0	1.47 ± 0.29
24:0	1.02 ± 0.25

B. free fatty acid (6 mM)	relative first-order transfer rate (k^{FFA}/k^{none})
none	1.00 ± 0.09
16:0	1.91 ± 0.27
18:1	2.02 ± 0.25

[a] LUV and SUV were prepared and mixed as described in Table 1, ±FFA at 3 mM (5 mol %, A) or 6 mM (10 mol %, B), and rates of cholesterol transfer were measured.

rates were observed with C12:0, C14:0, C16:0, and C18:0, with no significant differences ($t_{1/2} = 3.3 \pm 0.1$ h at 3 mM and $t_{1/2} = 2.4 \pm 0.1$ h at 6 mM) between them. In measuring lipid order, we found that FFA C12:0, C14:0, C16:0, C18:0 C18:1, C18:2, and C18:3 decreased line widths of PC and cholesterol similar to OA, while C8:0, C10:0, and C24:0 had no effect.

Effects of Headgroup versus Carbon Chain. We used two approaches to determine if the charge on FFA molecules and movement of FFA across membranes was required for their observed effects on cholesterol transfer. First, at physiological pH 7.4, FFA within membranes are approximately 50% ionized, and un-ionized FFA flip-flop rapidly across lipid bilayers (*28*). To increase the membrane-bound pools of un-ionized FFA approximately 10-fold, we repeated experiments at pH 6.5; cholesterol transfer rates (±OA) were not affected (Table 3). Second, we substituted two fatty acyl-CoAs, C18:1- and C20:4-CoA, for FFA. Because of the large, charged CoA group, these molecules cannot flip-flop across bilayers (*29*). Five mol % of either fatty acyl-CoA increased rates of cholesterol transfer by approximately 1.5-fold, similar to OA (Table 4). In addition, like FFA, fatty acyl-CoAs induced sharper line widths of PC and cholesterol peaks in LUV.

Effect of SM on Cholesterol Transfer. Since SM plays important roles in biological membranes and has a high affinity for cholesterol (*7*, *8*, *30*), we investigated if FFA had similar effects on cholesterol transfer in the presence of SM in an equal molar ratio to PC. As expected, SM in donor LUV vesicles decreased transfer rates by 45% (Table 5) (*21*, *31*). Five mol % OA increased the rate of cholesterol transfer by almost the same factor as for LUV made only with PC (1.3-fold vs 1.4-fold). In addition, OA decreased the line width of the C_4-cholesterol resonance in LUV containing SM. Incorporation of SM into acceptor vesicles (SUV) had no effect on transfer rates.

Table 3: Effect of pH on Cholesterol Transfer from LUV to SUV[a]

pH	18:1 (mM)	relative first-order transfer rate (k^{FFA}/k^{none})
7.4	0	1.00 ± 0.09
6.5	0	1.02 ± 0.04
7.4	3	1.44 ± 0.19
6.5	3	1.55 ± 0.15

[a] LUV were prepared at the molar ratio of 4:1 PC/cholesterol. LUV and SUV (100% PC) were each adjusted to pH 6.5 or 7.4 and mixed ±OA (18:1) at 3 mM (5 mol %), and rates of cholesterol transfer were measured. The experimental mean (±SD) without FFA at pH 7.4 was set at 1.00 ($k = 0.15 \pm 0.01$ h^{-1}), and all other experiments (±SD) are represented relative to this value.

Table 4: Effects of Fatty Acyl-CoAs on Cholesterol Transfer from LUV to SUV in Comparison to Oleic Acid (18:1)[a]

free fatty acid (3 mM)	relative first-order transfer rate (k^{FFA}/k^{none})
none	1.00 ± 0.09
18:1	1.44 ± 0.19
18:1-CoA	1.50 ± 0.10
20:4-CoA	1.55 ± 0.07

[a] LUV and SUV were prepared and mixed, and transfer was monitored as described in Table 1.

Table 5: Effect of Oleic Acid on Cholesterol Transfer from LUV Containing SM[a]

oleic acid	LUV	
	PC	PC + SM
−	1.00 ± 0.09	0.55 ± 0.02
+	1.44 ± 0.19	0.73 ± 0.05
fold increase	1.44 ± 0.05	1.33 ± 0.04

[a] LUV were prepared at molar ratios of either 4:1 PC/cholesterol (PC column) or at 2:2:1 mM PC/SM/cholesterol (PC + SM column). LUV were mixed with PC SUV, ±3 mM OA (5 mol %), and rates of cholesterol transfer were measured. The experimental mean (±SD) without OA and using PC/cholesterol LUV was set at 1.00 ($k = 0.15 \pm 0.01$ h^{-1}), and all other experiments (±SD) are represented relative to this value.

DISCUSSION

Our studies describe a novel, NMR-based method to monitor cholesterol transfer between lipid vesicles. We observed first-order kinetics in all of our transfer experiments, with a half-time of cholesterol transfer from LUV to SUV of 4.7 ± 0.7 h, similar to previous work in other laboratories, considering differences in vesicle sizes and other experimental conditions (*31*−*33*). In addition, this rate of transfer closely resembles the rate of cholesterol transfer from cell surfaces to cholesterol-depleted HDL particles (*34*). Ap-

proximately 40% of LUV cholesterol transferred to SUV at equilibrium, corresponding to 9 mol % cholesterol in the SUV, but we detected no changes in vesicle size of SUV or LUV. It is likely that SUV size is one of the factors that limit the total amount of cholesterol transferred (*35*). For LUV, it may be that small decreases in size are occurring but not within the experimental variation of our dynamic light scattering studies.

Our method not only monitors the kinetics and equilibrium of cholesterol transfer but also simultaneously observes physical effects of FFA on membrane cholesterol. We demonstrate that FFA have substantial effects on the mobility of both cholesterol and PC within membranes, which likely contributes to, if not accounts for, enhanced rates of cholesterol transfer between membranes. More specifically, OA dose-dependently increased rates of cholesterol transfer, with a corresponding dose-dependent increase in the mobility of cholesterol and PC fatty acyl chains within LUV. Several previous studies have shown that cholesterol primarily interacts with the fatty acyl chains of phospholipids, excluding the two terminal carbons, and leads to a higher degree of order of these chains (*7*, *26*, *36*−*38*). Since the narrowing of PC peaks in the present study were primarily observed in the fatty acyl chain resonances (except for the very mobile terminal carbons, CH_3 at 14.1 ppm and CH_2 at 22.9 ppm), we suggest that fatty acids could be interfering with the close packing of cholesterol and PC. Interestingly, other amphiphiles, such as lysophosphatidylcholine, increased the rates of cholesterol efflux from macrophage and CHO cells, and from model systems, by altering membrane structure (*39*, *40*).

It is interesting to note that in other studies—in model systems and plasma membrane vesicles—alterations in cholesterol distribution between two different lipid phases have been observed with increasing temperature or addition of exogenous lipases (*21*, *41*). Furthermore, two C_4 signals were resolved in unsonicated (multilamellar) phospholipids with >20 mol % cholesterol, and these signals were attributed to more- and less-ordered states (*41*). The disordered state was represented by the downfield peak, which in our studies would correspond to the peak at 41.9 ppm. OA dose-dependently increased peak height (decreased line width) of this C_4-cholesterol peak (Figure 4A). We do not have direct evidence that the peak at 41.9 ppm represents a more disordered state, but it is a possibility that may warrant further verification.

Similar sequential additions of OA had no effect on the heights (line widths) of any of the resonances in SUV spectra (data not shown). The cholesterol in SUV already possesses a relatively high mobility as compared to LUV, due in part to the more rapid tumbling of the SUV. In addition, the tight curvature of SUV alters the packing of lipids in both leaflets as compared to more planar membranes. It is possible that FFA intercalate between the lipids in SUV without significantly altering their already limited interactions (*42*). The larger LUV have markedly decreased membrane curvature, which permits tighter packing of lipids, and FFA may disrupt some of the cholesterol−phospholipid interactions. This would translate to greater effects of FFA on enhancing mobility of cholesterol within large, low-curvature membranes (e.g., plasma membrane) relative to smaller, tightly curved intracellular membranes (e.g., lysosomes, peroxisomes).

Factors other than FFA—such as changing vesicle sizes, initial vesicle PC/cholesterol ratios, or relative concentrations of LUV to SUV—could each affect cholesterol transfer kinetics. However, our primary goal was to determine the effects of FFA on cholesterol transfer and lipid mobilities. Therefore, except where specified, experiments were performed with LUV at a starting PC/cholesterol molar ratio of 4:1 and with diameters of approximately 100 nm. SUV were made with 100% PC, with diameters of approximately 40 nm. Additionally, using equimolar amounts of LUV and SUV (in terms of PC) for all experiments ensured that addition of FFA was the perturbation studied.

The observed differences between medium and long chain FFA are likely because of their varying solubilities in the lipid membrane. Long chain fatty acids (C12:0, C14:0, C16:0, and C18:0) increased the rate of cholesterol transfer from LUV to SUV in a similar manner as OA, whereas medium (C8:0 and C10:0) and very long (C24:0) chain fatty acids had no effect on transfer rate. Long chain FFA rapidly associate with membranes because of their low solubility in water and their high solubility in an oily environment, and the membrane/aqueous FFA concentration ratios are on the order of 10^7 (*43*−*47*). In our experiments, this would translate to essentially all long chain FFA added to vesicles (0−10 mol % relative to PC or 0−6 mM) being present in the membrane bilayers. In contrast, medium chain FFA have a high solubility in aqueous solution and therefore would be present at much lower concentrations within membranes (*48*, *49*). Of all the FFA studied, the very long chain FFA C24:0 will partition most favorably into the phospholipids, but it is longer than one leaflet of the bilayer, which alters the way C24:0 interacts with surrounding lipids (*48*−*50*). Such alterations of membrane-bound FFA concentration, or of FFA-lipid interactions, may explain the lack of effect on cholesterol transfer observed with C8:0, C10:0, or C24:0. This suggests that the increase in the rate of cholesterol transfer observed with addition of FFA with 12−18 carbons is due to FFA-induced changes in the interactions between PC and cholesterol within the membranes versus effects in the aqueous milieu.

Two additional lines of evidence suggest that the observed FFA effects on cholesterol transfer are more dependent on their hydrophobic acyl chains than their hydrophilic headgroups. First, at physiological pH, approximately half of membrane-bound FFA is ionized. Our studies compared a pH of 6.5 with 7.4, providing a nearly 10-fold change in FFA ionization. Over this range, there was no observed dependence of cholesterol transfer on the ionization of the membrane bound FFA. Second, fatty acyl-CoAs had similar effects on cholesterol transfer rates as FFA. This also suggests that the observed FFA effects on cholesterol mobility and transfer do not require dynamic movement of FFA across the bilayer.

FFA with varying degrees of saturation and chain lengths between 12 and 18 carbons had similar effects on lipid mobilities and transfer rates. Previous work has shown that cholesterol orders membranes primarily through transient van der Waals interactions of the rigid steroid ring system with surrounding fatty-acyl chains. (*51*−*54*). The cholesterol polycyclic ring spans the first 8−10 methylene segments of

fatty-acyl chains in phospholipids and FFA, leading to increased ordering primarily of chain carbons 1−8, with sharply decreasing effects further down the chains (*51*, *53*, *55*−*57*). In agreement with these studies, we observe that heights of peaks assigned to most of the acyl chains of PC were increased with FFA addition, while the two terminal carbon peaks (CH_3 at 14.1 ppm and CH_2 at 22.9 ppm) were not altered. We speculate that FFA intercalate between cholesterol and PL in membranes, disrupting these interactions and increasing lipid mobilities. Since varying biological effects are often observed with unsaturated versus saturated FFA, other FA-related factors must influence these differences.

SM and cholesterol-rich domains, or rafts, have putative roles in multiple cellular functions, including protein sorting through the trans-Golgi network, specific membrane targeting of proteins, and in assembly and function of proteins involved in multiple signaling cascades. The presence of SM within donor vesicles (LUV) decreased the rate of transfer by 45%, whereas SM in acceptor vesicles (SUV) had no effect. This is consistent with previous studies showing that the cholesterol transfer rate is desorption-dependent and that SM incorporation into donor, but not acceptor, membranes results in decreased desorption rates (*20*, *21*, *31*, *33*, *58*, *59*). Our finding that FFA increased the rates of cholesterol transfer from LUV containing SM (Table 5) is in agreement with preliminary data showing that FFA partition similarly into membrane domains with and without SM (*12*) and suggests that FFA could facilitate release of raft-associated cholesterol into the more fluid phospholipid regions of the membrane, which would allow for enhanced release of cholesterol from the membrane.

In cholesterol transfer studies by Bar et al., the higher affinity of cholesterol for SM over other phospholipids was shown to decrease the total amount of cholesterol available for transfer (from approximately 90 to 20%) (*60*). While we did not directly measure changes in the total amount of cholesterol transferred from LUV made with SM to SUV, it is possible to gain some qualitative information from the plateau values of the kinetic curves. The plateau values correlate to the total amount of cholesterol within SUV at transfer equilibrium. With the addition of SM to LUV, we consistently observed a significant decrease of approximately 50% in the plateau values (preliminary data, not shown). This observation suggests that cholesterol may be preferentially distributing into SM domains in our LUV (*60*).

Many laboratories have investigated the phase properties of various lipids in the presence of changing cholesterol concentrations, and for a detailed discussion of lipid phases the reader is directed to various reviews and papers by O. G. Mouritsen, including the provided reference (*61*). While we were unable to find a temperature-dependent phase diagram of egg PC with cholesterol (either as multilamellar dispersion or in vesicles), egg PC with 20% cholesterol (as a multilamellar dispersion) at room temperature is in a single liquid disordered (Ld) state (*62*). In addition, studies of cholesterol and 1-palmitoyl-2-oleoly-*sn*-glycero-3-phosphatidylcholine (POPC)—the two predominate side chains present in egg PC—demonstrated that the main transition temperature was approximately −7 °C with 20% cholesterol present. Therefore, the lipids are in the L_d state in our studies (*63*). While the L_d phase is probably the dominant phase, it is important to note that work by Silvius et al. suggests that L_d, liquid ordered (L_o), and solid ordered (S_o) phases could coexist in membrane mixtures containing phosphatidylcholine and cholesterol, if cholesterol concentrations are not high (*64*).

In contrast, mixtures of phosphatidylcholine, cholesterol, and sphingomyelin consistently form two coexisting phases, usually defined as L_d and L_o (*65*−*69*). It is speculated that these two phases represent a phospholipid-rich domain (L_d) and a sphingomyelin and cholesterol-rich (raft-like) domain (L_o). As discussed earlier, the decrease in the amount of cholesterol transferred from LUV containing SM is consistent with cholesterol being sequestered into a L_o domain with SM. The fact that FFA induced similar increases in the rate of cholesterol transfer when added to LUV with or without SM (Table 5) suggests that FFA are not altering the lipid phases but are simply facilitating easier release of cholesterol from the membrane.

The concentrations of FFA in most experiments herein (0−6 mM, or 0−10 mol % relative to PC) are higher than usual physiologic levels in human plasma (0.2−0.6 mM). However, higher plasma levels (0.8−2.5 mM) are commonly observed in certain physiological states (e.g., fasting, strenuous exercise) or pathological conditions (e.g., ischemia, diabetes, sepsis, cancer) (*70*−*73*). Importantly, in local environments of the vascular bed, FFA concentrations are often much higher. For example, 90−95% of long chain FFA released from very low-density lipoproteins or chylomicrons by lipoprotein lipase directly enter cells, leaving only 5−10% to equilibrate with plasma (*72*). While desorption of long chain FFA from lipid membranes is far more rapid than cholesterol transfer, triglyceride lipolysis after a single meal is an ongoing postprandial event lasting up to 6−8 h, leading to sustained FFA levels at cellular membranes. Similarly, in adipocytes large amounts of FFA are released during lipolysis of intracellular fat droplets. Therefore, sustained local membrane concentrations could reach or perhaps exceed FFA levels tested herein. Furthermore, observed FFA effects on cholesterol mobilities and transfer rates were linear relative to FFA concentration (Figures 2B and 4A), and therefore lower doses should also produce similar, but perhaps lesser, effects.

There have been numerous studies of the physical state of cholesterol within membranes and on the kinetics of transfer. Nonetheless, we were unable to find either a method that allowed direct measurements—without extensive handling of the sample—or that addressed the physical effects of FFA on membrane cholesterol. The work presented herein provides a novel ^{13}C NMR-based method for direct measurement of cholesterol mobility and transfer, without separation of donor and acceptor membranes. A distinct advantage of this NMR method is that alterations in the interactions of cholesterol and other lipids within membranes can be detected in the same experiment that monitors transfer. This may prove useful in future studies of membrane domains, such as rafts. This method also has the potential to be modified to study effects and transport of varying endogenous (e.g., cholesterol and proteins) and exogenous (e.g., specific amphiphiles) compounds, or therapeutic agents within solution or incorporated into model or isolated cellular membranes, and in intact cells.

Results obtained from our NMR analyses suggest that FFA decrease affinities between cholesterol and surrounding lipids, including SM. This could produce important changes in membrane structure and in formation and stability of rafts, which are important in multiple cell signaling pathways. Thus, monitoring the ability of FFA to modulate the properties and partitioning of cholesterol within cellular membranes may help further define their role in normal physiology, as well as their adverse effects in disease states, such as diabetes mellitus.

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