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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