Reprod. Nutr. Dev. 45 (2005) 559–579

DOI: 10.1051/rnd:2005046

Review

Docosahexaenoic acid affects cell signaling by altering lipid rafts

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(Received 3 January 2005; accepted 2 February 2005)

Abstract – With 22 carbons and 6 double bonds docosahexaenoic acid (DHA) is the longest and most unsaturated fatty acid commonly found in membranes. It represents the extreme example of a class of important human health promoting agents known as omega-3 fatty acids. DHA is particularly abundant in retinal and brain tissue, often comprising about 50% of the membrane's total acyl chains. Inadequate amounts of DHA have been linked to a wide variety of abnormalities ranging from visual acuity and learning irregularities to depression and suicide. The molecular mode of action of DHA, while not yet understood, has been the focus of our research. Here we briefly summarize how DHA affects membrane physical properties with an emphasis on membrane signaling domains known as rafts. We report the uptake of DHA into brain phosphatidylethanolamines and the subsequent exclusion of cholesterol from the DHA-rich membranes. We also demonstrate that DHA-induced apoptosis in MDA-MB-231 breast cancer cells is associated with externalization of phosphatidylserine and membrane disruption ("blebbing"). We conclude with a proposal of how DHA incorporation into membranes may control cell biochemistry and physiology.

apoptosis / docosahexaenoic acid / lipid rafts / membranes / phospholipids

Abbreviations: DHA: docosahexaenoic acid; DPH: 1,6-diphenylhexatriene; DRM: detergent resistant membranes; DSM: detergent soluble membrane; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PUFA: polyunsaturated fatty acid; SM: sphingomyelin.

1. INTRODUCTION

A vast number of health benefits are reputed for docosahexaenoic acid (DHA). If even a small fraction turn out to be true, this simple fatty acid is a most remarkable, perhaps even magical compound. For years we have been investigating possible modes of action for DHA with a goal of discerning what makes it so "special" [1]. A cursory glance of DHA's structure (Fig. 1) would indicate that with 22 carbons and 6 double

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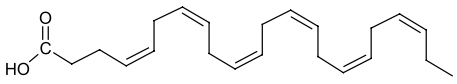

Figure 1. Structure of docosahexaenoic acid (DHA, 22:6$^{\Delta 4, 7, 10, 13, 16, 19}$).

bonds this molecule is the longest and most unsaturated, and therefore perhaps the most influential, of the omega-3 group of polyunsaturated fatty acids (PUFA) that are classified according to the location of the final double bond relative to the terminal methyl. Here we discuss some of the properties of DHA in the context of its involvement in membranes.

2. DHA: HEALTH EFFECTS

A search on DHA and literally any human health problem will generate a plethora of hits. Primarily via the diet, DHA has been reported to have positive effects on an enormous variety of human afflictions including cancer [2, 3], heart disease [4], rheumatoid arthritis [5], asthma [6], lupus [7], alcoholism [8], visual acuity [9], kidney disease [10], respiratory disease [11], peroxisomal disorders (Zellweger's Syndrome) [12], dermatitis [13], psoriasis [14], cystic fibrosis [15], Crohn's Disease [16], schizophrenia [17], depression [18], aggression [19l and brain development [20], malaria [21], multiple sclerosis [22], migraine headaches [23] and even suicide [18]. In fact, it is hard to find any human disorder that has not been tested with DHA. Of particular interest here are the large number of neurological afflictions improved by DHA. The multitude of potential health benefits generates the fundamental question of how one seemingly simple compound can affect so many different processes, and more importantly how this dietary compound can be employed effectively in improving human health.

3. DHA: POSSIBLE MODES OF ACTION

Since DHA appears to affect so many different biological systems, it can be assumed that the molecule must be acting at a fundamental level, common to most, perhaps all, cells. Although details of the mode of action remain elusive, five basic themes have emerged. DHA has been shown to affect: hormone (eicosanoid) production [24], formation of potent lipid peroxidation products [25], the conformation and hence activity of specific enzymes [26], transcription events [27], and membrane structure and function [1, 28]. These general mechanisms are not mutually exclusive. We have concentrated our efforts on how DHA affects membrane structure and function.

4. DHA: LOCATION IN MEMBRANES

Numerous dietary and cell culture studies have demonstrated that DHA can be rapidly taken up into cells and incorporated into membrane phospholipids. In mammals, there appear to be two distinctly different paradigms. DHA comprises a major portion of membrane acyl chains (occasionally approaching 50% [29]) in synaptosomes [30], sperm [31, 32] and the retinal rod outer segment [33, 34]. Membrane phospholipids have even been identified with DHA in both acyl chains in these specialized cells [35, 36]. Moreover, the already high levels in these membranes are not further augmented by diet and once incorporated, DHA is tenaciously retained at the expense of other fatty acids [29]. One unusual feature of these three high DHA-content membranes is their facility to fuse or exfoliate membrane vesicles ("blebs"). In sharp contrast to the few tissues containing high levels are the other tissues where DHA is often found below 5 mol% of the total phospholipid acyl chains. DHA levels in these tissues can be enriched several fold through the diet [29, 37, 38] and the fatty acid is primarily

found in the *sn*-2 chain with the *sn*-1 chain mainly composed of the saturated fatty acids palmitic and stearic acid [39, 40].

Incorporation of DHA into phospholipids also varies with the tissue type. In most reported studies DHA has been shown to be primarily esterified to PE (phosphatidylethanolamine) with lesser amounts to PC (phosphatidylcholine) and other phospholipid classes [38, 41]. For example with T27A leukaemia cells we found DHA initially preferred PE over PC by about 5.7 times [3]. DHA-accumulation into PE, however, is not a fast rule as Salem et al. [29] have reported that in synaptosomal membranes DHA is often found associated with PS (phosphatidylserine). The importance of this observation will be discussed later in the context of DHA's reputed role in inhibiting apoptosis in neuronal tissue. Furthermore, DHA-containing PE has been shown to preferentially accumulate in the inner, (PE- and PS-rich) leaflet of several membranes [41–43].

In a recent diet study on Wistar Rats (Harlan, Indianapolis, IN), we measured the accumulation of DHA into phospholipids isolated from different organs. The rats were fed either a corn oil (omega-6/omega-3, 18:1), a balanced corn oil/fish oil (omega-6/omega-3, 1:1) or fish oil (omega-6/omega-3, 1:3) supplemented diet (Research Diets, Inc., New Brunswick, NJ) for 3 weeks prior to harvesting the organs. Each diet contained similar quantities of protein (59% of calories), carbohydrates (20% of calories), lipids (21% of calories), vitamins and minerals. They only differed in the type of supplemented lipid. The acyl chain composition of PS, PC and PE for brain are reported in Table I. Table II presents results comparing the fatty acid content of heart and brain for rats maintained on the fish oil (omega-3-rich) diet. As expected, the percent DHA in all phospholipid classes tested increased with the level of DHA in the diet (Tab. I). In brain the percent DHA increased in the order PC << PS < PE while in the heart the order was altered with PS << PC < PE (Tab. II). In both tissues PE was the major receptacle for DHA and, in agreement with other reports [29], DHA content in brain PS was enriched relative to heart.

Table I. Composition of major fatty acids from phospholipids extracted from the brains of rats fed one of three different diets: corn oil (omega-6/omega-3, 18:1), a balanced corn oil/fish oil (omega-6/omega-3, 1:1) or fish oil (omega-6/omega-3, 1:3) supplemented diet.

PHOSPHATIDYLSERINE (PS)			
	Corn oil	Corn oil/ fish oil	Fish oil
16:0 + 18:0[1]	51:0 ± 4.6[2]	48.0 ± 3.0	52.5 ± 2.6
18:1n-9	22.0 ± 0.7	24 ± 1.0	17.4 ± 0.9
18:2n-6	3.5 ± 0	2.0 ± 0.1	1.4 ± 0.0
20:4n-6	4.5 ± 0.2	3.5 ± 0.5	2.8 ± 0.0
22:6n-3	12.0 ± 0.3	16.5 ± 0.8	18.3 ± 1.0
PHOSPHATIDYLCHOLINE (PC)			
	Corn oil	Corn oil/ fish oil	Fish oil
16:0 + 18:0	48.5 ± 1.5	50.0 ± 2.0	53.5 ± 3.0
18:1n-9	24.5 ± 1.5	22.2 ± 1.2	21.0 ± 1.7
18:2n-6	3.3 ± 0.1	2.2 ± 0.0	1.0 ± 0.0
20:4n-6	4.0 ± 0.2	4.4 ± 0.1	4.3 ± 0.3
22:6n-3	3.5 ± 0.0	4.5 ± 0.2	4.7 ± 0.1
PHOSPHATIDYLETHANOLAMINE (PE)			
	Corn oil	Corn oil/ fish oil	Fish oil
16:0 + 18:0	24.0 ± 2.5	23.0 ± 1.8	26.0 ± 2.0
18:1n-9	23.0 ± 1.0	20.0 ± 2.0	15.0 ± 0.7
18:2n-6	3.5 ± 0.5	2.0 ± 0.0	1.5 ± 0.3
20:4n-6	16.0 ± 0.8	12.4 ± 1.0	14.0 ± 1.5
22:6n-3	22.5 ± 1.6	24.7 ± 1.8	30.0 ± 1.2

[1] The number before and after the colon represent, respectively, the number of carbons and double bonds in the fatty acid chain.
[2] Expressed as a percentage of total fatty acid esterified to phospholipids species.

Table II. Docosahexaenoic acid (DHA) content in phospholipids extracted from the brains and hearts of rats fed a fish oil (omega-6/omega-3, 1:3) supplemented diet.

	PS	PC	PE
Brain	18.3 ± 1.9[1]	4.7 ± 0.1	30.0 ± 1.2
Heart	2.4 ± 0.0	16.6 ± 1.2	37.0 ± 0.2

[1] Expressed as a percentage of total fatty acid esterified to phospholipids species.

5. DHA: PHYSICAL PROPERTIES, AFFECT ON MEMBRANES

5.1. Molecular shape

As a first guess one would probably assume that membranes enriched in DHA with 22 carbons would be very thick. On the contrary, the rod outer segment membrane (where DHA levels approach 50% of the total acyl chains) is actually quite thin [44]. In agreement, on the basis of ^{2}H NMR order parameters measured for the perdeuterated stearoyl *sn*-1 chain in PCs it was inferred that the thickness of the bilayer is about the same with DHA as stearic acid (18 carbons) at the *sn*-2 position [45]. A wedge shape becoming slightly fatter at the center of the hetroacid saturated-polyunsaturated bilayer is indicated for the saturated chain while, conversely, the DHA chain occupies an inverted wedge shape with higher volume density near the aqueous interface [46]. The origin is the tremendously flexible structure of DHA undergoing rapid inter-conversions between many torsional states such that the terminal methyl end often approaches the membrane surface [47–49]. As a result, the PUFA-containing phospholipid has an increased cross-sectional area and hence exhibits a decreased membrane thickness.

5.2. "Fluidity"

A commonly studied membrane parameter is "fluidity". Unfortunately this somewhat conceptual term means different things to different investigators and is often defined by the physical method employed. Also studies on relatively simple, protein-free lipid bilayer model systems are frequently compared to those from far more complex, heterogeneous biological membranes. As a result contradictory reports of DHA's effect on membrane "fluidity" abound. The most commonly employed method is steady state fluorescence of the probe DPH (1,6-diphenyl hexatriene) that yield polarization values depending upon both the rate of reorientation and degree order of the probe that are combined in the term "fluidity".

High "fluidity" (disorder and rates of molecular reorientation) characterize DHA-containing membranes by fluorescence polarization of DPH [50–53]. In an illustrative example, Salem and Niebylski [52] made small unilamellar vesicles (SUV) from PCs containing stearic acid (18:0[1]) in the *sn*-1 position and long chain fatty acids with 0 to 6 double bonds in the *sn*-2 position. Steady state fluorescence polarization and time resolved correlation times of DPH were measured. The steady state measurements showed a large increase in "fluidity" upon the addition of the first double bond, a smaller further increase with the second and a still smaller increase in fluidity with up to 4 double bonds. No further increase was measured for DHA with 6 double bonds. However, with time resolved anisotropy they reported a progressive graded decrease in correlation time (increase in "fluidity") for each additional double bond through DHA. The interpretation offered by Salem and Niebylski was that there is an increase in acyl chain free volume with double bonds maximizing at DHA. A similar conclusion was arrived at by Stubbs [51] and by Mitchell and Litman [53].

While only minor contradictions exist concerning the effect of DHA on "fluidity" in model membranes, DHA's effect on the

[1] The number before and after the colon refers to the number of carbons and double bonds, respectively, in the fatty acyl chain.

"fluidity" of biological membranes is much less certain. Many dietary studies have reported increases in membrane "fluidity" from animals fed DHA-rich fish oil diets [54, 55], as well as for cells cultured in DHA-rich media [56–59]. In contrast, some studies did not reveal any significant change in "fluidity" upon incorporation of DHA, despite using the same techniques (primarily steady state polarization of membrane fluorescent probes) that indicated DHA induces an increase in "fluidity" in the other systems [60–63]. For example, Treen et al. [64] were unable to see a difference in "fluidity" (fluorescence polarization of DPH and TMA-DPH) with intact Y-79 retinoblastoma cells cultured in DHA-enriched media despite observing a 4 to 5 fold increase in cellular DHA levels. Employing a different fluorescence method, several reports have indicated DHA does increase "fluidity" as assayed by probes sensing lateral mobility [60, 64, 65]. These observations are consistent with the hypothesis that DHA may play a major role in membrane lateral domain structure (discussed below). The conclusion from these many experiments is that whatever DHA's influence on membrane "fluidity" is, the changes in acyl chain mobility on biological membranes already rich in unsaturated fatty acids are subtle and are less likely to be detectable by measurements with an extrinsic probe of bulk membrane "fluidity" (i.e. steady state fluorescence polarization).

5.3. Lipid packing and elasticity

Pressure-area curves for PC monolayer films at the air-water interface demonstrate that, as anticipated, the cross-sectional area generally increases with increasing double bonds and is particularly large in the presence of DHA [66, 67]. The area/molecule for a series of 18:0-XPCs where X represents acyl chains with 0 to 6 double bonds was shown to increase dramatically upon the addition of a first, second and third double bond, but does not change significantly with subsequent double bonds [68]. Interestingly this pattern of a large alteration caused by the addition of a first few double bonds but little further change with subsequent double bonds is qualitatively analogous to that reported for "fluidity" as detected by steady state fluorescence depolarization and membrane order as probed by ^{2}H NMR of the perdeuterated [$^2H_{35}$]18:0 *sn*-1 chain [45], and for main melting transition temperature (T_m) as measured by DSC (differential scanning calorimetry) [57, 66] and moment analysis of solid state ^{2}H NMR spectra [45].

Another property derived from pressure-area isotherms on lipid monolayers is the lateral compressibility modulus C_S^{-1}. The measurements indicate that monolayers become more compressible (lower modulus) with increasing number of double bonds [68]. The effect is maximal with DHA. A lower energy requirement to deform a DHA-containing membrane is the implication. Koenig et al. [69] arrived at a similar conclusion using a combined ^{2}H NMR and X-ray diffraction approach on PC bilayers. This elegant method allowed the investigators to simultaneously estimate the compressibility of both the saturated *sn*-1 chain and the unsaturated *sn*-2 chain. The values measured (307 mN·m^{-1} for stearic acid and 121 mN·m^{-1} for DHA) establish that the DHA chain is much more compressible than the saturated chain. It was estimated that 75% of the compressibility of 18:0-22:6PC could be attributed to the DHA chain. On the other hand, control experiments on 18:0-18:1PC reveal similar compressibility for the saturated and monunsaturated chains. It is predicted that DHA's low compressibility modulus may facilitate structural transitions of certain membrane proteins.

The details of the mechanism by which incorporation of DHA into the various phospholipid types affects membrane protein function is not yet clear. In a review, Mitchell and Litman [26] suggested modulation of three overlapping factors: curvature stress [70, 71], membrane thickness (hydrophobic match) [72] and acyl chain

packing free volume (f_V) [73]. The packing free volume is of interest here since it is directly related to the tightness of lipid packing. The packing free volume increases with additional double bonds maximizing at DHA. Therefore DHA with its high disorder and associated large cross-sectional area would be expected to pack poorly with other membrane lipids. For more than a decade Litman and Mitchell [26] have studied the effect of DHA on the visual process, particularly the rhodopsin M_I to M_{II} conformational transition [74]. The transition was shown to be dependent on lipid type, favoring lipids with small head groups and wide acyl tails (i.e. DHA-rich PCs). In general, hexagonal phase-preferring lipids possessing high elastic curvature stress favor the M_{II} conformation [75]. In their M_I to M_{II} rhodopsin model Mitchell and Litman [26, 74, 76] found the packing free volume and M_{II} formation increased in the order 16:0-18:1PC < 16:0-22:6PC < 22:6-22:6PC. It is not yet clear the relationship between elasticity, packing free volume and protein activity for the vast majority of membrane proteins.

5.4. Permeability

It stands to reason that membranes whose lipids are poorly packed should also be highly permeable and indeed there have been numerous reports linking DHA to increases in membrane permeability. In an early report, Hendriks et al. [77] showed that vesicles made from lipid extracts of rod outer segment membranes (DHA-rich) were 10 times more permeable to $^{22}Na^+$ than were vesicles made from lipids extracted from other (DHA-poor) retinal membranes. Demel et al. [78] demonstrated that DHA incorporated into the *sn*-2 position of PC enhanced permeability to glucose, erythritol and glycerol. Measuring bilayer permeability to erythritol and carboxyfluorescein, Stillwell et al. [79] established that DHA enhanced bilayer permeability 2 to 3 fold more than oleic acid in identical phospholipids. Huster et al. [80] used ^{17}O NMR to follow water permeability across lipid bilayers. They determined that 18:0-22:6PC membranes are about 4× more permeable than those made of 18:0-18:1PC but are about 30% less permeable than those of 22:6-22:6PC. As a result of increased water penetration, DHA favors increased hydration of the head group and inter-chain region. In agreement, fluorescence measurements on lipid bilayers confirmed that water content in the bilayer hydrocarbon region increases with double bond content [53]. Increases in mitochondrial permeability have also been linked to DHA content [65, 81]. The resultant proton leakage in turn decimates the essential trans-membrane H^+ gradients, an event linked to mitochondrial-linked apoptosis [82] (see below).

5.5. Bilayer instability: vesicle fusion, exfoliation and flip-flop

The loose packing of DHA-containing phospholipids implies that high levels of this fatty acid may also lead to general membrane instability resulting in enhanced vesicle exfoliation ("blebs"), fusion and flip-flop. Perhaps not coincidentally, biological membranes that are naturally enriched in DHA (neuronal synaptosomes, rod outer segments and sperm) are partly characterized by their predisposition to undergo membrane vesicle formation and fusion. Ahkong et al. [83] and later Meers et al. [84] showed that PUFA promote the fusion of natural membranes. In a series of studies Stillwell and Jenski have fused DHA-containing phospholipid vesicles with several types of membranes and monitored the effect of fusion-augmented DHA levels on bilayer membrane [85], mitochondria [65] and T27A cell properties [3, 86–89]. DHA as either the free fatty acid or as part of a mixed chain PC (18:0-22:6PC) enhanced SUV fusion to a much larger extent than did PCs with other, less unsaturated fatty acids [85]. When T27A tumor cells were fused with vesicles containing 18:0-22:6PC, the tumor plasma membrane became leakier

[79], had altered expression of surface proteins [89], altered domain structure [90], decreased cell deformability [91], increased cytolysis by cytotoxic T-lymphocytes [86] and decreased cell longevity [3, 89]. Upon fusion with SUVs made from 18:0-XPC, where X is stearic, oleic, α-linolenic, arachidonic, eicosapentaenoic or DHA, only DHA was cytotoxic [88]. It is now well documented that membrane fusion is enhanced by the presence of lipids, like DHA, that promote inverted hexagonal phase and thus provide curvature stress to membranes [92].

Since exfoliation is the reverse of fusion, Williams et al. [93] isolated exfoliated vesicles (EV) from DHA-enriched T27A cells and measured their lipid content (phospholipid class, fatty acid, and cholesterol) and membrane molecular order. A comparison of these values between the DHA-induced exfoliated vesicles (EVs) and the parent plasma membrane led these authors to conclude that EV composition and structure was considerably different than that of the parent plasma membrane. After incubation in the presence of DHA, EVs exhibited higher levels of DHA and lower levels of cholesterol. Also fluorescence anisotropy of DPH, PA-DPH and TMA-DPH decreased, indicating a DHA-induced increase in membrane "fluidity" in the EVs. These results imply that DHA-driven microdomains are either large enough or numerous enough to influence a sizable portion of T27A membrane surface and that EVs reflect plasma membrane lipid domains.

Recently Armstrong et al. [94] employed fluorescence quenching of NBD-PE probes to monitor the effect of PC acyl chain double bond content on trans-membrane flip-flop. This study demonstrated that as the number of double bonds increases, so does the flip-flop rate of the NBD-PE probes. The increase was particularly marked in the presence of DHA. A half-life $t_{1/2}$ of 0.086 h was reported for bilayers composed of 22:6-22:6PC and 0.29 h for 18:0-22:6PC, whereas 18:0-18:1PC exhibited a much slower flip-flop of $t_{1/2}$ = 11.5 h. These authors concluded that PC membranes containing DHA support very fast flip-flop rates [94].

6. MEMBRANE STRUCTURE AND RAFTS

The structure of biological membranes remains one of the most difficult problems in the life sciences [95–97]. Since membrane structure was first described by the Fluid Mosaic Model in 1971 [98], it has become evident that membranes consist of very complex heterogeneous lipid and protein patches that are in constant flux and so far have been recalcitrant to precise analysis. For the past decade the concept of lipid rafts has been a focus of membrane research [97, 99].

Lipid rafts are liquid ordered, sphingomyelin (SM)-rich/cholesterol-rich microdomains that are postulated to serve as platforms for protein activity by accumulating specific lipidated proteins such as the src family of kinases in the inner leaflet and GPI-anchored proteins in the outer leaflet of plasma membranes [97, 99, 100]. The rafts are floating in a sea of liquid disordered, SM-poor/cholesterol-poor microdomains that must have its own array of proteins. In fact it is likely that numerous proteins may migrate between domains in response to alterations in lipid composition. Historically their resistance to dissolution in cold temperature detergents, particularly Triton X-100, has defined lipid rafts. While detergent resistant membranes (DRMs) mark the beginnings of raft studies, they are wrought with artifacts and are being replaced by gentler, detergent-free methods. Our initial guess was that DHA-phospholipids, being very "fluid" should accumulate in the detergent-soluble membrane (DSM) fractions and indeed, this is the case (Fig. 2). The question can then be posed, does DHA incorporation actually affect membrane architecture and if so how?

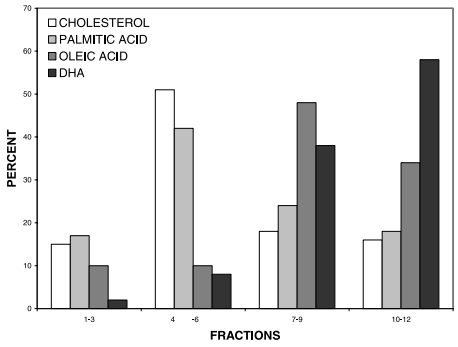

Figure 2. Fractionation of MDA-MB-231 breast cancer cells grown in culture with various radiolabeled lipids. The cells were disrupted in 1% Triton X-100 at 4 °C and incubated for 30 min after which the fractions were separated on a sucrose gradient by standard procedures [171]. Radioactive counts were determined for each of the 12 fractions. For ease of presentation fractions 1–5, 4–6, 7–9 and 10–12 were combined. The detergent-resistant rafts are in fractions 4–6. Cholesterol and palmitic acid are primarily found in the raft fractions while DHA is primarily found in the non-raft fractions 7–9 and 10–12.

7. DHA: INTERACTION WITH RAFT LIPIDS (CHOLESTEROL AND SPHINGOMYELIN)

One molecule seems to be at the heart of understanding the structure and function (and even existence!) of rafts – cholesterol. For many years it has been known that the major lipid comprising the plasma membrane of most animal cells is cholesterol and the primary role of the sterol is to modulate the physical properties of membranes [101]. The situation is well-documented in membranes composed of saturated acyl chains [101], but is far less understood in DHA-containing membranes. Upon its incorporation into cells, DHA is incorporated into membranes where it must be exposed to cholesterol and the interaction between these two species may profoundly affect membrane structure and function. This has been the premise of our research.

Early experiments showed that DHA-containing PCs were far less affected by cholesterol than were many other PCs [101, 102]. They showed that cholesterol demonstrated little monolayer "condensation" and had little effect on bilayer permeability of DHA-containing PCs compared to less unsaturated PC [78, 103, 104]. Using pressure-area techniques on monolayers, Smaby et al. [68] measured elasticity compression moduli for various PCs. The in-plane elasticity moduli of the PC species with higher *sn*-2 double bond content were found to be *less* affected by increased levels of cholesterol. Their work predicts that cholesterol-unsaturated fatty acid interaction results in localized regions in membranes that differ considerably in their in-plane elasticity. Partition coefficients (K_A^B) determined for cholesterol in unilamellar vesicles by a cyclodextrin assay follow the order 18:0-18:1PC > 18:1-18:1PC > 18:2-18:2PC > 16:0-22:6PC > 22:6-22:6PC [105]. Mitchell and Litman [26, 73] have employed a model system monitoring the effect of acyl chain packing free volume (f_V) on the M_I to M_{II} equilibrium (K_{eq}) of rhodopsin to determine the effect of cholesterol on a protein's function in a DHA-rich membrane. They incorporated rhodopsin into LUV made from 22:6-22:6PC; 16:0-22:6PC or 16:0-18:1PC with or without 30% cholesterol. Without cholesterol K_{eq} and f_v increased in the order: 16:0-18:1PC < 16:0-22:6PC < 22:6-22:6PC. Cholesterol reduced these values in the order: 16:0-18:1PC > 16:0-22:6PC > 22:6-22:6PC. Their results suggest that regulation of an integral membrane receptor (rhodopsin) function may be metabolically controlled by changes in phospholipid acyl chain composition and/or cholesterol content. They propose a primary role for phospholipid and a secondary role for cholesterol [73].

DSC has been extensively employed to investigate cholesterol-phospholipid interactions. Many DSC experiments have demonstrated that cholesterol induces phase separations in PC bilayers [1, 102, 106, 107]. The addition of up to 50 mol% cholesterol

to 22:6-22:6PC has little impact upon the temperature or enthalpy of the gel-to-liquid phase transition [108], whereas with 16:0-18:1PC or 16:0-22:6PC the transition is eliminated by ≥ 35 mol% cholesterol [109]. Regardless of unsaturation, the response to incorporation of cholesterol is a disruption of acyl chain packing in the organized gel state while in the fluid liquid crystalline state acyl chain motion is restricted [110–112] (i.e. cholesterol increases the "fluidity" of solid membranes while decreasing the "fluidity" of fluid membranes).

The implication of the biophysical studies outlined above is that the solubility of cholesterol in phospholipid membranes may be dependent on double bond content. Recently we have directly tested this hypothesis using a combination of solid state ^{2}H NMR spectroscopy and XRD (X-ray diffraction) [113–116]. The NMR technique identifies a narrow spectral component due to cholesterol incorporation into the membrane superimposed upon a broad component from solid crystalline monohydrate cholesterol form outside the membrane. The XRD technique relies upon the detection of diffraction peaks from cholesterol monohydrate crystals excluded from the membrane once the solubility limit is exceeded. Both methods established that when forced to interact with PUFA chains in *sn*-1, *sn*-2 dipolyunsaturated PC membranes, cholesterol precipitates out of the membrane at concentrations of a factor of 3–5 less than in the corresponding *sn*-1, *sn*-2 saturated-polyunsaturated membrane where close proximity to the PUFA chain may be avoided. Profoundly reduced affinity of the sterol for PUFA was thus quantified. It is the extraordinarily high disorder of PUFA chains that we propose on steric grounds is incompatible with near approach from the rigid steroid moiety.

In companion ^{2}H NMR experiments we measured the most probable orientation (tilt angle) for [3a-$^2H_1$]cholesterol in bilayers composed of various PCs and PEs [113–115, 118]. We discovered that, irrespective of the degree of *sn*-2 chain unsaturation, the tilt angle ($\alpha_0 = 16°$) was the same for all the hetero-acid phospholipids studied with a saturated *sn*-1 chain. Only for dipolyunsaturated phospholipids with DHA or 20:4 acid at both *sn*-1 and -2 positions was the value substantially greater ($\alpha_0 = 24°$). Our interpretation is that cholesterol will laterally organize within membranes to minimize contact with a DHA *sn*-2 chain and preferentially associate with a saturated *sn*-1 chain. Others have made the same proposal [53, 73], and both experimental [80] and computer simulated [118] data lend further support.

The interaction of cholesterol with DHA-containing PEs has received much less attention than with the equivalent PCs. Early DSC studies found that cholesterol-lipid interaction follows the following sequence: SM >> PS, PG > PC >> PE [119]. In agreement, by a cyclodextrin assay Niu and Litman [105] determined partition coefficients in the order SM > PS > PC > PE for cholesterol in unilamellar vesicles. Although most PE localizes to the inner leaflet, nearly 17% is outer leaflet and so should directly act on rafts [120]. Also PE is the major receptacle of DHA in most membranes. In Table III we compare cholesterol solubility in *sn*-1 saturated, *sn*-2 unsaturated PE and PC bilayers with 18:1 acid or DHA at the *sn*-2 position. The

Table III. Solubility of cholesterol in PEs obtained from XRD measurements. Values for PCs are included for purposes of comparison. Samples were in the lamellar liquid crystalline state.

PE	Cholesterol solubility mol%	PC	Cholesterol solubility mol%
16:0–18:1	51 ± 3[1]	16:0–18:1	65 ± 3[3]
16:0–22:6	31 ± 3[2]	18:0–22:6	55 ± 3[4]

[1] 40 °C, value taken from Shaikh et al. [116].
[2] 7.5 °C, value taken from Shaikh et al. [116].
[3] 24 °C, value taken from Huang et al. [170].
[4] 20 °C, value taken from Brzustowicz et al. [114].

results demonstrate that there is a greater DHA-associated reduction in solubility for PE than for PC bilayers, which we attribute to its smaller headgroup [116].

8. DHA AND MEMBRANE DOMAINS

Although details have not been forthcoming, it has been known for a long time that lipid-lipid interactions must play an active role in organizing membranes into domains [1, 121]. We [104, 115–117, 122] and others [74, 111, 123] have shown that a reduced affinity between cholesterol and polyunsaturated fatty acids may drive lateral phase separation into cholesterol-rich/PUFA-poor and cholesterol-poor/PUFA-rich membrane microdomains.

There have been a few examples of DHA-induced lipid phase separations described for model bilayer membranes. Stillwell et al. [28, 90] have demonstrated that bilayers composed of 16:0-16:0PC and 18:0-22:6PC can phase separate. Niebylski and Litman [124] employed fluorescence probes to show that bilayers composed of 16:0-16:0PC and 22:6, 22:6PC phase separate. While both of these reports clearly establish the formation of DHA-enriched domains in model membranes, each suffers from the same shortcoming. They are examples of liquid crystalline/gel separations. What are needed are biologically relevant fluid/fluid (liquid ordered/liquid disordered) separations. For this reason, Stillwell et al. [1, 28] have looked for fluid/fluid phase separation in more biologically relevant bilayer model membranes composed of either PCs or PEs with palmitic or stearic acid in the *sn*-1 position and DHA in the *sn*-2 position mixed with the raft lipids SM and cholesterol. Surface elasticity measurements on monolayers, and DSC, detergent extraction and solid state ^{2}H NMR measurements on bilayers support the idea that DHA-containing phospholipids enhance the lateral segregation of cholesterol into sterol-rich/SM-rich liquid ordered regions away from sterol-poor/DHA-PE- or DHA-PC-rich liquid disordered domains [117, 122, 125]. Estimates of the size of PUFA-induced domains are small. From an analysis of ^{2}H NMR spectra, an upper limit of ~ 160 Å was placed on microdomain size in 18:0-20:4PC/20:4-20:4PC/cholesterol (1/1/2 mol) [115]. A comparable estimate (≤ 250 Å) for microdomains produced by cholesterol-triggered segregation in 18:0-22:6PC/18:0-22:6PE/18:0-22:6PS (4/4/1 mol/mol/mol) membranes was deduced on the basis of NMR data by Huster et al. [80]. These values are much less than that typically quoted for lipid rafts (≥ 50 nm). As raft studies continue to evolve, however, the estimated raft size in biological membranes continues to decrease, making their direct observation less likely [96].

Indirect observations supporting an effect of DHA on lateral organization within membranes are abundant. For example, Clamp et al. [60] reported there is greater inter-domain variation in hepatocyte plasma membranes from rats maintained on a fish oil diet. Jenski et al. [126] added DHA to cultured splenic lymphocytes or lymphoma cells and measured fatty acid uptake and membrane structure alteration (protein clustering). Protein clustering rate (determined by fluorescence resonance energy transfer between fluorophore-labeled antibodies bound to membrane proteins) was 2-fold higher in DHA-rich than DHA-poor cells. Williams et al. [93] isolated exfoliated vesicles (EV) from DHA-enriched T27A cells and measured their lipid content (phospholipid class, fatty acid, and cholesterol) and membrane "fluidity". A comparison of these values between the DHA-induced EVs and the parent plasma membrane led these authors to conclude that EV composition and structure was considerably different than that of the plasma membrane implying that DHA-driven microdomains are either large enough or numerous enough to influence a sizable portion of T27A membrane surface and that EVs reflect plasma membrane lipid domains. These are but a few examples of many that indicate DHA may affect membrane structure and hence function.

The best studied DHA-membrane system is the rhodopsin model of Litman and Mitchell [26, 73, 74, 76]. Their model is based on strong interactions between the saturated *sn*-1 chain of PCs containing DHA in the *sn*-2 position. The *sn*-1 chains are oriented towards the tightly packed domain interior with the *sn*-2 chains oriented toward the domain boundary [73]. The DHA-rich *sn*-2 chain is proposed to primarily determine the lateral packing of the system that is responsible for the M_I to M_{II} equilibrium. Cholesterol partitions into the saturated chain-rich domain interior where it reduces the acyl chain packing free volume. This model also predicts that 22:6-22:6PC will pack between the domains to maintain maximal saturated chain interactions. Therefore, there are two levels of interaction in the Litman/Mitchell model, primary regulation of rhodopsin by acyl chain composition and a secondary regulation by cholesterol content. In model membrane studies it was reported that coexisting 16:0-16:0PC and 22:6-22:6PC lateral domains were found only when both cholesterol and rhodopsin were also included and that the protein was preferentially enriched into 22:6-22:6PC domains about 6× greater than into 16:0-16:0PC domains [127]. Furthermore it was proposed by Litman et al. [128] that DHA-containing phospholipids can buffer the inhibitory effects of cholesterol in the visual receptor signalling pathway.

We have recently begun an AFM (atomic force microscopy) study of the effect of DHA on raft structure [125]. Our very initial imaging studies compared bilayers composed of either 16:0, 18:1 PE/SM/cholesterol (1:1:1) or 16:0, 22:6 PE/SM/cholesterol (1:1:1). Large differences in raft (SM/cholesterol-rich) domains were readily detected between the two systems. Raft domains comprised 26 ± 2% of the 16:0, 18:1 PE/SM/cholesterol membrane and 59 ± 10% of the 16:0, 22:6 PE/SM/cholesterol membrane indicating that DHA drives greater phase separation than does oleic acid. Also the individual domains observed in the PUFA-containing membrane were much bigger and connected compared to those observed in the oleic acid-containing membrane.

While lateral phase separation has been the focus of most lipid microdomain (raft) studies, biological membranes also exhibit substantial trans-bilayer heterogeneity. It is well documented that highly unsaturated species of PE and PS are found primarily on the inner leaflet of many membranes [29, 43, 129] including murine synaptosomal plasma membranes [130]. However DHA distribution is not absolute as rats fed a fish oil diet exhibited an increase in DHA-containing species of PC in the outer leaflet of erythrocyte membranes [131]. It was proposed that membrane enrichment of PUFA might result in relocation of acylated raft proteins between raft and non-raft domains.

Addition of PUFA to membranes has also been shown to alter trans-bilayer sterol localization [132, 133]. Although the transmembrane distribution of cholesterol is not known with absolute certainty [134, 135], the outer leaflet of plasma membranes may contain more cholesterol and therefore be more rigid than the inner leaflet. This is consistent with the current idea of cholesterol- and sphingolipid-rich lipid rafts residing in membrane outer leaflets [100]. Also, it is known that cholesterol has a higher affinity for sphingolipids than for other common membrane lipids [120, 136, 137] and sphingolipids clearly are primarily located in the outer leaflet [100]. However, very rapid cholesterol flip-flop [138, 139] makes precise cholesterol asymmetry measurements difficult. Upon addition of PUFA in culture, a decrease in molecular order and a redistribution of cholesterol with more than 70% going to the outer leaflet was reported by Schroeder and co-workers [132, 133]. Dusserre et al. [140] reported cholesterol efflux from plasma membranes remained the same after incorporation of oleate, linoleate or arachidonate but increased with EPA and DHA. They suggested that incorporation of DHA into the membrane inner leaflet forces

cholesterol into outer leaflet where it is more readily lost from the membrane.

9. DHA: AFFECT ON CELL SIGNALING AND APOPTOSIS

At present there is a large and ever expanding list of proteins whose activities have been shown to be affected by DHA. The most thoroughly studied of the proteins by far has been rhodopsin (see discussion above) [26, 73, 74). The important signaling protein, PKC (protein kinase C) has also been shown to be activated by fatty acids like DHA that exhibit large negative curvature stress [141–143]. In one example, Giorgione et al. [144] found that DHA acyl chains produced the highest level of PKC activity when incorporated into PE but not into PC and the activity was correlated with increased partitioning of PKC into the membrane. Their results indicate that the formation of membrane domains can be important for the activation of PKC and, furthermore, the activation can be inhibited by disrupting the domains.

Since nervous tissue is known to be highly enriched in DHA [29], it was logical to investigate the effect of this fatty acid on channel activity. Using patch clamp techniques, Poling et al. [145, 146] reported that non-esterified DHA interacts with an external channel domain and regulates the activity of certain voltage-gated K^+ channels in a Zn^{2+}-dependent fashion. PUFA have additionally been shown to modulate L-type Ca^{2+} channels [147, 148], suggesting a role in cell signaling. Hasler et al. [149] reported that 50 μM DHA inhibited gap junction intercellular communication by 18% while several other fatty acids had no measurable effect. Finally, Jenski et al. have studied the expression of MHC I molecules as modified by DHA. They demonstrated altered expression of MHC I, CD8, and CD90 (Thy-1) on murine lymphocytes and leukemia cells enriched in DHA through diet or cell culture [89, 150]. These are but a few of a wide variety of reports linking DHA to cell signaling events through membrane alteration.

The effect of DHA on apoptosis or programmed cell death has received considerable scrutiny. It is well established that many events associated with apoptosis are linked to membrane structure and function [151]. For example, externalization of PS to the outer leaflet of the plasma membrane [152, 153] and formation of "blebs" [154] are considered hallmarks of apoptosis. However, there remains a distinct dichotomy of opinions concerning whether DHA *enhances* or *inhibits* apoptosis.

The preponderence of reports coming from a vide variety of primarily cancer cell types [155–159] indicate that DHA induces apoptosis and it has been suggested that DHA's anticancer properties are not due directly to cytotoxicity, but rather to the fatty acid's ability to induce apoptosis [160–162]. Siddiqui et al. [163] have recently reviewed the role of DHA in enhancing both the cytosol-linked and mitochondrial-linked apoptotic pathways. We [164] have compared the effect of DHA on breast cancer cell proliferation in vivo (in nude mice, nu/nu) and in vitro (cultured MDA-MB-231 cells). We reported that DHA caused a profound inhibition of cancer cell growth and increased apoptosis. Associated with this was an increase in N-SMYase activity (by ~ 40%) in the breast cancer tissues from mice raised on the omega-3 containing diets and by 70–75% ($P < 0.05$) in the MDA cells that had been treated with DHA. The DHA-induced increase in N-SMYase activity was further analyzed by formation of ceramide, the product of sphingomyelin hydrolysis. Ceramide is a lipid that is regarded as a universal component of apoptosis [165, 166]. We have also reported extensive DHA-induced "blebbing" of the tumor cell membranes (Fig. 3a) and appearance of PS on the outer membrane surface (Fig. 3b), both strong indicators of apoptosis. In addition, DHA-induced apoptosis and "bleb" formation was inhibited (~ 40%) by the N-SMYase inhibitor GW4869. The conclusion of these experiments was that inhibition of breast

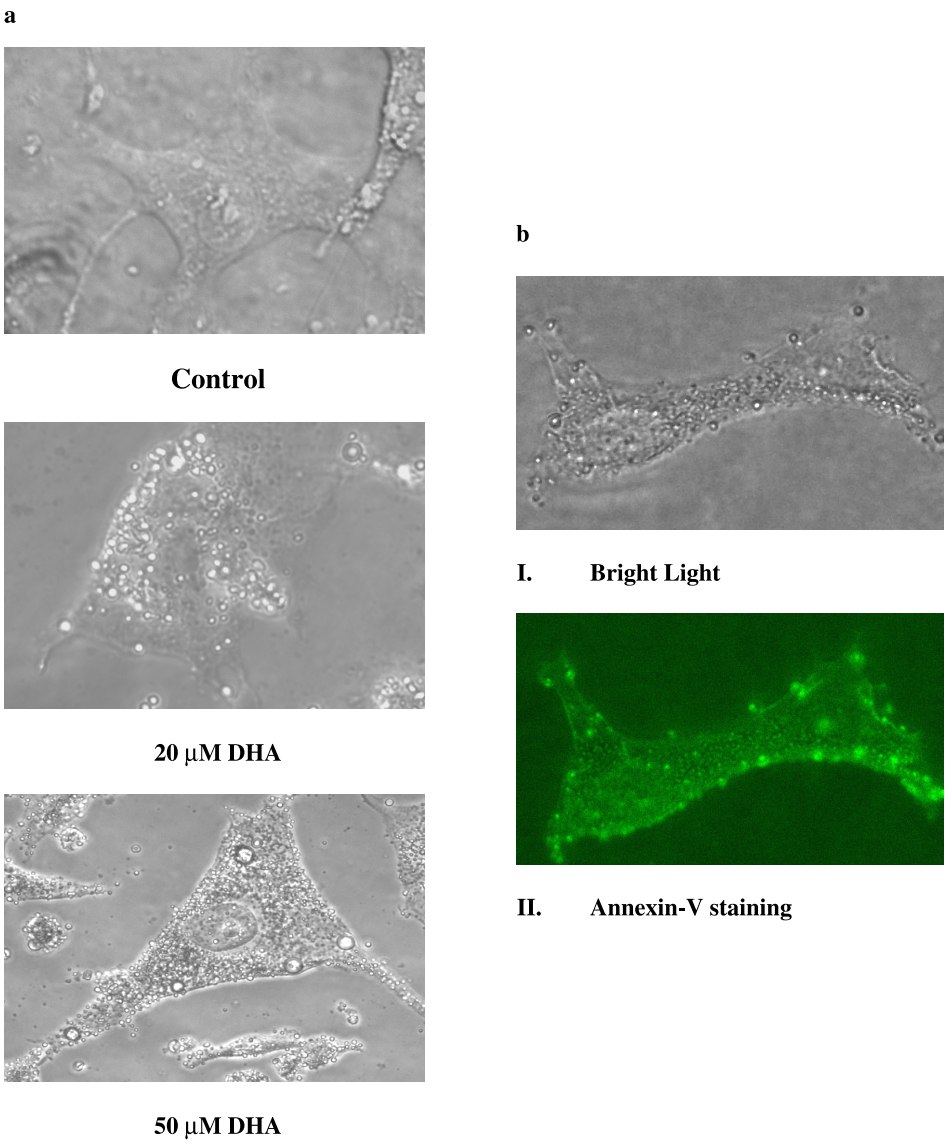

Figure 3. MDA-MB-231 cells were cultured in the presence of various concentrations of DHA (**a**). DHA-dependent "blebs" were observed under a microscope using a 40X objective. The "blebs" from cells grown in 50 μM DHA were analyzed for externalized PS using a fluorescent annexin binding kit (**b**).

cancer growth in nude mice by fish oil or in culture by treatment with DHA appear to be mediated by generation of ceramide through enhanced N-SMYase activity.

In sharp contrast to the many reports demonstrating that DHA induces apoptosis are fewer reports, usually on neuronal cells, indicating that DHA actually *inhibits* apoptosis

[167–169]. Kim and coworkers have reported that DHA inhibits serum deprived- [168] and stautosporine-induced [167] apoptosis in mouse neuroblastoma Neuro 2A cells using the very same assays (cleavage of procaspase-3, DNA laddering etc.) others have used to demonstrate that DHA induces apoptosis. Clearly there is a discrepancy between the affect of DHA on neuronal vs. other types of cells. Kim [168] explains this in terms of DHA increasing the PS content in membranes of neuronal cells. However increased PS levels would enhance the chance of PS appearing in the membrane outer leaflet, a well-established trigger for apoptosis [152, 153]. Also it has been demonstrated that increasing the DHA content of membranes substantially increases phospholipids flip-flop [94], again increasing the likelihood of moving PS to the outer leaflet. The important relationship between DHA and apoptosis will require far more study.

10. CONCLUSIONS

There is abundant evidence that DHA can be rapidly accumulated into membrane phospholipids and there, potentially affect a variety of membrane physical properties including: membrane thickness, lipid packing, "fluidity", elasticity, permeability, flip-flop, protein activity, fusion, "blebbing" and the structure and function of lateral and trans-membrane heterogeneities (domains) [1]. The implication of many diverse observations, moreover, is that membrane perturbations are either the cause or the result of apoptosis. Thus, it is interesting to note many of the membrane properties that change during apoptosis are similar to those that change upon accumulation of DHA into membranes. Our focus has been on the affect of DHA on one important and very controversial type of membrane domain, the cell signaling lipid raft.

We hypothesize that DHA is incorporated mainly into PEs (primarily in the membrane inner leaflet) and secondarily into PCs (primarily in the membrane outer leaflet). It is DHA's aversion to cholesterol that will segregate the DHA-containing phospholipids away from cholesterol, resulting in enhanced phase separation into DHA-rich/cholesterol and sphingolipid-poor liquid disordered (non-raft) domains and cholesterol and sphingolipid-rich/DHA-poor liquid ordered (raft) domains. The large difference in physical properties between the two domains will alter the membrane location of important signaling proteins, thus modulating cellular functions. Also accumulation of DHA into inner leaflet PEs will enhance cholesterol's flip-flop to the outer leaflet where its increased concentration would further add to the size and stability of rafts. In support of an enhancement of cholesterol flip-flop, Dusserre et al. [140] reported that cholesterol efflux from plasma membranes increased with DHA.

We conclude that although the mechanism of action of DHA is likely multifaceted, one major function is its affect on membrane structure and function.

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