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Molecular Organization of Cholesterol in Unsaturated Phosphatidylethanolamines: X-ray Diffraction and Solid State ^{2}H NMR Reveal Differences with Phosphatidylcholines

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Abstract: The major mammalian plasma membrane lipids are phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), and cholesterol. Whereas PC−cholesterol interactions are well studied, far less is known about those between PE and cholesterol. Here, we investigated the molecular organization of cholesterol in PEs that vary in their degree of acyl chain unsaturation. For heteroacid sn-1 saturated (palmitoyl), sn-2 unsaturated (various acyl chain) PEs, cholesterol solubility determined by X-ray diffraction was essentially identical with 1 (oleoyl, 51 ± 3 mol %) and 2 (linoleoyl, 49 ± 2 mol %) double bonds before decreasing progressively with 4 (arachidonyl, 41 ± 3 mol %) and 6 (docosahexaenoyl, 31 ± 3 mol %) double bonds. With 6 double bonds in each chain, cholesterol solubility was further reduced to 8.5 ± 1 mol %. However, ^{2}H NMR experiments established that the orientation of cholesterol in the same heteroacid PE membranes was unaffected by the degree of acyl chain unsaturation. A tilt angle of 15 ± 1° was measured when equimolar [3α-$^2H_1$]cholesterol was added, regardless of the number of double bonds in the sn-2 chain. The finding that solubility of cholesterol in sn-1 saturated PEs depends on the amount of polyunsaturation in the sn-2 chain of PE differs from the equivalent PCs that universally incorporate ∼50 mol % sterol. Unlike PCs, a differential in affinity for cholesterol and tendency to drive lateral segregation is inferred between polyunsaturated PEs. This distinction may have biological implications reflected by the health benefits of dietary polyunsaturated fatty acids that are often taken up into PE > PC.

Introduction

Cholesterol is found in high abundance in the mammalian plasma membrane where its concentration can exceed 50 mol % of the total lipid.[1] As a major constituent of the membrane, cholesterol influences acyl chain order and dynamics and may significantly influence signal transduction pathways. There is some suggestion, moreover, that select integral membrane proteins respond directly to cholesterol signals.[2] Much of the understanding of cholesterol's role in membrane organization and function has come from model membrane studies that have often employed phosphatidylcholine (PC) lipids with saturated and/or monounsaturated acyl chains.[3] Far less is known about the sterol's interaction with other phospholipids, including phosphatidylethanolamines (PEs), and especially those containing polyunsaturated acyl chains. We[4−6] and others[7−9] have suggested that a reduced affinity between cholesterol and polyunsaturated fatty acids (PUFA) may drive lateral phase separation into microdomains that are sterol-rich/PUFA-poor, such as lipid rafts that are enriched in sphingolipids and cholesterol,[10] and sterol-poor/PUFA-rich. We have speculated that changes in the location and function of signaling proteins resulting from modification of the molecular architecture of

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microdomains when the amount of PUFA-containing phospholipids, in particular docosahexaenoic acid (DHA)-containing PE, in the plasma membrane increases may be in part responsible for the diverse health benefits associated with dietary PUFA.[6,11,12]

The aim of the present study was to explore the molecular interactions between PE lipids containing varying degrees of unsaturation and cholesterol in order to better understand the molecular mechanism by which PUFA-driven phase separation into microdomains may occur. Molecular organization of cholesterol in PE membranes was systematically investigated as a function of acyl chain unsaturation using low- and wide-angle X-ray diffraction (XRD) and solid-state ^{2}H NMR spectroscopy. We specifically evaluated the solubility and orientation of cholesterol in a series of heteroacid sn-1 saturated, sn-2 unsaturated PEs and in a homoacid dipolyunsaturated PE with XRD and ^{2}H NMR. The solubility (determined by XRD) is representative of the affinity for the sterol of each phospholipid while the orientation (determined by ^{2}H NMR) of the steroid moiety within the membrane is indicative of interaction with the saturated sn-1 vs unsaturated sn-2 chain.[12] The results are compared with those of PCs. They demonstrate a substantially greater dependence of sterol solubility on the number of double bonds in the sn-2 position of heteroacid saturated−unsaturated PEs compared to PCs. In contrast, like PC, the tilt angle for cholesterol incorporated into PE membranes is relatively insensitive to the degree of sn-2 unsaturation. The implication is that a balance between acyl chain and headgroup structure modulates the interactions that determine membrane organization of the sterol and thereby influence lateral phase separation and membrane functional profile.

Materials and Methods

Materials. 1-palmitoyl-2-oleoylphosphatidylethanolamine (16:0−18:1 PE), 1-palmitoyl-2-linoleoylphosphatidylethanolamine (16:0−18:2 PE), 1-palmitoyl-2-arachidonylphosphatidylethanolamine (16:0−20:4 PE), 1-palmitoyl-2-docosahexaenoylphosphatidylethanolamine (16:0−22:6 PE), and 1,2-didocosahexaenoylphosphatidylethanolamine (22:6−22:6 PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO) while Cambridge Isotope Laboratories (Andover, MA) was the source of [3α-^{2}H$_1$]cholesterol, an analogue of cholesterol selectively deuterated at the 3α position. Deuterium-depleted water used for ^{2}H NMR studies was obtained from Isotec Inc. (Miamisburg, OH). All lipids were checked for purity on HPTLC plates (Alltech, Deerfield, IL). To minimize oxidation, samples were prepared under dark conditions with either a gentle stream of argon or within an argon-filled glovebox. Water and buffer solutions were degassed.

XRD Sample Preparation. XRD sample preparation and experimentation were as previously described.[4] Samples were prepared from 20 mg of PE/cholesterol mixtures at varying mol ratios. The lipids were codissolved in chloroform, the organic solvent was evaporated under a gentle stream of argon, and samples were immediately placed under vacuum for 12 h to remove trace solvent. Lipid mixtures were then hydrated to 50 wt % with 50 mM Tris buffer (pH 7.5) and vigorously vortexed for ∼5 min at room temperature. Excess water (∼1.5 mL) was added to the samples to enable pH adjustment. Following pH correction, the samples were frozen in dry ice and lyophilized once at ∼50 mTorr. Final homogenization with 50 wt % water was achieved by forcing the sample back and forth between the narrow constrictions of two Hamilton syringes using a lipid mixing device, as described by Cheng et al.[13] The resultant aqueous dispersions were transferred to 1 mm inner diameter X-ray capillary tubes (Charles Supper, Co., Natick, MA) that were flame-sealed followed by a bead of quick-drying epoxy to ensure hermetic sealing. All samples were stored in dry ice and equilibrated at the appropriate temperature for 1 h prior to conducting XRD experiments.

Huang et al.[14] found a variable reduction in cholesterol solubility in samples prepared by the conventional protocol outlined above. The problem was attributed to lipid demixing in the intermediate dry state. Their answer was to trap the lipids in a well-mixed state by lyophilizing at low temperature (−20 °C). We have performed both conventional and low-temperature trapping methods of sample preparation and achieved identical results within experimental error.[4]

XRD Measurements. XRD experiments were performed on PE/cholesterol mixtures with varying mol fractions to determine the sterol solubility via the observation of membrane-excluded cholesterol monohydrate crystals. All measurements, except for those on 22:6−22:6 PE, were conducted in the lamellar liquid crystalline phase. In excess of the solubility limit, cholesterol phase separates from the membrane and forms monohydrate crystals that are detected by characteristic second-order diffraction peaks.[4,14] Their respective reciprocal and real spacings are (002) 0.3701 Å^{-1}, 17.0 Å; (020) 1.033 Å^{-1}, 6.079 Å; and (200) 1.044 Å^{-1}, 6.015 Å.[15] The combined integrated intensities of these reflections (normalized with respect to the integrated intensity of the lipid wide-angle peak at $q \approx 1.4$ Å^{-1}) are then plotted against the total concentration of cholesterol added. Linear extrapolation to zero intensity gives the solubility of cholesterol. The slope, although dependent upon solubility, is rendered an imprecise measure by the normalization applied to intensity values.

^{2}H NMR Sample Preparation. PE/[3α-^{2}H$_1$]cholesterol in equimolar mixtures (100 mg of total lipid) were dissolved in chloroform, dried overnight and adjusted for pH as described for the XRD samples. Samples were then frozen in dry ice, and lyophilized three times in deuterium-depleted water to remove naturally abundant ^{2}HHO. After hydrating the powders to 50 wt %, the resultant aqueous dispersions were transferred to 5 mm glass NMR tubes that were sealed with a Teflon-coated plug. Samples were stored at −80 °C when not in use and were allowed to equilibrate at the appropriate temperature for ∼1 h prior to experimentation.

Solid State ^{2}H NMR Spectroscopy. ^{2}H NMR spectroscopy experiments were conducted as described.[4] Spectra were acquired on a home-built spectrometer operating at a resonant frequency of 27.6 MHz. A home-built probe with a 5 mm transverse mounted coil and temperature control was utilized. Temperature regulation with a precision of ± 0.5 °C was achieved employing a Love Controls (1600 series) temperature controller (Michigan City, IN).

Determination of Cholesterol Tilt Angle. ^{2}H NMR experiments with [3α-^{2}H$_1$]cholesterol allow for the determination of the orientation of the sterol molecule in membranes.[16] The most probable orientation of the steroid moiety, the tilt angle α_0, is obtained on the basis of the following theory.[17] Figure 1 illustrates the disposition of axes and angles. Fast axial rotation occurs about the long molecular axis that, in addition, wobbles with respect to the bilayer normal. The powder pattern observed is dominated by two intense peaks with splitting $\Delta\nu_r$ defined by the equation:

$$\Delta\nu_r = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)|S_{CD}| \quad (1)$$

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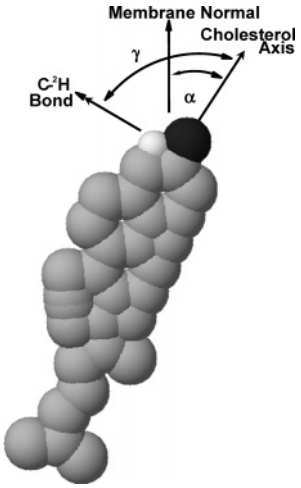

Figure 1. Angle and axis designation for membrane-incorporated [3α-^{2}H$_1$]cholesterol.

where $e^2qQ/h = 170$ kHz is the static quadrupolar splitting constant and S_{CD} is an order parameter describing the motionally averaged angular fluctuations of the C−^{2}H bond with respect to the bilayer normal. The order parameter S_{CD} can be expressed as

$$S_{CD} = S_\alpha S_\gamma \tag{2}$$

The term S_γ is a geometric factor

$$S_\gamma = \frac{1}{2}(3\cos^2\gamma - 1) \tag{3}$$

dependent on the angle $\gamma = 79°$[18] between the C−^{2}H bond and the molecular axis. The term S_α is the order parameter of the cholesterol molecule

$$S_\alpha = \frac{1}{2}\langle 3\cos^2\alpha - 1\rangle \tag{4}$$

with α representing the instantaneous angle between the long molecular axis of cholesterol and the bilayer normal, and the angular brackets designating a time average. Assuming a Gaussian distribution applies to α, the tilt angle α_0 of the steroid moiety may be extracted by numerical integration from

$$S_\alpha = \frac{\frac{1}{2}\int_0^\pi \sin\alpha e^{-\alpha^2/2\alpha_0^2}(3\cos^2\alpha - 1)d\alpha}{\int_0^\pi \sin\alpha e^{-\alpha^2/2\alpha_0^2}d\alpha} \tag{5}$$

Results

We determined the solubility and orientation of cholesterol in heteroacid PEs that contained saturated palmitic (16:0) acid at the sn-1 position, while a series of increasingly unsaturated fatty acids consisting of oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic (22:6) acids were esterified to the sn-2 position. Heteroacid PEs were our focus because phospholipids usually exist as sn-1 saturated, sn-2 unsaturated lipids in the plasma membrane of most tissues.[19] We also examined 22:6−22:6 PE, a homoacid PE with DHA for both sn-1 and -2 chains, as an extreme example of a dipolyunsaturated phospholipid found in select tissues (e.g., neuronal membranes).[20] All measurements, with the exception of 16:0−18:1 PE, were conducted at 7.5 °C. This temperature was chosen to conform to the narrow temperature range (6−10 °C) over which 16:0−22:6 PE is in the lamellar liquid crystalline state that mimics the in vivo condition for most phospholipids.[21] Employing 7.5 °C with 16:0−18:2 PE and 16:0−20:4 PE, which form a lamellar liquid crystalline phase over a wider span of temperatures, facilitates comparison under equivalent conditions. In the case of 16:0−18:1 PE, the measurements were not performed at 7.5 °C because, at this temperature, the lipid is in the lamellar gel phase. As a compromise, we opted for 40 °C, a temperature that approximates physiological conditions and where 16:0−18:1 PE is also lamellar liquid crystalline. Experiments on 22:6−22:6 PE/cholesterol samples were conducted in the reverse hexagonal phase (H_{II}) at 7.5 °C, as no other phase was observed with the dipolyunsaturated system over the temperature range studied.

XRD. The XRD method of measuring the solubility of cholesterol relies upon detecting the diffraction peaks produced by solid sterol that crystallizes outside the membrane in monohydrate form when the solubility limit has been exceeded.[14] Figure 2 presents integrated radial intensity profiles as a function of reciprocal space ($I-q$ plots) for PE/cholesterol samples. The data were recorded at a series of concentrations of added cholesterol employed to determine the solubility in 16:0−18:1 PE (Figure 2A), 16:0−18:2 PE (Figure 2B), 16:0−20:4 PE (Figure 2C), and 16:0−22:6 PE (Figure 2D). They are cropped from $q = 0.25-2.00$ Å^{-1} to focus upon the region where the three second-order diffraction peaks arise from monohydrate crystals, which were preferred over the first-order peaks to avoid potential overlap with more intense reflections at low-angles associated with the lipid phase.[4] To illustrate the low-angle region and serve as an example of phase assignment, the entire spectrum for a 16:0−20:4 PE/cholesterol sample is also shown (Figure 2E). The first-order (chol(001)) peak from solid cholesterol lies between the larger first- and second-order (L(001) and L(002)) peaks from the lipid that establish lamellar phase.

Inspection of the $I-q$ plots in Figure 2 reveals a dependence upon sn-2 unsaturation for the solubility of cholesterol in 16:0 sn-1 saturated PE. In the case of 16:0−18:1PE (Figure 2A), monohydrate crystalline reflections due to cholesterol excluded from the bilayer begin to emerge at $\chi_{CHOL} = 52.5$ mol % and grow in intensity as the mol fraction cholesterol increases. A similar concentration, $\chi_{CHOL} = 51.0$ mol %, for the emergence of crystalline peaks applies to 16:0−18:2 PE (Figure 2B). 16:0−20:4 PE (Figure 2C) and 16:0−22:6 PE (Figure 2D) are quite different. They display the presence of monohydrate crystals at lower cholesterol contents with reflections appearing at $\chi_{CHOL} = 44.0$ and 35.0 mol %, respectively, before growing in intensity at higher χ_{CHOL}.

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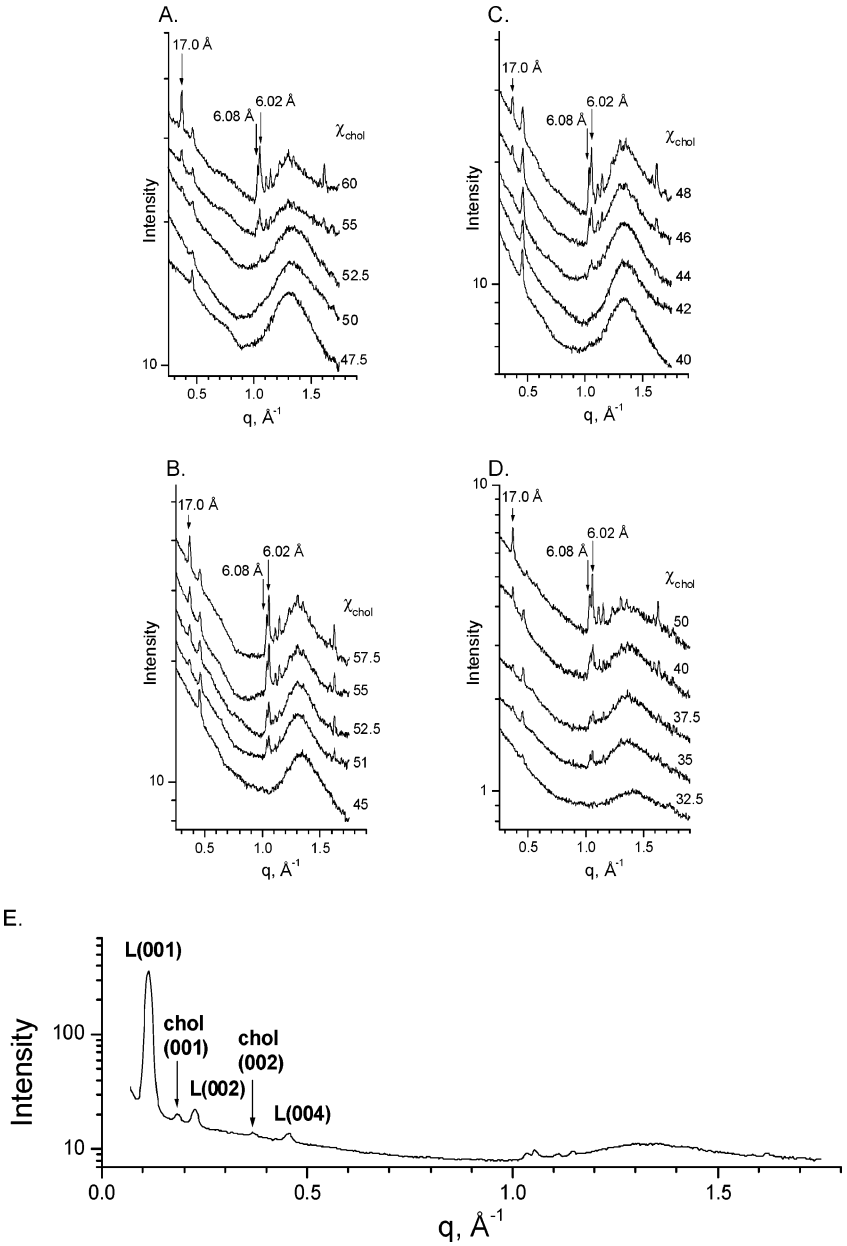

Figure 2. Integrated XRD radial intensity profiles for 50 wt % aqueous dispersions in 50 mM Tris (pH 7.5) of (A) 16:0−18:1 PE/cholesterol, (B) 16:0−18:2 PE/cholesterol, (C) 16:0−20:4 PE/cholesterol, and (D) 16:0−22:6 PE/cholesterol. Data were acquired in the lamellar liquid crystalline phase at 40 °C for (A) and 7.5 °C for (B−D). Concentration of added cholesterol (χ_{chol}) is indicated in mol % to the right of each profile. Plots are cropped from q = 0.25−2.00 Å^{-1} in reciprocal space to highlight the 002 (17.0 Å), 020 (6.08 Å), and 200 (6.02 Å) reflections (indicated by arrows) from cholesterol monohydrate crystals. (E) Diffractogram that extends to low angles showing the lamellar L(001) and L(002) reflections from 16:0−20:4 PE/cholesterol (χ_{chol} = 46 mol %).

$I-q$ diffractograms for 22:6−22:6 PE/cholesterol samples are plotted in Figure 3. They are distinct from those for the heteroacid saturated−unsaturated PE/cholesterol systems (Figure 2) in two respects. The low angle reflections (H(01), H(11) and H(02)) in the example of an entire spectrum shown for the dipolyunsaturated PE/cholesterol system signify the presence of an inverted hexagonal H_{II} phase (Figure 3B). Additionally, second-order diffraction peaks due to monohydrate crystals of cholesterol are discernible at χ_{CHOL} = 10 mol % in the $I-q$ plots observed as a function of the amount of added sterol (Figure 3A). Thus, placing DHA at both the sn-1 and -2 positions greatly reduces the concentration of sterol that PE can accommodate.

The sum of the integrated intensities of the three second-order reflections produced by cholesterol monohydrate crystals from the membrane (Figures 2 and 3) is plotted as a function of the concentration of added χ_{CHOL} in Figure 4. Linear extrapolation to zero intensity represents the solubility limit for the sterol in each PE species, and the values obtained are listed in Table 1. The highest solubility is observed for 16:0−18:1 PE. The monounsaturated PE can accommodate 51 ± 3 mol % cholesterol, consistent with a previous measurement.[14] The earlier published value was obtained at room temperature, suggesting that the solubility of cholesterol in 16:0−18:1 PE is relatively insensitive to temperature. Inclusion of a second double bond (16:0−18:2 PE) does not significantly alter the

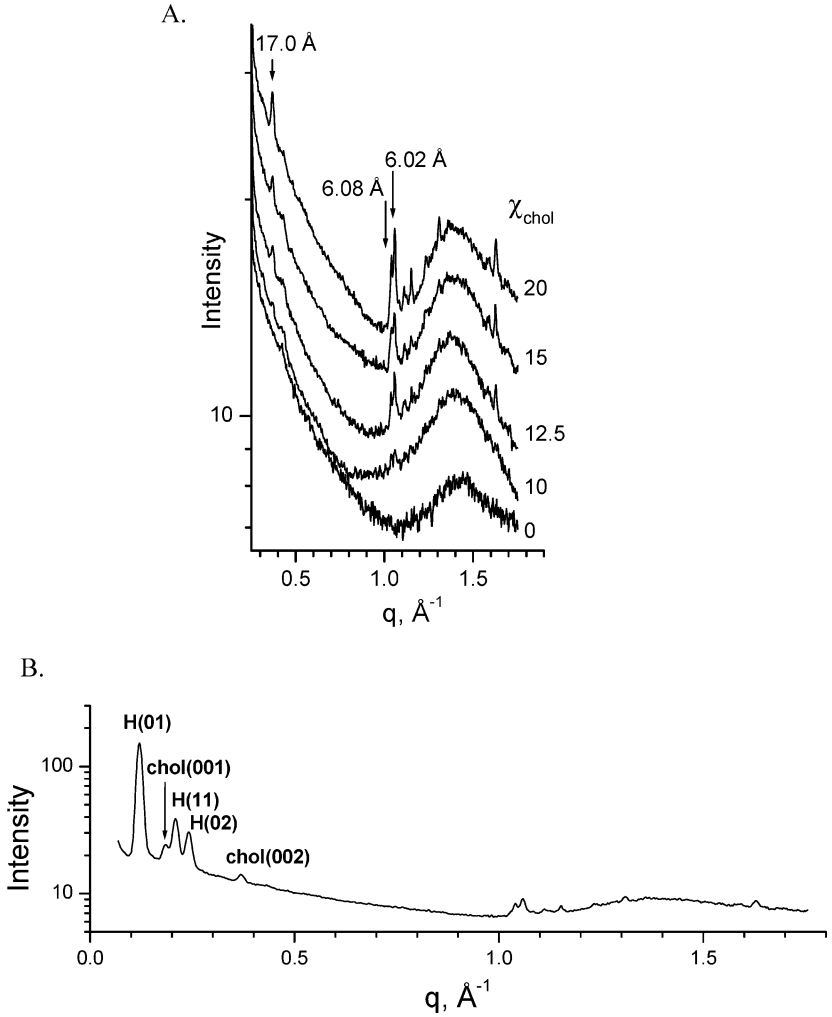

Figure 3. Integrated XRD radial intensity profile for 50 wt % aqueous dispersions in 50 mM Tris (pH 7.5) of (A) 22:6−22:6 PE. Data were acquired in the inverted hexagonal H_{II} phase at 7.5 °C. Concentration of added cholesterol (χ_{chol}) is indicated in mol % to the right of each profile. All other details as in Figure 2 with the exception that the reverse hexagonal H(01), H(11), and H(02) reflections for 22:6−22:6 PE/cholesterol (χ_{chol} = 20 mol %) are indicated in the diffractogram presented to low angles (B).

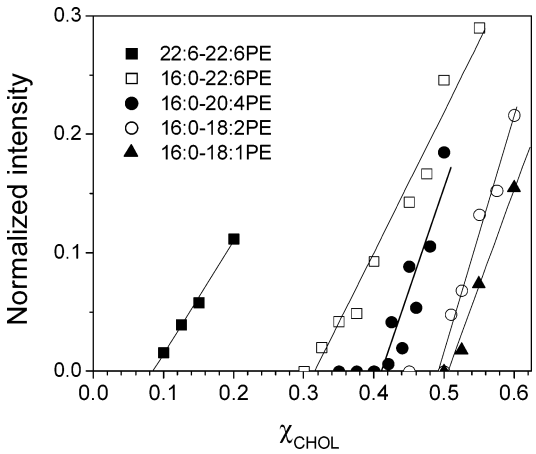

Figure 4. Combined integrated intensity of second-order XRD peaks (002, 020, 200) from cholesterol monohydrate crystals as a function of concentration of cholesterol (χ_{chol}) for 50 wt % aqueous dispersions in Tris (pH 7.5) of 16:0−18:1 PE/cholesterol, 16:0−18:2 PE/cholesterol, 16:0−20:4 PE/cholesterol, 16:0−22:6 PE/cholesterol, and 22:6−22:6 PE/cholesterol. The heteroacid saturated, unsaturated PEs were in the lamellar liquid crystalline phase whereas the homoacid dipolyunsaturated PE was in the inverted hexagonal H_{II} phase. Data were recorded at 7.5 °C for all PEs except 16:0−18:1 PE where 40 °C was used.

Table 1: Solubility of Cholesterol in PEs Obtained from XRD Measurements[a]

PE	cholesterol solubility mol %	PC	cholesterol solubility mol %
16:0−18:1	51 ± 3[b]	16:0−18:1	65 ± 3[e]
16:0−18:2	49 ± 2[c]		
16:0−20:4	41 ± 3[c]	18:0−20:4	49 ± 1[f]
16:0−22:6	31 ± 3[c]	18:0−22:6	55 ± 3[g]
22:6−22:6	8.5 ± 1[d]	22:6−22:6	11 ± 3[g]

[a] Values for PCs are included for purposes of comparison. Unless otherwise stated, the samples were in the lamellar liquid crystalline state. [b] 40 °C [c] 7.5 °C [d] 7.5 °C, H_{II} phase [e] 24 °C, value taken from Huang et al.[14] [f] 20 °C, value taken from Brzustowicz et al.[4] [g] 20 °C, value taken from Brzustowicz et al.[5]

solubility limit. The value of 49 ± 2 mol % obtained is the same, within experimental uncertainty, as that in 16:0−18:1 PE. Incorporation of four double bonds in the sn-2 position (16:0−20:4 PE) depresses the solubility of the sterol to 41 ± 3 mol %. The amount of cholesterol that can be incorporated into the membrane is further reduced with six double bonds for the heteroacid 16:0−22:6 PE where a solubility of 31 ± 3 mol % was measured. Dipolyunsaturated 22:6−22:6 PE with six double bonds in the sn-1 as well as sn-2 chain displays a markedly lower solubility for cholesterol. The value is 8.5 ± 1 mol %, a

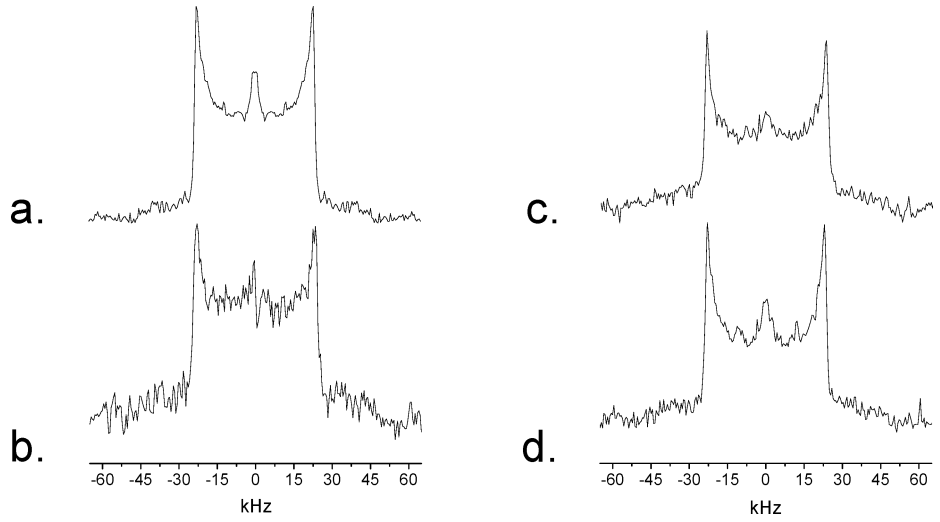

Figure 5. ^{2}H NMR spectra for 50 wt % aqueous dispersions in 50 mM Tris (pH 7.5) of (a) 16:0−18:1 PE, (b) 16:0−18:2 PE, (c) 16:0−20:4 PE, and (d) 16:0−22:6 PE in the presence of equimolar [3α-^{2}H$_1$]cholesterol. The temperature was 7.5 °C except for 16:0−18:1PE, where 40 °C applied. All spectra were acquired in the lamellar liquid crystalline phase.

factor of about four less than in the sn-1 saturated, sn-2 polyunsaturated heteroacid equivalent

^{2}H NMR. We utilized solid state ^{2}H NMR spectroscopy to determine the orientation of [3α-^{2}H$_1$]cholesterol incorporated into heteroacid PE membranes with increasing levels of unsaturation in the sn-2 chain. The tilt angle α_0, representing the most probable orientation of the long molecular axis of the steroid moiety (Figure 1), was obtained by measuring the quadrupolar splitting $\Delta\nu_r$ from the powder patterns recorded (Figure 5) and subsequently calculating the order parameter S_{CD} of the 3α C−^{2}H bond (eq 1). From S_{CD}, a molecular order parameter S_α was derived (eq 2) and then matched with numerically integrated values (eq 5) to obtain the tilt angle α_0. The model employed applies a weighted Gaussian distribution to describe the wobbling motion of the sterol molecule relative to the bilayer normal.[17]

^{2}H NMR spectra acquired for aqueous dispersions of the heteroacid PEs in the presence of equimolar [3α-^{2}H$_1$]cholesterol are shown in Figure 5. To comply with the conditions of the XRD experiments, they were obtained in the lamellar liquid crystalline phase at 40 °C for 16:0−18:1 PE (Figure 5a) and 7.5 °C for 16:0−18:2 PE (Figure 5b), 16:0−20:4 PE (Figure 5c), and 16:0−22:6 PE (Figure 5d). The delay of 75 ms employed between repetition of pulse sequences ensured full recovery and detection of the signal from membrane-intercalated [3α-^{2}H$_1$]cholesterol, which is characterized by a short spin−lattice relaxation time ($T_1 \approx 3$ ms).[4] Crystalline [3α-^{2}H$_1$]-cholesterol monohydrate excluded from the membrane, although present in the 16:0−20:4 PE and 16:0−22:6 PE samples according to XRD, does not contribute to the spectra because it relaxes much more slowly ($T_1 \approx 3$ s) and was essentially completely attenuated.[4] The resultant spectra are single component powder patterns produced by labeled sterol undergoing anisotropic reorientation in the bilayer.[16] A small central peak is, in addition, apparent in each spectrum (Figure 5). This peak, indicating the presence of lipid structures in which motion is isotropic and/or of residual ^{2}HHO, constitutes <5% of the total spectral intensity and was ignored.

For all four samples examined in Figure 5, the quadrupolar splitting for [3α-^{2}H$_1$]cholesterol inside the membrane is $\Delta\nu_r = 45 \pm 1$ kHz irrespective of whether there are 1 (Figure 5a), 2 (Figure 5b), 4 (Figure 5c), or 6 (Figure 5d) double bonds. As exemplified by the corresponding tilt angle $\alpha_0 = 15 \pm 1°$, the implication is that the orientation of the sterol does not vary with the degree of unsaturation in the sn-2 chain of heteroacid PEs containing a saturated palmitoyl chain at the sn-1 position. Table 2 catalogues the order parameters S_{CD} measured from the spectra in Figure 5 and the values calculated for the tilt angle α_0.

Table 2: Order Parameters S_{CD} and Derived Tilt Angles α_0 Obtained from ^{2}H NMR Spectra for [3α-^{2}H$_1$]Cholesterol Incorporated into PE Membranes[a]

PE	S_{CD}	α_0	PC	S_{CD}	α_0
16:0−18:1	0.37[b]	15 ± 1°	18:0−18:1	0.36[d]	16 ± 1°
16:0−18:2	0.36[c]	15 ± 1°			
16:0−20:4	0.36[c]	15 ± 1°	18:0−20:4	0.35[e]	16 ± 1°
16:0−22:6	0.36[c]	15 ± 1°	18:0−22:6	0.35[d]	16 ± 1°
			22:6−22:6	0.27[f]	24 ± 1°

[a] Values for PCs are included for purposes of comparison. Unless otherwise stated, the samples were in the lamellar liquid crystalline state. [b] 40 °C. [c] 7.5 °C. [d] 20 °C, value taken from Brzustowicz et al.[16] [e] 20 °C, value taken from Brzustowicz et al.[4] [f] 20 °C, value taken from Brzustowicz et al.[5]

Discussion

With 22 carbons and 6 double bonds, DHA is the longest and most unsaturated fatty acid found in abundance in living systems.[22] This fatty acid is the extreme example of the family of biologically active compounds known as ω3 PUFAs. Its positive link to the alleviation of a wide variety of human afflictions ranging from cancer and heart disease to neurological abnormalities[11] has prompted us to investigate the molecular mode of action of this influential but seemingly simple molecule. Dietary DHA has been shown to be rapidly incorporated primarily into the sn-2 acyl chain of plasma membrane phospholipids (usually PE > PC > phosphatidylserine (PS))[11,23] where it must encounter the most prevalent polar lipid in these

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membranes, cholesterol. Our initial work focused on cholesterol/DHA−PC interactions.[4,5,12,16,24] Because PE, rather than PC, represents the more typical metabolic fate of DHA consumed in the diet, we then turned our attention to DHA−PE/cholesterol interactions[6,21,25] and here characterize the dependence upon degree of unsaturation of the molecular organization of the sterol in PE membranes.

Prior Cholesterol Studies. The effect that introducing cholesterol has on the phase behavior of lipid bilayers has long been recognized.[3] It disrupts the regular packing of the rigid acyl chains that exists in the lamellar gel, L_β, state (also known as solid ordered or s_o), whereas in the lamellar liquid crystalline, L_α, state (also known as liquid disordered or l_d), which resembles the native state of a large fraction of membrane lipids, the sterol restricts rotational isomerization within the melted chains. A feature of phase diagrams constructed for homoacid disaturated and heteroacid saturated−monounsaturated PC membranes containing >25 mol % cholesterol is the adoption of a liquid-ordered (l_o) state, an intermediate between s_o and l_d states.[26−28] The l_o phase is characterized by a high degree of acyl chain order that, except for fast axial rotation, resembles the s_o phase and, by rapid lateral diffusion, that is similar to the l_d phase.[29] As judged by ^{2}H NMR of PCs with a saturated [$^2H_{31}$]palmitoyl ([$^2H_{31}$]16:0) sn-1 chain, the response to cholesterol is largely independent of the level of unsaturation present at the sn-2 position.[27,28,30,31] Although PEs constitute the second most abundant phospholipid type after PCs found in the mammalian plasma membrane,[19] few phase diagrams have been described for PE/cholesterol systems. PE has a lower affinity than PC for the sterol.[32] Nevertheless, PE/cholesterol membranes can exist in a l_o state. ^{2}H NMR spectra from 1-[$^2H_{31}$]palmitoyl-2-oleoylphosphatidylethanolamine ([$^2H_{31}$]16:0−18:1 PE) have identified a l_o phase at cholesterol concentrations >35 mol %.[33] The existence of the l_o phase has also been inferred on the basis of the sharpness of the peaks resolved in ^{2}H NMR spectra for polyunsaturated [$^2H_{31}$]16:0−22:6 PE/50 mol % cholesterol.[21]

A series of studies has established that highly disordered PUFAs are sterically incompatible with close proximity to the rigid steroid moiety.[11,12] Definitive evidence is provided by the reduced solubility of the sterol in PUFA-containing phospholipid membranes,[7,12,34] notably 16:0−22:6 PE.[21] This phospholipid has been a focus of attention in our recent work.[6,21] We have hypothesized that incompatibility of cholesterol for DHA may promote phase separation of the sterol into sphingolipid- and cholesterol-rich rafts away from DHA-rich microdomains. Supporting our hypothesis, we showed in mixtures of PE with sphingomyelin (SM) and cholesterol that 16:0−22:6 PE phase separates from the lipid raft molecules far more than 16:0−18:1 PE containing monounsaturated oleic acid.[6] Concomitant changes in protein location, we propose, may have implications for cellular signaling. What remains unknown, however, are details of the interactions of the sterol with PEs possessing other degrees of acyl chain unsaturation and their potential to drive phase separation. In an earlier investigation, we observed by moment analysis of ^{2}H NMR spectra for [$^2H_{31}$]16:0−22:6 PE that the addition of equimolar cholesterol does not abolish the gel-to-liquid crystalline transition.[21] On the other hand, equimolar sterol broadened the transition beyond detection in [$^2H_{31}$]16:0−18:1 PE. A reduced solubility for the sterol in the DHA-containing phospholipid was proposed.

Cholesterol Solubility in PEs vs PCs. Inspection of Table 1 reveals that the solubility measured for cholesterol in heteroacid PEs varies with the number of double bonds in the sn-2 chain. It can be seen that whereas the mono and diunsaturated PEs can accommodate approximately 50 mol % cholesterol, the amount of the sterol that can be accommodated in the PUFA-containing PEs (16:0−20:4 PE and 16:0−22:6 PE) is reduced. We attribute the drop to the high disorder of the polyunsaturated chain, the accompanying increase in the number of sn-2 chain carbons presumed to have little effect. The low-energy barrier to rotational isomerization about the pairs of single C−C bonds that separate the multiple unsaturated carbon atoms facilitates rapid interconversion between torsional states.[35−37] A highly disordered structure that is incompatible with intimate contact with the rigid steroid moiety results, thereby limiting the concentration of cholesterol that may be incorporated into the bilayer. The increase in conformational disorder that accompanies the additional two double bonds in 16:0−22:6 PE vs 16:0−20:4 PE explains the diminished solubility of cholesterol in the DHA-containing PE. In the case of 16:0−18:1 PE and 16:0−18:2 PE, the unsaturations are located at the Δ9 position and at the Δ9 and Δ12 positions, respectively. Their depth within the membrane is lower than that to which the sterol ring system penetrates. The close approach of unsaturated fatty acid and cholesterol does not then necessitate unfavorable interactions between the steroid moiety and double bonds, agreeing with our measurement of higher but similar solubility in the mono- and di-unsaturated PEs.

Table 1 shows that placing DHA at both sn-1 and -2 positions dramatically lowers the amount of cholesterol that can be incorporated into PE membranes. The solubility measured for cholesterol in 22:6−22:6 PE is only 8.5 mol % sterol, which is a factor of 4 reduction relative to 16:0−22:6 PE that can incorporate 31 mol % sterol. As illustrated by the data also presented for 22:6−22:6 PC and 18:0−22:6 PC in Table 1, a similarly appreciable drop in solubility of cholesterol is seen in dipolyunsaturated PC membranes compared to the mixed chain saturated-polyunsaturated PC equivalents.[12] This enhanced exclusion of cholesterol from PE and PC membranes where it cannot avoid PUFA chains unequivocally demonstrates poor affinity for sterol.

Our findings regarding the solubility of cholesterol for PE lipids contrast with those on PC membranes.The solubility of cholesterol in 18:0−18:1 PC, 18:0−20:4 PC, and 18:0−22:6

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PC membranes determined by us[4,5] and others[14] in earlier work are included in Table 1 for purposes of comparison. In each case, the values for PC are higher than in the heteroacid PE with the same sn-2 chain. The poorer affinity of sterol for PE as opposed to PC agrees with published work. Early differential scanning calorimetry (DSC) of lipid mixtures indicated that cholesterol associates with SM ≫ PS, phosphatidylglycerol (PG) > PC > PE.[32] More recently, partition coefficients in the order SM > PS > PC > PE were determined for cholesterol in unilamellar vesicles by a cyclodextrin assay.[38] Miscibility of the sterol in saturated lipids possessing myristoyl (14:0) sn-1 and -2 chains as ascertained by XRD, DSC, and/or neutron scattering decreased in the following manner PC > PG ≈ PE > PS.[39]

The differential solubility of cholesterol in PE and PC membranes demonstrates that the headgroup structure in addition to acyl chain unsaturation influences the interaction of the sterol with phospholipids. Although energy minimization studies indicate that van der Waals forces make the largest contribution to sterol−lipid interactions,[40] hydrogen bonding is also considered to play an important role. One suggestion is that intermolecular hydrogen bonds between the NH_3^+ hydrogens of the PE headgroup and the neighboring phosphate oxygens stabilize the bilayer and serve to reduce the solubility of cholesterol in PE relative to PC.[41] Recent molecular dynamics (MD) simulations of PC bilayers with cholesterol, ergosterol, and lanosterol found that the sterol forms hydrogen bonds with oxygen atoms in the phospholipid either directly or via a water bridge in which water molecules form hydrogen bonds with both sterol and phospholipid.[42] Complexation between sterol and phospholipid, as judged by the tendency to hydrogen bond, was affected by the modest differences in structure of the three sterols. It is reasonable to infer that differences in headgroup structure and hydration of the headgroup would lead to a change in the hydrogen bonding of cholesterol to PE vs PC. A smaller area per molecule, reflecting tighter lipid packing,[33] is another structural parameter with the potential to affect the interaction with sterol that distinguishes PE from PC.

An alternative view that reconciles the diminished solubility seen for cholesterol in PE as opposed to PC membranes is offered by the umbrella model.[43] This model links membrane lateral organization and cholesterol solubility. It shows that at select phospholipids/sterol ratios, the lipids organize laterally in highly regular rectangular or hexagonal arrangements to minimize cholesterol multibody interactions that are energetically unfavorable. The polar phospholipid headgroups, acting as "umbrellas", shield the cholesterol molecules from water at the membrane surface. A slight increase in sterol content, however, is followed by a steep jump in the potential energy of cholesterol that favors exclusion from the bilayer because the headgroups no longer shelter the membrane-incorporated cholesterol from interfacial water. Accordingly, a smaller headgroup for PE would provide less protection than PC, so that the solubility limit for cholesterol would be reached at a lower level of incorporation. Because increased molecular area is associated with the high disorder of PUFA chains, the exclusion of sterol from polyunsaturated membranes seen at lower concentration of incorporation may similarly be explained on the basis of the umbrella model. Reservations concerning the umbrella concept exist. From a consideration of the relative size of the ethanolamine and choline moieties, for example, PE might be expected to provide far less cholesterol coverage than PC and, in terms of the umbrella model, to predict a much greater difference between cholesterol solubility than is measured.

The dependence upon the number of double bonds of the solubility of cholesterol in heteroacid PCs catalogued in Table 1 is also less pronounced than in PE. All of the values come in at ∼50 mol % or more. Our interpretation is that the rigid steroid moiety preferentially associates with the saturated 18:0 sn-1 chain of the PCs and is largely insensitive to the presence of polyunsaturation in the sn-2 chain.[4,5,16] A plateau region of almost constant order parameter S_{CD} in the upper portion of a saturated sn-1 chain is universally reported for PC membranes irrespective of the degree of unsaturation at the sn-2 position.[44] It is attributed to a preponderance of trans isomeric states that align segments parallel to the bilayer normal, allowing the rigid steroid moiety to come into intimate contact. In marked contrast, PUFA chains are highly flexible and undergo rapid transitions between torsional states that redistribute the mass of the chain toward the lipid−water interface.[45] Near approach of sterol is, thus, deterred by the entropically unfavorable severe restriction to chain motion that would ensue.

A compilation of the solubility of cholesterol in phospholipids with a variety of acyl chains and headgroups was reported in a recent review.[39] As in our work, the onset of the observation of cholesterol crystals defined the limit of the amount of sterol that a membrane can accommodate. Egg PE, a natural lipid extract predominantly comprised of 16:0 and 18:0 acids at the sn-1 position and 18:1, 18:2, and 20:4 acids at the sn-2 position offers the closest comparison to a PE studied here. Although the solubility of 35−40 mol % stated for sterol is less than the value of 51 ± 3 mol % that we obtained in 16:0−18:1 PE (Table 1), a lowering in the heterogeneous PE mixture is conceivable due to the presence of polyunsaturated 20:4 acid.

Molecular Orientation of Membrane-Incorporated Cholesterol. The ^2H NMR spectra shown in Figure 5 for [3α-$^2H_1$]-cholesterol added in equimolar amount to heteroacid PE membranes were recorded to ascertain whether the differences in solubility are related to a change in orientation of the sterol within the membrane. Spectra were collected at temperatures that matched the XRD work, ensuring that each sample was in the lamellar liquid crystalline phase. The experiments were designed to solely observe membrane-incorporated sterol. Because equimolar cholesterol approximates or exceeds the solubility limit of each membrane, as determined on the basis of the XRD data, the spectra characterize the molecular organization of the maximum content of [3α-$^2H_1$]cholesterol that the membrane can contain. From the quadrupolar splittings measured, the tilt angle α_0 that represents the most probable orientation of the long molecular axis was determined. According to our earlier work on PCs, the tilt angle is symptomatic of

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the level of unsaturation of the acyl chain with which the sterol predominantly interacts.[12] Interaction with a saturated vs polyunsaturated chain is predicted by a smaller ($\alpha_0 \approx 15°$) vs larger ($\alpha_0 \approx 24°$) value.

Table 2 summarizes the tilt angles obtained for [3α-$^2H_1$]-cholesterol in the heteroacid PEs and, for comparison, the corresponding PCs. A tilt angle for [3α-$^2H_1$]cholesterol in 22:6−22:6 PC is also included, but not in 22:6−22:6 PE because the dipolyunsaturated PE adopted an inverted hexagonal phase at all temperatures investigated. Inspection of the values demonstrates that the tilt angle for [3α-$^2H_1$]cholesterol remains the same ($\alpha_0 = 15 \pm 1°$) in 16:0−18:1 PE, 16:0−18:2 PE, 16:0−20:4 PE, and 16:0−22:6 PE membranes. This value, within experimental uncertainty, matches that measured for the analogous PCs where the tilt angle ($\alpha_0 = 16 \pm 1°$) was similarly independent of the number of double bonds in the sn-2 chain (Table 2). The same tilt angle, moreover, was reported in the complete absence of double bonds for 1,2-dipalmitoylphosphatidylcholine (16:0−16:0 PC).[46] Only when multiple double bonds are present in both sn-1 and -2 chains in 22:6−22:6 PC, and also as in 20:4−20:4 PC,[12] is the orientation ($\alpha_0 = 24 \pm 1°$) different (Table 2). Thus, the data reinforce our interpretation that the sterol molecule organizes within the membrane to minimize contact with the polyunsaturated sn-2 chain and preferentially associates with the saturated sn-1 chain in both heteroacid PEs and PCs. In homoacid dipolyunsaturated 22:6−22:6 PC, the tilt angle increases because the membrane-intercalated cholesterol molecule is forced to interact with highly disordered PUFA chains that allow a greater amplitude of angular fluctuation. Perhaps the identical tilt angle measured for all heteroacid PCs and PEs argues against the umbrella model,[43] as the larger "umbrella" provided by the larger PC headgroup would allow a greater range of angular fluctuation for the steroid moiety than the smaller PE headgroup.

There is other experimental evidence in support of the preferential affinity of cholesterol for saturated over polyunsaturated chains. Closer contact with the 18:0 sn-1 chain in 18:0−22:6 PC/[25,26,26,27,27,27-$^2H_7$]cholesterol (1:1 mol) membranes was revealed by a higher rate of chain-to-sterol nuclear Overhauser enhancement spectroscopy (NOESY) cross-relaxation in ^{1}H MAS NMR experiments.[7] This result was reproduced in subsequent MD simulations on an 18:0−22:6 PC/cholesterol (1:3 mol) bilayer that corroborated that the sterol favors solvation by saturated over polyunsaturated chains.[47] A similar increase in orientational order of the saturated [$^2H_{31}$]-16:0 sn-1 chain was observed by ^{2}H NMR irrespective of the presence of polyunsaturation in the sn-2 chain when cholesterol was introduced into PC membranes.[27,28,30,31]

Physiological Implications. Poor affinity for highly disordered PUFA chains is the molecular origin of the mechanism that we propose promotes the segregation of cholesterol away from DHA-rich/sterol-poor domains into sphingolipid-rich/sterol-rich rafts.[11] Accompanying changes in the location of signaling proteins would then have the potential to modulate cellular signaling events, perhaps enhancing immunosuppression.[6] Other profound implications for normal and pathological cellular conditions of cholesterol-rich domains have been discussed (see a recent review by Mason et al.[48]). Cholesterol-rich domains have been postulated to serve as nucleation sites for cholesterol crystals, which may promote atherosclerotic lesions. In contrast, cholesterol-rich domains in fiber cell plasma membranes of human ocular lens may have a role in maintaining light transparency. We believe differential interactions between fatty acids and cholesterol may explain how PUFAs, most influentially DHA, alleviate a vast array of chronic disease states and promote human health.[49]

The results presented here may have additional connotations for cell membrane function. It is well established that cholesterol associates strongly with sphingolipids primarily in outer leaflet lipid rafts[50] whereas DHA preferentially accumulates in PEs that are primarily located in the inner leaflet.[51] Cholesterol is also known to exhibit rapid flip-flop between the leaflets.[52] Therefore, it can be proposed that dietary DHA, first appearing in inner leaflet PEs, would enhance the transfer of cholesterol to the outer leaflet where it would affect raft composition and function. Indeed, Dusserre et al. reported cholesterol efflux from plasma membranes remained the same after incorporation of oleate, linoleate, or arachidonate but increased with eicosapentaenoic acid (EPA) and DHA.[53] Sweet and Schroeder saw, moreover, that PUFA accumulation into plasma membranes resulted in a substantial redistribution of cholesterol to the outer leaflet.[54] Interactions between DHA and cholesterol may then affect both lateral and trans-membrane domain structure.

Conclusion.

In the present study, we demonstrate that increasing the number of double bonds in the sn-2 chain of heteroacid PE molecules possessing a saturated sn-1 chain renders the bilayer less soluble to cholesterol. The findings contrast with PCs, where the number of double bonds in the sn-2 chain has little influence on sterol solubility. However, the orientation of cholesterol remains the same in both PE and PC membranes regardless of the degree of unsaturation at the sn-2 position. We conclude that both headgroup structure and acyl chain unsaturation modulate cholesterol solubility within membranes, which may have implications for lipid phase separation, raft formation, and membrane function.

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