Interaction of Polyunsaturated Fatty Acids with Cholesterol: A Role in Lipid Raft Phase Separation

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Summary: Unequal affinity between lipids has been hypothesized to be a mechanism for the formation of microdomains/rafts in membranes. Our studies focus upon the interaction of cholesterol with polyunsaturated fatty acid (PUFA)-containing phospholipids. They support the proposal that steric incompatibility of the rigid steroid moiety for highly disordered PUFA chains, in particular docosahexaenoic acid (DHA), provides a sensitive trigger for lateral segregation of lipids into PUFA-rich/sterol-poor and PUFA-poor/sterol-rich regions. Solid state ^{2}H NMR and x-ray diffraction (XRD) demonstrate that the solubility of cholesterol is reduced in 1-palmitoyl-2-docosahexaenoyl-phosphatidylethanolamine (16-0:22:6PE) bilayers. In mixed membranes of phosphatidylethanolamine (PE) with the lipid raft forming molecules egg sphingomyelin (SM) and cholesterol, diminished affinity of the sterol for 16:0-22:6PE relative to 1-palmitoyl-2-oleoylphosphatidylethanolamine (16:0-18:1PE) is identified by ^{2}H NMR order parameters and detergent extraction. Phase separation of the PUFA-containing phospholipid from SM/cholesterol rafts is the implication, which may be associated with the myriad of health benefits of dietary DHA.

Keywords: cholesterol; docosahexaenoic acid (DHA); lipid domains; polyunsaturated fatty acid (PUFA)

Introduction

There is tremendous interest in understanding the properties of membranes containing polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA).[1] This PUFA belongs to the ω3 series in which the ultimate unsaturation is 3 carbons from the terminal methyl or ω end and, with 22 carbons and 6 double bonds, constitutes the most unsaturated fatty acid commonly found in nature. A major issue that motivates the research is the enormous range of health benefits including the prevention of cancer and heart disease that accompany consumption of DHA found in fish oils. The diversity of the

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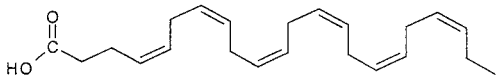
physiological processes affected suggests a fundamental mode of action common to most cells. Our experiments target the plasma membrane as a likely site of action and focus upon the interaction of cholesterol with DHA-containing phospholipids. They support the idea that steric incompatibility of the rigid steroid moiety for highly disordered PUFA chains promotes lateral segregation of lipids into DHA-rich/sterol-poor and DHA-poor/sterol-rich regions, such as lipid rafts that are enriched in cholesterol and sphingolipids.[2,3] We speculate that concomitant changes in lipid raft-mediated cellular signaling events may be responsible in part for the dietary advantage conferred by DHA.



Structure of DHA

A flexible structure with rapid inter-conversion between torsional states is revealed for DHA chains esterified to phospholipids by molecular dynamics (MD) simulations.[4-5] The low energy barrier to rotational isomerization about the pairs of single C-C bonds that separate the unsaturated carbon atoms more than offsets the rigidity of the multiple double bonds. It is the high degree of conformational flexibility that distinguishes the molecular organization of PUFA from saturated and less unsaturated fatty acids. Besides producing a disordered membrane interior[6-8], the impact of DHA upon membranes includes increased permeability[9], phospholipid flip-flop[10] and in-plane elasticity.[11] The high conformational disorder of the DHA chain also prevents the close approach from the rigid steroid moiety of cholesterol that is allowed by the predominantly all-trans configuration of the upper portion of saturated chains. Our measurement of a factor of 3-4 reduction in the solubility of cholesterol in membranes composed of PC (phosphatidylcholine) with DHA or arachidonic acid at both sn-1 and-2 positions compared to more typical PC's with a saturated sn-1 chain exemplifies the poor affinity of the sterol for PUFA.[12-14] Partition coefficients (K_A^B) measured for cholesterol in unilamellar vesicles corroborate.[15]

Biological membranes are no longer considered to be a homogeneous mixture of lipid and protein but are envisaged as a complex arrangement of domains of differing composition.[16] Unequal affinity between different lipid species or between lipids and membrane proteins is responsible for the formation of these patches. Differential interaction with cholesterol provides an influential lipid-driven mechanism whereby sterol-rich/liquid ordered (l_o) and sterol-poor/liquid disordered (l_d) lipid domains segregate

within lipid/cholesterol mixtures. Lipid rafts that are enriched in cholesterol and saturated sphingolipids and serve as platforms for signaling proteins, in particular, have recently attracted considerable interest. The formation of these domains reflects a preference of the sterol for sphingolipids. Their size, typically quoted ~ 50 nm, is the subject of debate.

The poor affinity of PUFA for cholesterol has been hypothesized to drive lateral phase separation into PUFA-poor/sterol-rich and PUFA-rich/sterol-poor microdomains.[12-14,17,18] We extend this hypothesis to a possible explanation of the health benefits of DHA. In most human cells DHA levels are low (typically < 5 % of the total acyl chains) and dietary supplementation primarily incorporates DHA into the sn-2 position of membrane phospholipids, often phosphatidylethanolamine (PE), while the sn-1 chain remains saturated.[19] We propose that DHA-containing phospholipids will be excluded from l_o sphingomyelin-rich/sterol-rich lipid rafts and will locate as DHA-rich microdomains within the l_d remainder of the membrane.[2,3] The partitioning into these regions of proteins that require a l_o vs. l_d environment to function will then be promoted and cellular signaling altered.

Here we present evidence in support of our proposal from predominantly solid state ^2H NMR spectroscopy supplemented by x-ray diffraction (XRD). The data amassed on 1-palmitoyl-2-docosahexaenoylphosphatidylethanolamine (16:0-22:6PE) demonstrate that this DHA-containing phospholpipid has low affinity for cholesterol and suggest that it separates from lipid raft molecules in mixed membranes.

Interaction of a DHA-Containing Phospholipid with Cholesterol

DHA Prohibits Close Contact with Cholesterol

Figure 1 shows solid state ^2H NMR spectra that are representative of the phases formed by [$^2H_{31}$]16:0-22:6PE in aqueous dispersion without and with equimolar cholesterol. Each spectrum is a superposition of powder patterns from all positions along the perdeuterated [$^2H_{31}$]16:0 sn-1 chain.[20] At -5 °C, the spectrum for [$^2H_{31}$]16:0-22:6PE is a broad featureless pattern with edges at ±63 kHz that reflects the slow reorientational motion undergone by acyl chains in the lamellar gel phase (Fig. 1a). The melting of the chains that accompanies the gel to liquid crystalline phase transition is apparent in the much narrower spectrum at 7.5 °C (Fig. 1 b). A plateau region of almost constant order in the upper part of the [$^2H_{31}$]16:0 sn-1 chain produces the well-defined edges at ±17 kHz. Increasingly more disordered methylenes in the lower portion of the chain give rise to the individual peaks,

while the central pair of peaks is due to the highly mobile terminal methyl group. The spectrum for [$^2H_{31}$]16:0-22:6PE at 40 °C with edges at ±6 kHz is characteristic of the inverted hexagonal (H_{II}) phase (Fig. 1c).[21] Rapid diffusion of lipid molecules around the cylindrical structures that comprise this phase is responsible for an additional reduction by a factor of ½ in spectral width relative to the lamellar phase.

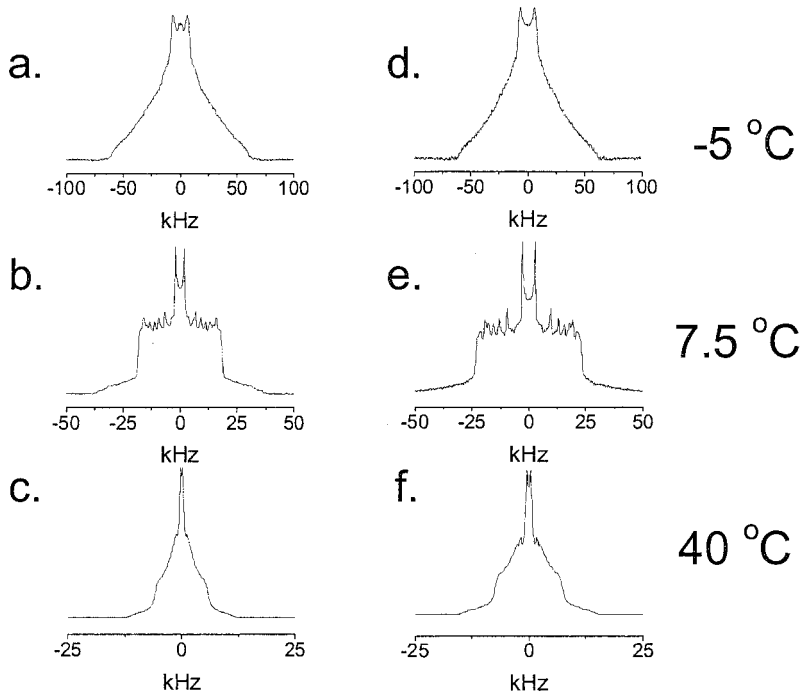


Figure 1. ^2H NMR spectra for 50 wt% aqueous dispersions in 50 mM Tris (pH 7.5) of a-c. [$^2H_{31}$]16:0-22:6PE and d-f. [$^2H_{31}$]16:0-22:6PE/cholesterol (1:1 mol).

The spectra for [$^2H_{31}$]16:0-22:6PE in the presence of equimolar cholesterol, remarkably, reveal the same phases. They are symptomatic of gel, liquid crystalline lamellar and H_{II} phases at –5 (Fig. 1d), 7.5 (Fig. 1e) and 40 °C (Fig. 1f), respectively. Apart from a slight narrowing at -5 °C and a broadening at 7.5 and 40 °C that are consistent with the sterol disrupting chain packing in the gel state and obstructing chain motion in the liquid crystalline phase, there is little perturbation to the spectra. Such a modest change is unusual. For most phospholipids the gel to liquid crystalline phase transition is broadened beyond detection with equimolar cholesterol.[22]

The variation with temperature of the first moments M_1 calculated from spectra that are plotted in Figure 2 elaborates upon the difference between the response to equimolar sterol of [$^2H_{31}$]16:0-22:6PE and [$^2H_{31}$]16:0-18:1PE. This quantity, defined by

$$M_1 = \frac{\int_{-\infty}^{\infty} |\omega| f(\omega) d\omega}{\int_{-\infty}^{\infty} f(\omega) d\omega} \qquad (1)$$

where $f(\omega)$ is the lineshape as a function of the frequency ω relative to the central Larmor frequency ω_0, is a sensitive indicator of membrane phase.[20] There are two discontinuities in the graph for [$^2H_{31}$]16:0-22:6PE (Fig. 2a). The transition from lamellar gel to liquid crystalline state gives rise to a sharp drop in the value of M_1 from $> 10 \times 10^4$ to $\sim 6.0 \times 10^4$ s^{-1} at 5 °C, and the transition to H_{II} phase results in a further drop to $< 3.0 \times 10^4$ s^{-1} centered at ~13 °C. Discontinuities associated with these phase changes, although somewhat modified, are still seen in the plot for [$^2H_{31}$]16:0-22:6PE/cholesterol (1:1 mol). In contrast, the M_1 values for our more typical [$^2H_{31}$]16:0-18:1PE control sample slowly decrease with temperature in the presence of cholesterol and do not exhibit the abrupt drop at 23 °C that in the absence of the sterol signifies the gel to liquid crystalline transition (Fig. 2b). We attribute the retention of a gel to liquid crystalline phase transition despite the addition of equimolar cholesterol to diminished solubility of the sterol in the DHA-containing phospholipid.

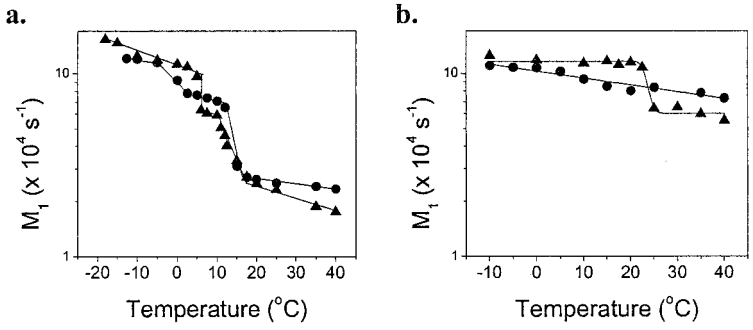


Figure 2. First moment M_1 vs. temperature for 50 wt% aqueous dispersions in 50 mM Tris (pH 7.5) of a. [$^2H_{31}$]16:0-22:6PE (▲) and [$^2H_{31}$]16:0-22:6PE/cholesterol (1:1 mol) (●), and b. [$^2H_{31}$]16:0-18:1PE (▲) and [$^2H_{31}$]16:0-18:1PE/cholesterol (1:1 mol) (●).

XRD confirms that the solubility of cholesterol is reduced in 16:0-22:6PE. Profiles of integrated radial intensity against reciprocal space ($q = 4\pi \sin\theta / \lambda$) for 16:0-

22:6PE/cholesterol and 16:0-18:1PE/cholesterol samples with a series of contents of added cholesterol χ_{chol} are presented in Figure 3. The method relies on detecting the second-order 002, 020 and 200 diffraction peaks from cholesterol monohydrate crystals that are formed outside the membrane when sterol in excess of the solubility limit is excluded.[13] Their respective reciprocal and real spacings are 0.3701 $Å^{-1}$, 17.0 Å (002); 1.033 $Å^{-1}$, 6.079 Å (020) and 1.044 $Å^{-1}$, 6.015 Å (200).[23] Inspection of Figure 3 reveals that sharp peaks (labeled ↓) from solid cholesterol are barely discernible at χ_{chol} = 32.5 mol% and subsequently grow in intensity as χ_{chol} increases for 16:0-22:6PE (left), whereas for 16:0-18:1PE they do not begin to emerge from the broad background due to membrane lipid until χ_{chol} exceeds >50 mol% (right).

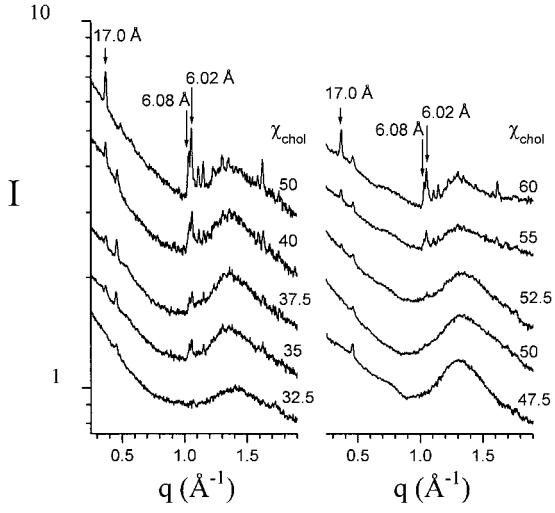


Figure 3. Integrated radial intensity profiles (I-q plots) for 50 wt% aqueous dispersions in 50 mM Tris (pH 7.5) of 16:0-22:6PE/cholesterol at 7.5 °C (left) and 16:0-18:1PE/cholesterol at 40 °C (right). The concentration of sterol χ_{chol} is listed in mol% to the right of each plot. At each temperature the systems are in the lamellar liquid crystalline state.

A precise measurement of the concentration at which cholesterol begins to precipitate from the membrane is obtained in Figure 4 by plotting the sum of the intensities of the three second-order reflections produced by solid sterol (normalized relative to the lipid wide-angle peak centered at $q \sim 1.4$ $Å^{-1}$) against χ_{chol}. Linear extrapolation to zero integrated intensity then gives a solubility of 32 ± 3 mol% for 16:0-22:6PE, substantially

less than the value of 51 ± 2 mol% measured in 16:0-18:1PE that is comparable to previous estimates for many phospholipids.[24] Our explanation is that the highly disordered DHA chain is responsible for the smaller solubility of cholesterol in 16:0-22:6PE. This view may be reconciled with the "umbrella" model for cholesterol-phospholipid mixing proposed by Huang and Feigenson.[25] According to their model lipid head groups shield cholesterol from unfavorable contact with water and the solubility limit is reached when the head groups can no longer cover additional sterol molecules. The shielding would be rendered less effective for DHA-containing PE by the increased molecular area that is associated with the high disorder of the polyunsaturated chain. The poor affinity for cholesterol that the reduced solubility in 16:0-22:6PE implies may also be interpreted in terms of another model suggested by McConnell and coworkers.[26] The model has cholesterol and phospholipid forming complexes that can separate into a complex-rich phase. Because the high disorder of DHA prevents close approach from sterol molecules, the formation of such complexes would be compromised in the case of polyunsaturated phospholipids.

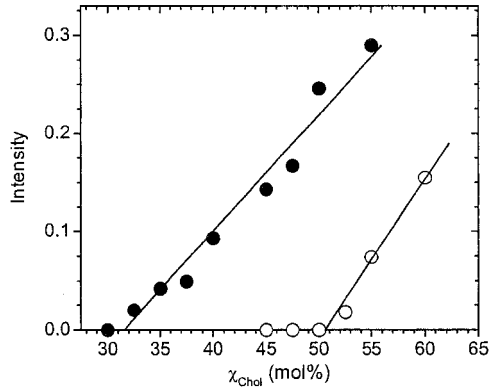


Figure 4. Combined integrated intensity of the second-order XRD peaks 002, 020 and 200 from cholesterol monhydrate excluded from the membrane for 50 wt% aqueous dispersions in 50 mM Tris (pH 7.5) of 16:0-22:6PE/cholesterol at 7.5 °C (●) and 16:0-18:1PE/cholesterol (○) at 40 °C.

DHA Promotes Phase Separation from Lipid Raft Molecules

To support our hypothesis that the introduction of DHA into a membrane enhances the segregation of cholesterol into lipid rafts, we compare the interaction of 16:0-22:6PE vs. 16:0-18:1PE with the sterol in mixtures containing egg sphingomyelin (SM). Figure 5

shows smoothed profiles of order parameter against carbon position for [$^2H_{31}$]16:0-22:6PE/SM (1:1 mol) and [$^2H_{31}$]16:0-18:1PE/SM (1:1 mol) in the absence and presence of cholesterol (1:1:1 mol) at 40 °C. At this temperature both DHA and oleic acid-containing systems are in the lamellar liquid crystalline phase, since SM stabilizes bilayer structure.[27] The order parameter profiles were generated from depaked spectra (inset) on the basis of integrated intensity assuming monotonic dependence.[28] The FFT depaking procedure deconvolutes powder pattern signals to spectra representative of a planar membrane of single alignment.[29] They consist of well-resolved doublets with splittings $\Delta\nu(\theta)$ that equate to order parameters S_{CD} via

$$\Delta\upsilon(\theta) = \frac{3}{2}\left(\frac{e^2qQ}{h}\right)|S_{CD}|P_2(\cos\theta) \qquad (2)$$

where $\theta = 0^\circ$ is the angle the membrane normal makes with respect to the magnetic field and $P_2(\cos\theta)$ is the second order Legendre polynomial.

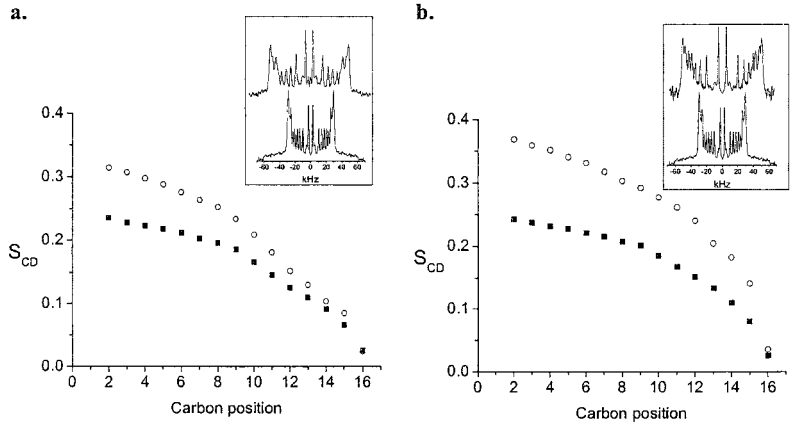


Figure 5. Smoothed order parameter profiles at 40 °C for 50 wt% aqueous dispersions in 50 mM Tris (pH 7.5) of a. [$^2H_{31}$]16:0-22:6PE/SM (1:1 mol) (■) and [$^2H_{31}$]16:0-22:6PE/SM/cholesterol (1:1:1 mol) (○), and b. [$^2H_{31}$]16:0-18:1PE/SM (1:1 mol) (■) and [$^2H_{31}$]16:0-18:1PE/SM/cholesterol (1:1:1 mol) (○). Insets show the depaked spectra (lower and upper without and with cholesterol, respectively) from which the profiles are constructed.

All of the profiles plotted in Figure 5 possess the same characteristic shape. A plateau region of virtually constant order in the top part of the chain is followed by progressively lower order in the bottom part. The profiles demonstrate that cholesterol orders the

[$^2H_{31}$]16:0 sn-1 chain of the PE component in both mixed membranes, but it is clear that the change is markedly less throughout the entire chain for [$^2H_{31}$]16:0-22:6PE (Fig. 5a) than [$^2H_{31}$]16:0-18:1PE (Fig. 5b). The differential corresponds to respective increases of 26% and. 61% in average order parameter. We deduce that the DHA-containing PE separates from the lipid raft molecules SM and cholesterol much more than the oleic acid-containing PE.

Additional evidence is garnered from detergent extraction experiments. This approach identifies sphingolipid/cholesterol-enriched lipid rafts by their insolubility in cold non-ionic detergents such as Triton X-100.[30] Phase separation between detergent resistant membranes (DRM), designated raft-rich, and detergent soluble membranes (DSM), designated non-raft, in 16:0-22:6PE/SM/cholesterol (1:1:1 mol) and 16:0-18:1PE/SM/cholesterol (1:1:1 mol) membranes at 4 °C is assessed in Figure 6. Very little SM or cholesterol (<10%) is found in the DSM fraction for both mixtures, consistent with raft molecules being detergent resistant. A significant difference, on the contrary, exists in the amount of 16:0-22:6PE (70%) vs. 16:0-18:1PE (22%) in the DSM fraction. Greater phase separation from rafts of the DHA-containing phospholipid is again the implication.

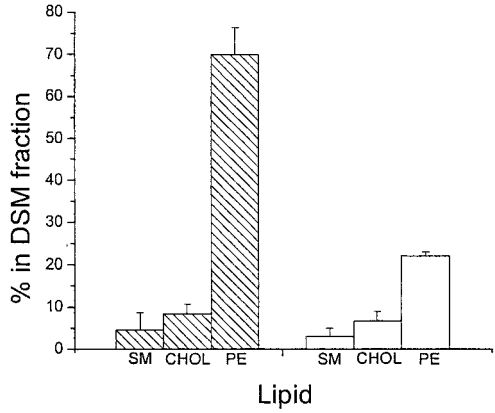


Figure 6. Percentage (mean + standard deviation from three separate experiments) of SM, cholesterol (CHOL) and PE in the DSM fraction of Triton X-100 treated aqueous multilamellar dispersions of 16:0-22:6PE/SM/cholesterol (1:1:1 mol) (shaded) and 16:0-18:1PE/SM/cholesterol (1:1:1 mol) (no pattern) at 4 °C.

We postulate that changes in the lateral architecture of plasma membranes that are driven by the steric incompatibility of PUFA for cholesterol constitute a possible mechanism by

which DHA from the diet is able to alleviate a multitude of diseases. A cartoon depicting enhanced segregation of cholesterol into a SM-rich/sterol-rich raft and formation of a DHA-rich/sterol-poor microdomain following the introduction of a DHA-containing phospholipid is drawn in Figure 7. The alteration in local environment has the potential, we propose, to cause signaling proteins to move from a raft to a non-raft region (or vice versa) and consequently undergo conformational modification that modulates cellular signaling events. DHA is known to displace phospholipase D1, for instance, from rafts.[31] The protein is activated in non-raft domains, which disrupts signal transduction and may be the origin of immuno-suppressive effects attributed to DHA.

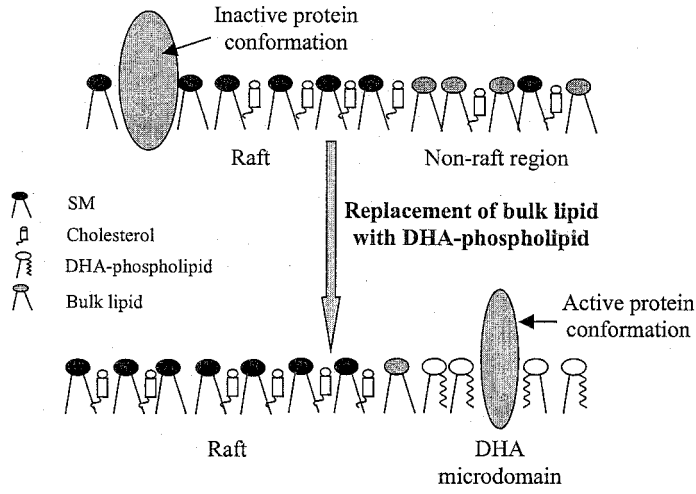


Figure 7. Cartoon rendition of how the introduction of DHA into the outer leaflet of the plasma membrane is proposed to promote the exclusion of cholesterol into SM-rich/sterol-rich rafts and form DHA-rich/sterol-poor microdomains. Concomitant changes in protein location and conformation may have implications for cellular signaling.

Conclusion

The diminished solubility of cholesterol in 16:0-22:6PE (Fig. 3 and 4) and the modest impact upon the phase behavior of [$^2H_{31}$]16:0-22:6PE that equimolar cholesterol elicits (Fig. 1 and 2) unequivocally establish that a highly disordered DHA sn-2 chain deters close contact with the sterol. An appreciably greater tendency for polyunsaturated 16:0-22:6PE than monounsaturated16:0-18:1PE to phase separate from lipid raft molecules SM and cholesterol is furthermore indicated. The sterol-associated increase in order of the sn-1

[$^2H_{31}$]16:0 chain of PE in mixtures with SM is much less for [$^2H_{31}$]16:0-22:6PE compared to [$^2H_{31}$]16:0-18:1PE (Fig. 5), and detergent extraction of PE/SM/cholesterol mixtures at 4 °C finds substantially more of the DHA than the oleic acid-containing PE in the non-raft DSM fraction (Fig. 6). We speculate that these findings may in part explain the diverse health benefits associated with dietary DHA and present a model whereby cellular signaling events may be mediated by DHA-induced relocation of signaling proteins between raft and non-raft regions (Fig. 7).

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