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Eicosapentaenoic acid reduces membrane fluidity, inhibits cholesterol domain formation, and normalizes bilayer width in atherosclerotic-like model membranes

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ABSTRACT

Cholesterol crystalline domains characterize atherosclerotic membranes, altering vascular signaling and function. Omega-3 fatty acids reduce membrane lipid peroxidation and subsequent cholesterol domain formation. We evaluated non-peroxidation-mediated effects of eicosapentaenoic acid (EPA), other TG-lowering agents, docosahexaenoic acid (DHA), and other long-chain fatty acids on membrane fluidity, bilayer width, and cholesterol domain formation in model membranes. In membranes prepared at 1.5:1 cholesterol-to-phospholipid (C/P) mole ratio (creating pre-existing domains), EPA, glycyrrhizin, arachidonic acid, and alpha linolenic acid promoted the greatest reductions in cholesterol domains (by 65.5%, 54.9%, 46.8%, and 45.2%, respectively) compared to controls; other treatments had modest effects. EPA effects on cholesterol domain formation were dose-dependent. In membranes with 1:1 C/P (predisposing domain formation), DHA, but not EPA, dose-dependently increased membrane fluidity. DHA also induced cholesterol domain formation without affecting temperature-induced changes in–bilayer unit cell periodicity relative to controls (*d*-space; 57 Å–55 Å over 15–30 °C). Together, these data suggest simultaneous formation of distinct cholesterol-rich ordered domains and cholesterol-poor disordered domains in the presence of DHA. By contrast, EPA had no effect on cholesterol domain formation and produced larger *d*-space values relative to controls (60 Å–57 Å; $p < 0.05$) over the same temperature range, suggesting a more uniform maintenance of lipid dynamics despite the presence of cholesterol. These data indicate that EPA and DHA had different effects on membrane bilayer width, membrane fluidity, and cholesterol crystalline domain formation; suggesting omega-3 fatty acids with differing chain length or unsaturation may differentially influence membrane lipid dynamics and structural organization as a result of distinct phospholipid/sterol interactions.

1. Introduction

Atherosclerosis is the product of endothelial dysfunction, inflammation, and excessive lipid accumulation in the arterial wall [1]. The intracellular accumulation of cholesterol promotes increased deposition of free cholesterol in the plasma membrane, resulting in the formation of membrane-restricted cholesterol crystalline domains [2,3]. Such changes in membrane lipid structural organization are associated with an increase in membrane permeability, generation of reactive oxygen species (ROS), and lipid peroxidation, all of which further degrade membrane structure and interfere with its proper function [4,5]. The continued concentration of cholesterol in the plasma membrane eventually promotes the formation of extracellular cholesterol crystals—jagged, microscopic shards that can expand rapidly and puncture the protective fibrous cap of an atherosclerotic lesion [2,6,7]. These crystals are also believed to trigger inflammatory pathways that result in both necrotic and apoptotic forms of cell death [8].

Unesterified or free cholesterol is a key component of caveolae that serve as important signaling domains on the surface of normal cellular membranes. Caveolin, the principal structural protein associated with

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; ApoB, apolipoprotein B; C/P, cholesterol-to-phospholipid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; HTG, hypertriglyceridemia; HTG, hypertriglyceridemia; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LUV, large unilamellar vesicles; MLV, multilamellar vesicle; *n.s.*, not statistically significant; O3FA, omega-3 fatty acid; oxLDL-C, oxidized low-density lipoprotein cholesterol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; ROS, reactive oxygen species; sdLDL, small dense low-density lipoprotein; TG, triglyceride; T/P, treatment-to-phospholipid; VLDL, very low-density lipoprotein.

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caveolae, efficiently binds cholesterol and modulates the activity of various receptor tyrosine kinases, G protein-coupled receptors, glycophosphatidylinositol (GPI)-linked proteins, ion channels, and endothelial nitric oxide synthase (eNOS) [5,9]. Through these proteins, caveolae mediate endothelial function, smooth muscle cell migration, cytokine expression, energy metabolism, and innate immunity, including the recruitment and activation of inflammatory cells. Cholesterol is a critical structural component of the caveolar membrane and must be maintained within narrow homeostatic limits in order for caveolae to perform these critical activities. Excessive accumulation of cholesterol and the subsequent formation of cholesterol domains may lead to endothelial dysfunction, localized inflammation, and potentiation of the atherogenic process [5]. Even minor structural disruptions to cellular membranes and caveolae, through deposition of excess cholesterol or oxidative modification, have been shown to adversely affect normal cell function [9,10].

Treatment of hypercholesterolemia with statins results in significant reductions in circulating cholesterol and cardiovascular risk; however, individuals with well-controlled low-density lipoprotein cholesterol (LDL-C) levels, but persistent hypertriglyceridemia (HTG) and elevated levels of other circulating lipoproteins, remain at increased risk for cardiovascular disease [11–16]. Medical management of HTG with marine-derived, long-chain, polyunsaturated omega-3 fatty acids (O3FAs) has been shown to significantly reduce plasma triglycerides (TG) but O3FA treatment, as shown in a number of well-controlled clinical trials, has not resulted in consistent reduction of cardiovascular risk [17–30]. One potential confounder in assessing the clinical utility of O3FAs in reducing cardiovascular risk is the heterogeneity of O3FA formulations and doses used in these various studies. Some O3FA formulations contained both eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3), and at different ratios, while others contained only EPA. It is generally assumed that EPA and DHA behave in similar ways; however, increasing evidence suggests otherwise. Indeed, recent data indicate that EPA and DHA segregate into separate domains in cell membranes, which may differentially affect membrane structure and function [31–33].

O3FAs play important roles in a variety of cellular processes and have been shown to reduce circulating triglycerides, cholesterol-containing remnant lipoproteins, oxidized LDL-C (oxLDL-C), monocyte and macrophage adhesion, and foam cell formation. Data also suggest that EPA has other direct benefits, such as improving endothelial function, stabilizing plaque to prevent rupture and thrombus formation, reducing plaque volume, and reducing inflammatory markers [4,34–42]. These benefits may be due in part to the unique physicochemical properties of EPA, which allow it to interact directly with plaque and cellular components. O3FAs are highly lipophilic and small enough to intercalate directly into lipoprotein particles and lipid bilayers where they play important roles in the maintenance of endothelial function, inflammation, activation of inflammatory cells, and platelet activation [43–46].

We recently showed that EPA, but not vitamin E, fenofibrate, niacin, or gemfibrozil, inhibits the oxidation of apolipoprotein B (ApoB)-containing lipid particles, including LDL, small dense low-density lipoprotein (sdLDL), and very low-density lipoprotein (VLDL), obtained from healthy subjects [47,48]. In addition, treatment with EPA resulted in a dose-dependent reduction in lipid peroxidation and cholesterol domain formation in model membranes exposed to glucose-induced oxidative stress. DHA also inhibited lipoprotein oxidation, but its effects were limited to a shorter time period. These data suggest that differences in hydrocarbon chain length or the number of double bonds may differentially effect how EPA and DHA interact with the lipoprotein, including their precise orientation and location in the particle lipid layer, leading to differences in their ability to inhibit lipid oxidation [48]. In this study, we expand our previous work by evaluating the direct, non-peroxidation-mediated effects of EPA, other TG-lowering agents, and DHA on membrane fluidity and membrane structural properties, including cholesterol crystalline domain formation and changes in membrane width in cholesterol-enriched model membranes. These data will provide additional insights as to the direct effects of EPA and DHA on membrane structure and function.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and monomeric cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and solubilized in HPLC-grade chloroform at 25 and 10 mg/mL, respectively. EPA, DHA, docosapentaenoic acid (DPA; 22:5, n-3), α-linolenic acid (ALA; 18:3, n-3), arachidonic acid (AA; 20:4, n-6), and glycyrrhizin were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and solubilized in ethanol to 1.0 mM under nitrogen atmosphere. Glycyrrhizin is a glycosylated sterol that has been shown to reduce the domain formation in model membranes [49] and was included in this study as a positive control. Fenofibrate, gemfibrozil, and nicotinic acid (niacin) were purchased from Toronto Research Chemicals (North York, Ontario, Canada) and solubilized in ethanol at 1.0 mM. DPH was obtained from Molecular Probes/Invitrogen (Eugene, OR). All test compounds were further diluted in ethanol or aqueous buffer as needed.

2.2. Preparation of multilamellar lipid vesicles

Multilamellar vesicles (MLVs) were prepared as binary mixtures of POPC (1.0 mg total phospholipid per sample) and cholesterol at C/P mole ratios of 1.1:1 and 1.5:1. Cholesterol has been observed, in a number of membrane lipid systems, to undergo lateral phase separation at C/P mole ratios greater than 1:1 [3,50,51]. We have observed similar effects in POPC prepared at C/P mole ratios ranging from 1:1 to 2:1.

Component lipids (in chloroform) were transferred to 13 × 100 mm borosilicate culture tubes and combined with vehicle (ethanol) or an equal volume of EPA, DHA, DPA, ALA, AA, fenofibrate, gemfibrozil, or niacin stock solutions, each adjusted to achieve desired treatment concentrations. Agents were tested at either 1:30 or 1:19 total treatment-to-phospholipid (T/P) mole ratios (3.2 and 5 mol%, respectively). EPA and DHA were also tested in combination after adjusting each to 50% of these target concentrations.

Samples were shell-dried under nitrogen gas and placed under vacuum for 3 h to remove residual solvent. After desiccation, each sample was resuspended in saline buffer (0.5 mM HEPES, 154 mM NaCl, pH 7.3, warmed to room temperature) to yield a final phospholipid concentration of 2.5 mg/mL. Lipid suspensions were then vortexed for 3 min at ambient temperature to form MLVs [52].

2.3. X-ray diffraction analysis

Membrane samples were oriented for x-ray diffraction analysis as previously described [53]. Briefly, a 100 μL aliquot (containing 250 μg of phospholipid) was aspirated from each MLV sample and transferred to a Lucite® sedimentation cell fitted with an aluminum foil substrate upon which a given membrane sample could be collected upon centrifugation. Samples were then loaded into a Sorvall AH-629 swinging bucket ultracentrifuge rotor (Dupont Corp., Wilmington, DE, USA) and centrifuged at 35,000*g*, 5 °C, for 90 min.

Following membrane orientation, sample supernatants were aspirated and aluminum foil substrates, each supporting a single membrane pellet, were removed from the sedimentation cells and mounted onto custom-designed, curved glass slides. The membrane samples were then placed in hermetically sealed containers in which temperature and relative humidity were controlled prior to and during x-ray diffraction analysis. Data reported in this study were collected at various temperatures (15 °C, 20 °C, and 30 °C) and 74% relative humidity. Experimental humidity conditions were established by exposing

membrane samples to saturated solutions of L-(+) tartaric acid ($K_2C_4H_4O_6 \cdot 1/2\ H_2O$). Samples were incubated at test conditions for at least 1 h prior to experimental analysis.

Each oriented membrane sample was aligned at grazing incidence with respect to a CuK_α x-ray beam ($K_{\alpha1}$ and $K_{\alpha2}$ unresolved; $\lambda = 1.54$ Å) produced by a Rigaku Rotaflex RU-200, high brilliance microfocus generator (Rigaku-MSC, The Woodlands, TX, USA) and collimated using a single Franks' mirror as previously described [54]. Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Hecus X-ray Systems, Graz, Austria) spaced 150 mm from the sample site. Detector calibration was performed by the manufacturer and verified using crystalline cholesterol monohydrate. Each sample was analyzed at least 12 h after membrane pellet preparation and was retested up to several days after initial analysis.

This technique allows for precise measurement of the unit cell periodicity, or *d*-space, of the membrane lipid bilayer, which is the distance from the center of one lipid bilayer to the next, including surface hydration. The *d*-space for any given membrane multibilayer is calculated from Bragg's Law,

$$h\lambda = 2dsin\theta \quad (1)$$

where *h* is the diffraction order, λ is the wavelength of the x-ray radiation (1.54 Å), *d* is the membrane lipid bilayer unit cell periodicity, and θ is the Bragg angle (equal to one-half the angle between the incident beam and scattered beam).

The presence of cholesterol domains in a given membrane sample results in the production of distinct Bragg (diffraction) peaks having singular periodicity values of 34 and 17 Å (first- and second-order cholesterol domain peaks, respectively) [55–57]. Under the specific temperature and relative humidity conditions established for these experiments, the second-order (17 Å) cholesterol domain peak is well-delineated from other, neighboring cholesterol and phospholipid diffraction peaks making it particularly useful for quantitating relative cholesterol domain peak intensity. Routines written in Origin 8.6 (OriginLab Corporation, Northampton, MA, USA) were used to quantitate second-order cholesterol domain peak intensity, which was normalized to the sum area associated with all phospholipid diffraction peaks in a given diffraction pattern.

2.4. Preparation of large unilamellar vesicles

Fluorescent anisotropy experiments were conducted using large unilamellar vesicles (LUVs) of 100 nm diameter, composed of either POPC alone or POPC/cholesterol binary mixtures, each containing increasing concentrations of EPA or DHA (0–10 mol%) and treated with the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), at 1 mol%. DPH is a cylindrically-shaped molecule with absorption and fluorescence emission transition dipoles aligned approximately parallel to its long axis. Consequently, the fluorescence polarization of DPH is high in the absence of rotational motion and very sensitive to reorientation along its long axis resulting from interactions with surrounding lipids. These properties have led to its extensive use in membrane fluidity measurements.

In this study, each sample was prepared by combining 320 nmol total lipid (POPC alone or POPC plus 50 mol% cholesterol), 3.2 nmol DPH, and dose-dependent aliquots of vehicle (ethanol), EPA, or DHA. Components were mixed well, dried under a stream of nitrogen at 35 °C, and then placed under high vacuum for at least 3 h to remove residual solvent. Each sample was then resuspended in 1 mL aqueous buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) and vortexed for 3 min to uniformly disperse the lipids and form homogeneous MLVs. The buffer was maintained at a temperature well above the phospholipid phase transition temperature during this and all subsequent lipid vesicle preparation steps.

LUVs of 100 nm diameter were prepared from MLVs by extrusion using a LiposoFast Extruder (Avestin, Inc., Ottawa, Ontario, Canada). The MLVs were freeze-thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in the extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV, USA). The samples were then subjected to 11 passes through polycarbonate filters to give the final LUV suspension. Samples were incubated in dark for 12 h at room temperature (~23 °C) to equilibrate before measuring fluorescence. All experiments were done in triplicate at room temperature.

2.5. Steady-state fluorescence anisotropy measurements

LUVs were transferred to quartz cuvettes (of 1 cm path length) and examined for changes in membrane fluidity using a Hitachi F-7000 spectrofluorometer (Tokyo, Japan) fitted with a Hitachi polarization accessory (Hitachi High Technologies America, Inc., Schaumburg, IL, USA), which was used to determine apparent rotational correlation time (ARCT) values for DPH in each sample. For monitoring DPH, the excitation wavelength was set at 358 nm and emission was monitored at 430 nm. Excitation and emission slits with bandpass of 1 and 5 nm were used for all measurements. The excitation slit was set to the lowest possible aperture to minimize photoisomerization of DPH during irradiation. Fluorescence was measured with a 30 s interval between successive openings of the excitation shutter to reverse any photoisomerization of DPH. Anisotropy values were calculated from the equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background correction) with the excitation and emission polarizers oriented vertically and horizontally, respectively. *G* is the grating factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}.

2.6. Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using IBH 5000F NanoLED equipment (Horiba JobinYvon, Edison, NJ) with DataStation software in the time-correlated single photon counting (TCSPC) mode. A pulsed light-emitting diode (LED) (NanoLED-16) was used as an excitation source. This LED generates optical pulse at 337 nm with pulse duration 1.2 ns and is run at 1 MHz repetition rate. The LED profile (instrument response function) was measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal-to-noise ratio, 10,000 photon counts were collected in the peak channel. All experiments were performed using emission slits with bandpass of 8 nm. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam, thereby avoiding any possible photo damage of the fluorophore. Data were stored and analyzed using DAS 6.2 software (Horiba JobinYvon, Edison, NJ). Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms

$$F(t) = \Sigma_i\, \alpha_i\, exp(-t/\tau_i) \quad (3)$$

where $F(t)$ is the fluorescence intensity at time *t* and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\Sigma_i\alpha = 1$. The program also includes statistical and plotting subroutine

packages. The goodness of fit for a given set of observed data and the chosen function was evaluated by the χ^2 ratio, the weighted residuals, and the autocorrelation function of the weighted residuals. A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation around zero with a minimum χ^2 value not > 1.6. Intensity-averaged mean lifetimes $<\tau>$ for biexponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the following equation:

$$<\tau> = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (4)$$

In order to ensure that the anisotropy values measured for DPH were not influenced by lifetime-induced artifacts, the apparent (average) rotational correlation times (ARCT) were calculated using Perrin's equation:

$$\tau_c = \frac{<\tau>r}{r_0 - r} \quad (5)$$

where r_0 is the limiting anisotropy of DPH, r is the steady state anisotropy, and $<\tau>$ is the mean fluorescence lifetime [58].

2.7. Statistical analyses

Data were presented as mean ± SD (fluorescence data) or SEM (x-ray diffraction data) for (N) separate samples or treatment groups. Differences between groups were analyzed using the two-tailed, Student's *t*-test (for comparisons between only two groups) or ANOVA followed by Dunnett or Student-Newman-Keuls multiple comparisons *post-hoc* analysis (for comparisons between three or more groups). Alpha error was set to 0.05 in this study.

3. Results

3.1. Comparative effects of EPA, DHA, and other test agents on the disruption of pre-existing cholesterol domains in model membranes

To assess whether EPA has a direct effect on domain formation independent of its antioxidant properties, we evaluated the comparative and disruptive effects of EPA, DHA, EPA + DHA combination treatment, as well as other polyunsaturated fatty acids (ALA, AA and DPA), lipid-lowering agents (fenofibrate, gemfibrozil, and niacin), and the positive control, glycyrrhizin, on cholesterol domain structural organization in model membranes prepared at 1.5:1 C/P—levels known to induce cholesterol domain formation even in the absence of other perturbations such as oxidative stress. Because cholesterol crystalline domains have a consistent unit cell periodicity (*d*-space) value that is substantially smaller than the typical plasma *d*-space value, the formation of these domains can be evaluated and quantitated using small angle x-ray diffraction [3,59,60]. As shown in Fig. 1, scattering data collected from each membrane preparation yielded up to four phospholipid diffraction orders having an average *d*-space value of 57.0 ± 0.6 Å (mean ± SD) and consistent with an homogenously-distributed, lipid bilayer phase. Each diffraction pattern also yielded an additional set of peaks with an average *d*-space of 34 Å and consistent with a cholesterol crystalline domain phase. Differences in the relative intensity of these peaks indicate material differences between the various treatments and are quantitated in Fig. 2.

Numeric reductions in cholesterol domain formation were observed in all samples, with the exception of gemfibrozil, relative to vehicle-treated controls (Fig. 2). EPA, glycyrrhizin, AA, and ALA had the most pronounced effects, reducing cholesterol domain levels by 65.5%, 54.9%, 46.8%, and 45.2%, respectively ($p < 0.05$ for all pairwise comparisons with vehicle-treated controls). Lesser nonsignificant reductions, and in some cases greater experimental variability, were observed for EPA + DHA combination treatment (29.6%), DPA (20.8%), DHA (11.1%), niacin (35.0%), and fenofibrate (1.4%) ($p > 0.05$ for all pairwise comparisons with vehicle-treated controls). Gemfibrozil increased cholesterol domain formation by a nonsignificant 10.9% as compared to vehicle-treated controls ($p > 0.05$). The statistical significance of EPA, glycyrrhizin, AA, and ALA effects were lost with ANOVA modeling across all treatment arms.

3.2. Dose-dependent effects of EPA on the disruption of pre-existing cholesterol domains in model membranes

EPA was observed to reduce cholesterol domains levels to a much greater extent than DHA, but was unable to exhibit that effect when tested in equimolar combination with DHA (Fig. 2). These data suggested that the cholesterol domain-lowering effects of EPA may be dose-dependent. To test this hypothesis, we evaluated EPA in the same model membrane system at additional (lower) doses. As shown in Fig. 3, EPA reduced cholesterol domain levels in a dose-dependent manner, beginning at 1:90 T/P mole ratio (15.0% reduction, *n.s.*), continuing through 1:60 T/P mole ratio (30.2% reduction, *n.s.*), and culminating in a 65.5% reduction ($p = 0.0139$) at the 1:30 T/P mole ratio.

3.3. Comparative and dose-dependent effects of EPA and DHA on membrane fluidity

EPA and DHA were first tested in membranes prepared in the absence of cholesterol; however, these membranes were so highly disordered that neither treatment was observed to have any substantive effect on DPH ARCT as compared to controls (data not shown). To resolve this issue, we added cholesterol to the membrane preparations at 50 mol%, which has been shown to significantly increase membrane order, as evidenced by a nearly 7-fold increase in ARCT measured for DPH (from 2.85 to 19.35 ns) [58], and to predispose cholesterol crystalline domain formation [61]. When EPA and DHA were tested in membranes prepared at 50 mol% cholesterol (Fig. 4), EPA was found to have no significant effect on DPH ARCT measured over 1–10 mol% as compared to vehicle-treated controls; however, DHA significantly reduced ARCT from 19.35 ns (control) to 15.56 ns (10 mol%) in a dose-dependent manner, consistent with a significant increase in membrane isotropy or fluidity. DHA was also observed to have significantly greater effects on membrane fluidity as compared to EPA at both 5 and 10 mol% treatment levels.

3.4. Comparative effects of EPA and DHA on membrane width and cholesterol domain formation in membranes prepared at cholesterol levels that predispose domain formation

To better understand the structural parameters associated with the fluidity changes measured for EPA and DHA in the anisotropy study, we also tested the comparative effects of EPA and DHA, each at 5 mol%, on changes in membrane width (*d*-space) and cholesterol domain formation in MLVs prepared at 50 mol% cholesterol and examined over a broad range of temperature conditions. As shown in Fig. 5, increasing the temperature from 15 °C to 30 °C in vehicle-treated controls reduced membrane bilayer width from 56.0 to 54.2 Å, consistent with a thermally-induced phospholipid acyl chain disordering effect. A similar pattern was observed in DHA-treated samples, with membrane *d*-space decreasing from 56.5 to 54.6 Å over the same temperature range, with no effects that were significantly different than those observed for vehicle-treated controls. EPA had a much more pronounced effect on bilayer width; EPA significantly increased membrane *d*-space at each temperature condition ($p < 0.05$) as compared to controls. This effect was also temperature-dependent, as membrane *d*-space decreased from 59.8 to 57.2 Å in EPA samples over the experimental temperature range. EPA also had a significantly greater effect on membrane bilayer width as compared to DHA at 20 °C ($p < 0.05$).

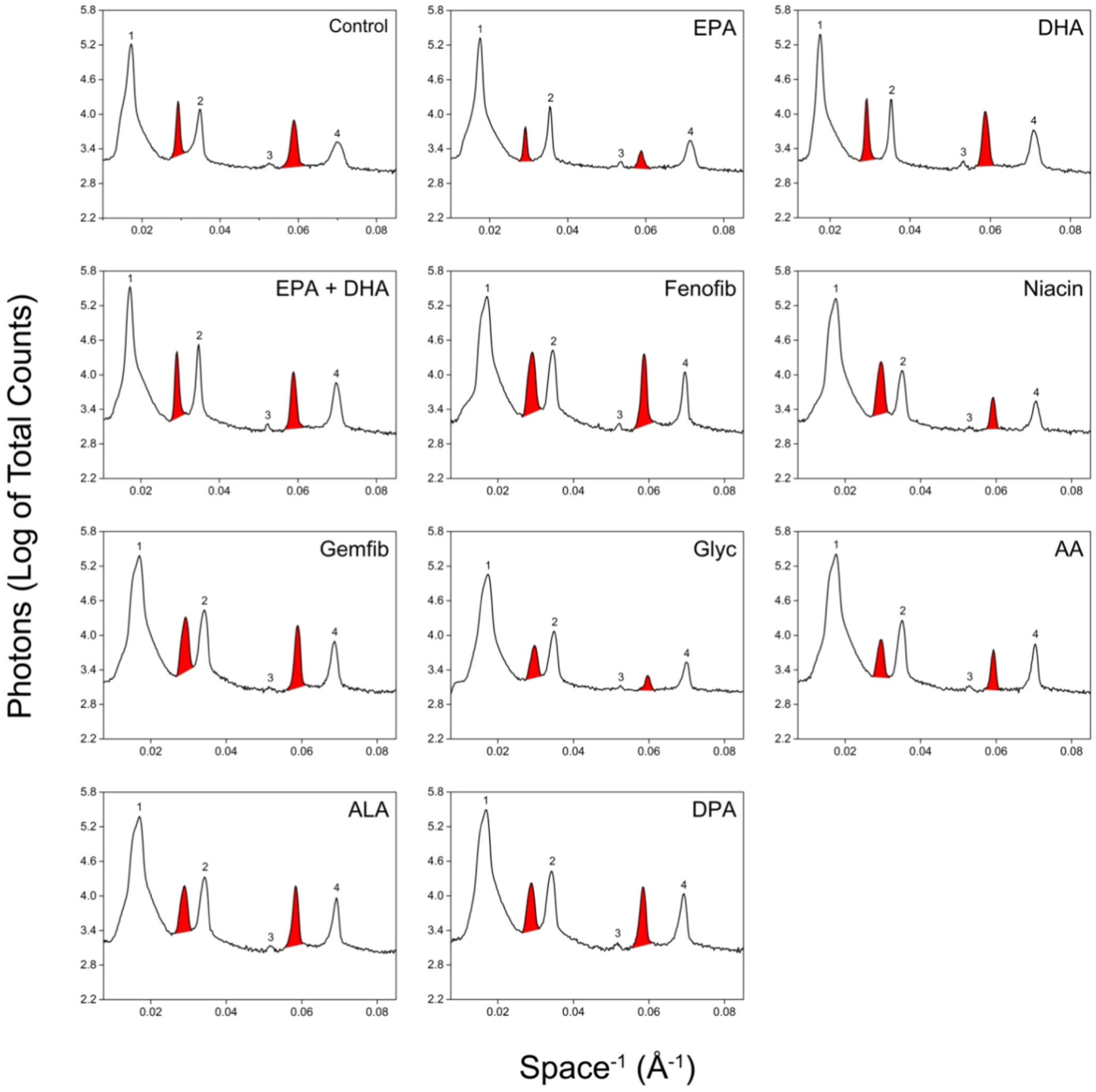

Fig. 1. Representative x-ray diffraction patterns collected from cholesterol-enriched model membranes treated with vehicle (control), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), EPA + DHA combination treatment, fenofibrate (Fenofib), nicotinic acid (Niacin), gemfibrozil (Gemfib), glycyrrhizin (Glyc), arachidonic acid (AA), α-linolenic acid (ALA), or docosapentaenoic acid (DPA). Membranes were reconstituted from palmitoyloleoylphosphatidylcholine (POPC) and cholesterol at a cholesterol-to-phospholipid (C/P) mole ratio of 1.5:1 and treated with each of the various agents to achieve a total drug-to-phospholipid (D/P) mole ratio of 1:30. In each panel, diffraction peaks highlighted in red correspond to a cholesterol crystalline domain phase, which has a characteristic periodicity (*d*-space value) of 34 Å; peaks labeled 1 through 4 correspond to the surrounding membrane phospholipid bilayer phase, which was observed to have an average periodicity of 57 Å.

These samples were also tested for cholesterol domain formation effects. As shown in Fig. 6, cholesterol domains were not observed in any of the vehicle- or EPA-treated samples and were not evoked by broad changes in temperature. In contrast, treatment with DHA induced the formation of prominent cholesterol crystalline domains, which remained stable over the entire experimental temperature range.

4. Discussion

We recently reported that EPA inhibits the formation of cholesterol domains in membranes exposed to oxidative stress [47]. The current data now show that EPA also inhibits the formation of cholesterol domains, independent of mechanisms associated with oxidative stress, in a dose-dependent manner, and under conditions of elevated cholesterol, suggesting that EPA has a direct physicochemical effect on membrane lipid organization under disease-like conditions. We first assessed whether EPA and other fatty acids or lipid-lowering agents could inhibit cholesterol domain formation in model membranes designed to form cholesterol domains spontaneously (*i.e.*, membranes prepared at a 1.5:1 C/P mole ratio). Compared to control, treatment with EPA, glycyrrhizin, AA, and ALA resulted in the greatest inhibition of cholesterol domain formation in membranes prepared in this fashion. Minor changes in cholesterol domain formation were observed with the lipid-lowering agents, fenofibrate, gemfibrozil, or niacin. The inhibitory

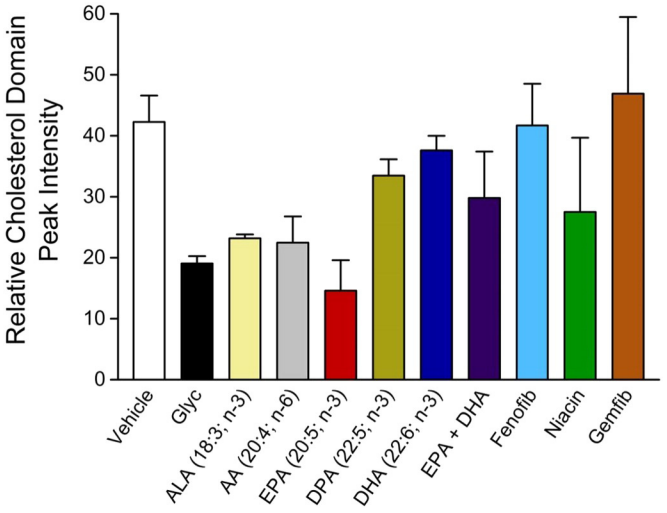

Fig. 2. Comparative effects of glycyrrhizin (Glyc), α-linolenic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), fenofibrate (Fenofib), nicotinic acid (Niacin), and gemfibrozil (Gemfib) on membrane cholesterol domain structural integrity. Values within parentheses after fatty acids denote carbon length and number of unsaturated bonds; omega-3 (n-3) or -6 (n-6) fatty acid classification. Each agent was tested at D/P mole ratio of 1:30. Values are mean ± SEM (N = 3–6). ANOVA: $p = 0.0946$, F = 1.864).

effects of the various long-chain fatty acids on cholesterol domain formation were observed to numerically increase with increasing chain length and degree of unsaturation from ALA to EPA and then to decrease with further increases in chain length and unsaturation (from EPA to DHA). As such, these agents could be ranked in order of their discrete effects on cholesterol domain formation as follows: ALA < AA < EPA > DPA > DHA. This suggests that EPA may possess the optimal combination of chain length and degree of unsaturation for mitigating changes in membrane structure, including cholesterol domain formation. When considered together with the dose-dependent, domain formation inhibitory effects observed for EPA, these data suggest that EPA may interfere with the deleterious effects of cholesterol enrichment at the membrane level, which could potentially translate, *in vivo*, to direct atheroprotective benefits.

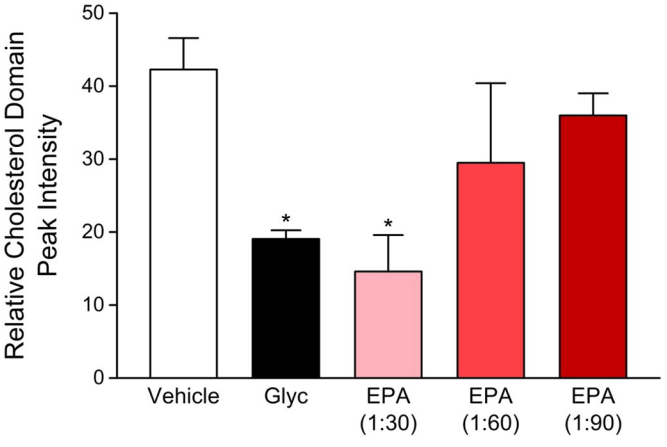

Fig. 3. Dose-dependent disruptive effects of EPA, versus vehicle and glycyrrhizin (Glyc), on the structural integrity of pre-existing membrane cholesterol domains. Model membranes were prepared as binary mixtures of POPC and cholesterol at a C/P mole ratio of 1.5:1 and treated with EPA at the D/P mole ratios indicated. Relative cholesterol peak intensity values were derived by integrating the second order cholesterol domain peak and normalizing to total phospholipid peak area associated with a given diffraction pattern. Values are mean ± SEM (N = 3–6). *$p < 0.05$ versus vehicle (Student-Newman-Keuls multiple comparisons analysis; overall ANOVA: $p = 0.0085$, F = 5.434).

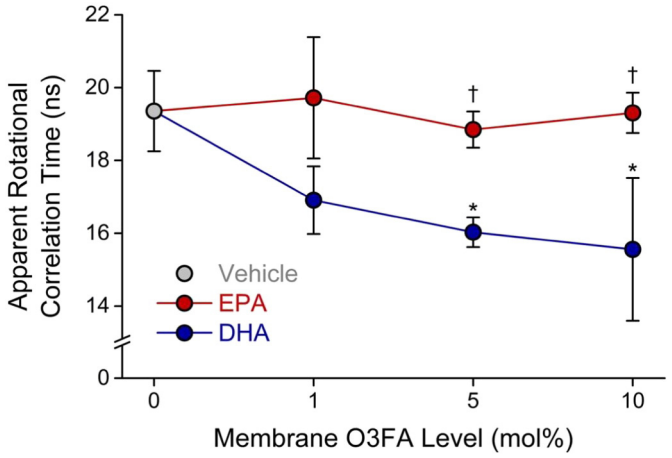

Fig. 4. Dose-dependent effects of EPA and DHA on the apparent rotational correlation time (ARCT) measured for diphenylhexatriene (DPH) in POPC membranes prepared at 50 mol% cholesterol. Values are mean ± SD (N = 3). *$p < 0.05$ versus control (vehicle) treatment; †$p < 0.05$ versus cognate (equimolar) DHA treatment (Student-Newman-Keuls multiple comparisons test; overall ANOVA: $p = 0.0016$; F = 6.778).

O3FAs are highly flexible and have been shown to increase the fluidity of endothelial cell membranes [62]. DHA, for example, has been shown to isomerize through each of its possible confirmations within 50 ns after being added to biological membranes [63]. High acyl chain flexibility and rapid conformational changes are thought to interfere with the close association of O3FAs with cholesterol molecules, which have a rigid steroid ring structure and are less flexible in their membrane disposition [64]. In this study, we evaluated the comparative effects of EPA and DHA on membrane structure and fluidity in model membranes prepared at cholesterol levels that predispose domain formation (*i.e.*, 1:1 C/P mole ratio). As expected, model membrane width was observed to decrease as a function of increasing temperature. Interestingly, similar temperature-dependent decreases in membrane width were observed in the presence of EPA, but an increase in membrane width was observed at all temperatures relative to vehicle-treated controls, and EPA did not alter membrane fluidity. In contrast, at all temperatures tested, DHA-treated membranes showed no change in membrane width compared to controls, but DHA did increase membrane fluidity in a dose-dependent manner. In addition, DHA *induced* the formation of distinct cholesterol domains at all temperatures, while EPA, like vehicle-treated controls, demonstrated membrane lipid homogeneity. Thus, EPA and DHA were distinguished in this study in

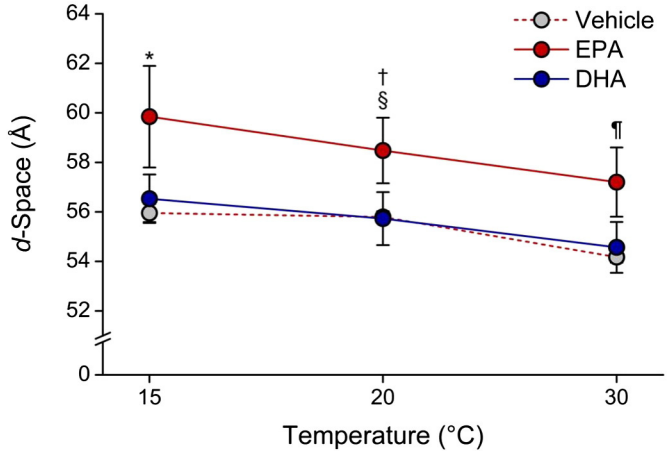

Fig. 5. Temperature-dependent effects of EPA and DHA on membrane unit cell periodicity (*d*-space) measured in POPC membranes prepared at 50 mol% cholesterol. Values are mean ± S.D. (N = 3). *$p = 0.0318$ versus vehicle (at 15 °C); §$p = 0.0259$ versus vehicle (at 20 °C); †$p = 0.0491$ versus DHA (at 20 °C);¶ $p = 0.0207$ versus vehicle (at 30 °C) (unpaired, two-tailed Student's *t*-test).

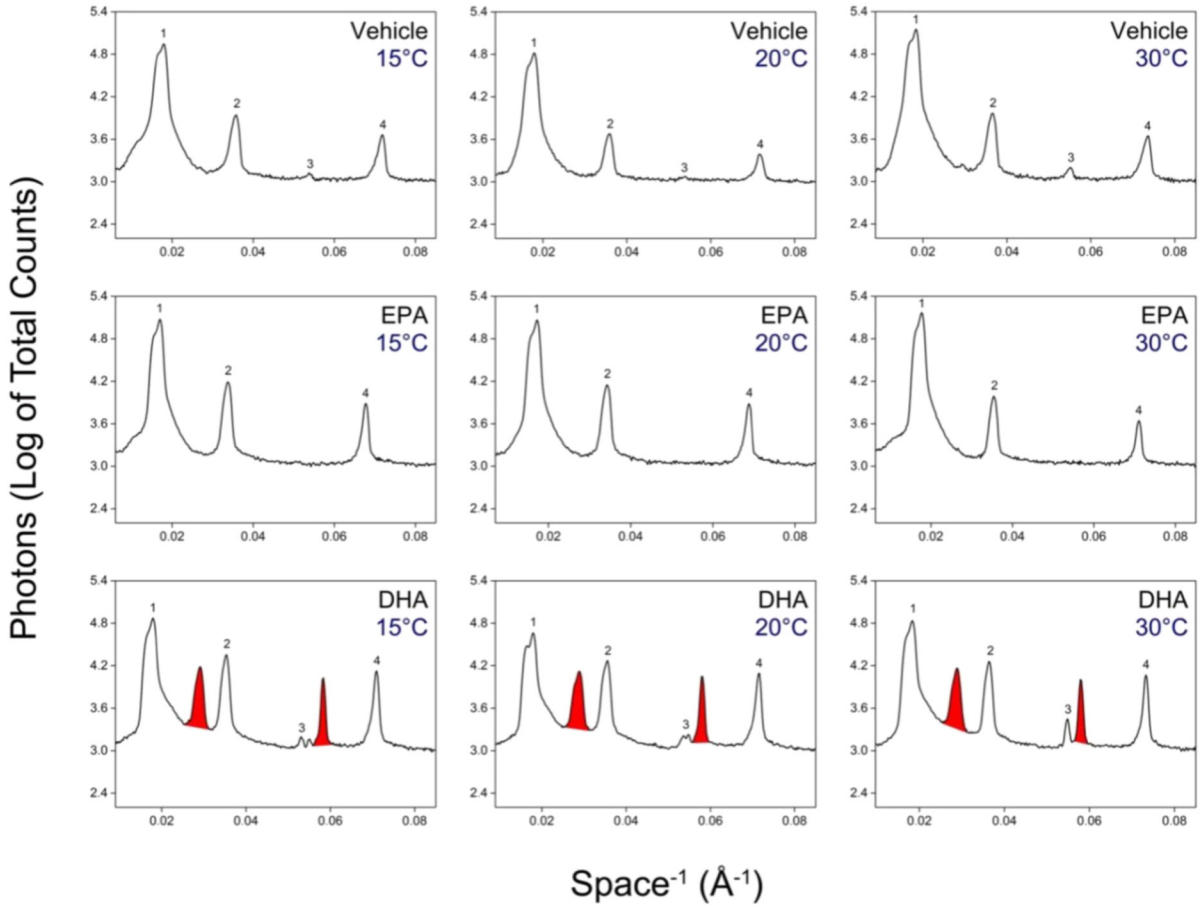

Fig. 6. Representative x-ray diffraction patterns collected from cholesterol-enriched model membranes treated with vehicle (control), EPA, or DHA and examined at 15 °C, 20 °C, and 30 °C. Membranes were reconstituted from POPC and cholesterol at 50 mol% cholesterol and treated with each of the various agents at 5 mol% (to match treatments used in corresponding lipid dynamics experiments). Each sample was maintained at 74% relative humidity by exposing the samples to a saturated solution of L-(+) tartaric acid. Diffraction peaks associated with the membrane phospholipid bilayer phase (labeled 1 through 4) were observed in all samples regardless of treatment or temperature condition. DHA, but not EPA or vehicle, induced the formation of cholesterol domains, which are indicated by a distinct set of diffraction peaks (shown in red fill) having a characteristic *d*-space value of 34 Å and shown to be stable over a broad range of temperatures.

that EPA prevented significant changes in membrane structure and fluidity while DHA increased the formation of cholesterol crystalline domains, had no overall effect on membrane width, and increased membrane fluidity (Fig. 7). These findings suggest that EPA allows cholesterol to remain more evenly distributed within the membrane while DHA promotes the simultaneous formation of highly-ordered, cholesterol-rich microdomains and DHA-rich, lipid-disordered domains. This dual domain motif appears to be balanced in regards to total membrane order, as demonstrated by a lack of change in membrane width, and seems to be in agreement with a model proposed by Shaikh et al. [32]. These findings are consistent with previous studies showing that DHA has a greater effect on endothelial membrane fluidity relative to EPA and suggests that acyl chain length or the number of double bonds differentially affects fluidity and the formation of membrane domains [62]. These results also suggest that DPH may preferentially locate within the more-fluid, DHA-rich domains as opposed to the more-ordered, cholesterol-rich domains. Despite experimental constraints, EPA prevented changes in cholesterol domain formation and membrane fluidity, suggesting an inherent ability to normalize membrane structural features despite shifts in temperature or cholesterol content.

The results reported herein showing that EPA confers stability to membranes is consistent with some previous studies [62,65]; however, as mentioned above, other studies have shown that EPA, like DHA, is able to increase membrane fluidity [66,67]. In addition, these data suggest that DHA induces the formation of cholesterol domains, but previous studies have shown that treatment with DHA decreases membrane cholesterol content [62]. These differences are likely explained by the different experimental models employed in each study. Unlike *in vivo* or cell culture (*in vitro*) experimental systems, the data presented in this study were generated in a closed, controlled model system in which cholesterol was restricted to the membrane alone; that is, there were no internal or external cholesterol acceptors, as in natural or cell culture systems, that might otherwise affect membrane cholesterol content. As such, this model system allows for a direct assessment of the structural effects of cholesterol, O3FAs, and other molecules, without the need to control extraneous effects; however, an important next step is to examine these effects in more advanced, cell culture or *in vivo*, systems.

These data also suggest that EPA may support normal cellular function even in a state of increased cholesterol load, which could have important implications in disease conditions such as atherosclerosis. For example, disruption of membrane structure and the formation of cholesterol-rich domains may affect endothelial function, vascular tone, cytokine expression, and immunity as well as recruitment and activation of inflammatory cells [5,9]. By reducing the volume or number of lipid rafts present in endothelial membranes, EPA may disengage stress-induced signaling complexes in caveolae, thus reducing endothelial dysfunction (*e.g.*, increasing eNOS secretion) [68] and vascular inflammation (*e.g.*, inhibiting NLRP3 inflammasome activation) [69], which may have clinically-significant cardiovascular benefits [9, 70–72]. Overall, by mitigating the formation of cholesterol-rich domains, treatment with EPA may provide positive benefits in a variety

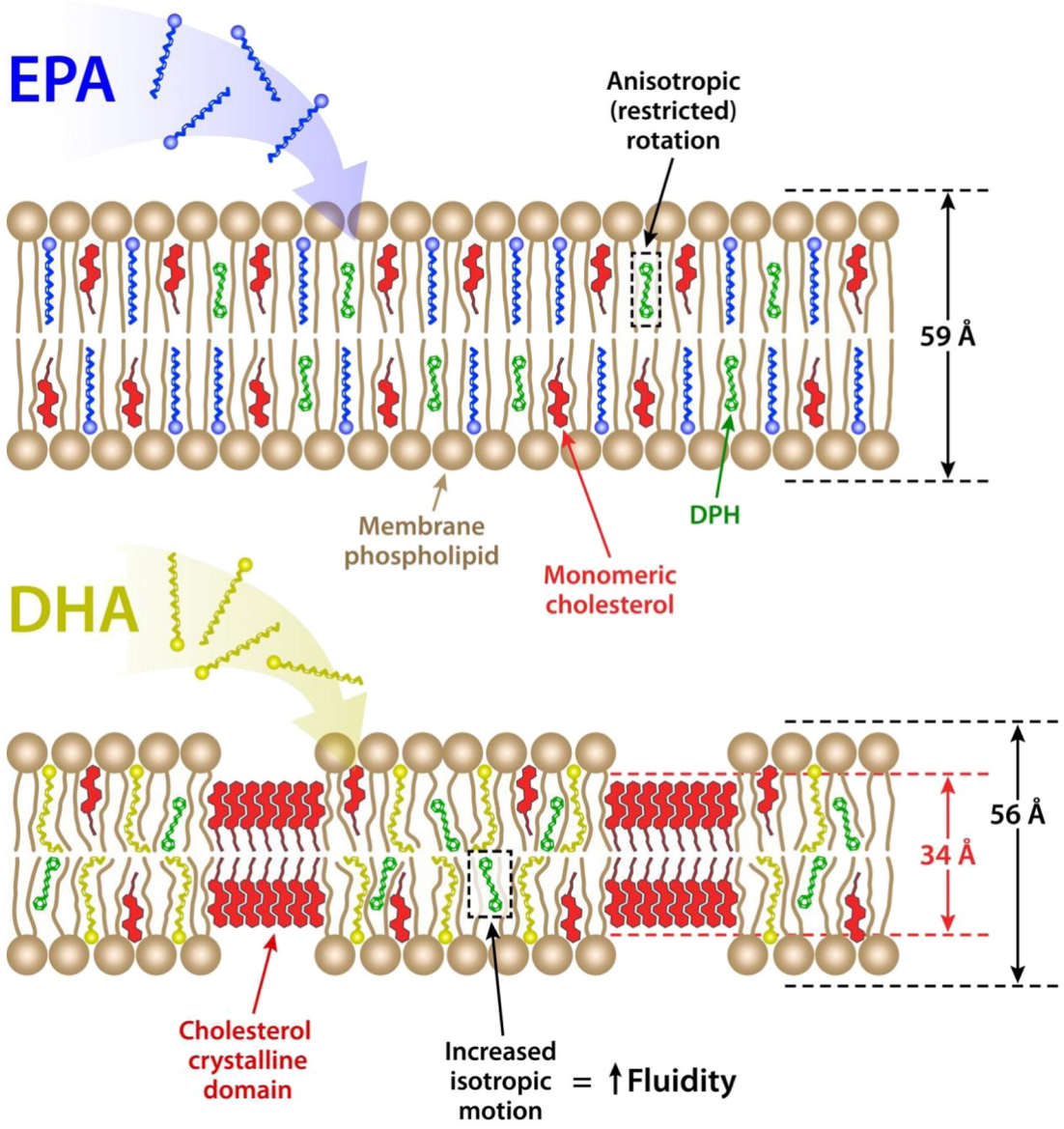

Fig. 7. Schematic illustration of the proposed effects of EPA and DHA on membrane structural and dynamic properties as determined in this study. EPA intercalates into the hydrocarbon core region of the membrane lipid bilayer where it provides important antioxidant benefits, as previously reported, but without inducing any significant changes in membrane fluidity, bilayer width, and cholesterol distribution. In contrast, DHA increases membrane fluidity, promotes the formation of discrete, cholesterol crystalline domains, and reduces the overall width of the membrane bilayer. The distinct effects of DHA as measured in this study are attributed to its greater molecular length and reduced saturation level, which alters membrane phospholipid packing constraints, effectively increasing molecular space in the hydrocarbon core, with subsequent effects on cholesterol redistribution and bilayer width. Note: The precise locations of the molecules depicted in this schematic are not known and are only suggested as a means of broadly illustrating the disparate effects of EPA and DHA on membrane lipid structural organization.

of conditions, including cardiovascular disease, type 2 diabetes mellitus, and autoinflammatory disease.

The structural-functional differences observed for EPA and DHA in this study suggest that both fatty acid chain length and the degree of unsaturation are important determinants of activity in cellular membranes. An important next step would be to test *in vivo* the comparative effects of EPA and DHA, against other anti-hypertriglyceridemic medications, ideally in a well-controlled, outcomes-based clinical trial. A few randomized, controlled clinical outcome studies with omega-3 fatty acids have demonstrated significant reductions in cardiovascular risk [17–30, 73] and coronary plaque regression [39]. Unfortunately, drawing an overall conclusion from the results of these studies is challenging because of limitations in study design such as the use of different formulations of EPA and DHA, subtherapeutic omega-3 dosing, potential underutilization of statin therapy, and lack of statistical power. In an effort to confirm whether an EPA-only formulation can reduce cardiovascular risk, particularly in a broad patient population, a well-controlled cardiovascular outcomes study is underway. This study, known as the *Reduction of Cardiovascular Events with Eicosapentaenoic Acid Intervention Trial* (REDUCE-IT), is evaluating the ability of a high purity prescription EPA-only formulation to reduce cardiovascular mortality and morbidity in a high-risk patient population with persistently high TG levels in spite of statin therapy [74].

5. Conclusion

The data presented in this report regarding model membranes suggests that EPA may provide atheroprotection through its direct effects on biological membranes. Conversely, these effects were not observed in model membranes treated with DHA. While treatment with EPA stabilized membranes in the presence of increasing temperature and cholesterol levels, treatment with DHA induced the formation of cholesterol

domains and altered membrane structure and fluidity, suggesting that EPA and DHA have very distinct membrane interaction effects. By reducing the accumulation of cholesterol-rich domains in atherosclerotic membranes, EPA may reduce inflammation and improve endothelial function. Further evaluation of the differential effects of omega-3 fatty acids on membrane physiology is warranted, and will be important to clarify the potential benefits of EPA relative to DHA in treating cardiovascular disease.

Transparency document

The Transparency document associated with this article can be found, in online version.

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