Eicosapentaenoic Acid (EPA), But Not Other TG-Lowering Agents, Reduces Cholesterol Domain Levels in Atherosclerotic-Like Model Membranes

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Abstract

Background: Cholesterol accumulation in vascular cell membranes promotes cholesterol aggregation and domain formation, which have been linked to free cholesterol crystal deposition and plaque instability. Lipid modifying agents, including omega-3 fatty acids, may disrupt such membrane cholesterol domains due to their specific interactions with constituent lipids.

Purpose: In this study, we tested the direct effects of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), equimolar EPA + DHA, and other TG-lowering agents on cholesterol domain structural organization in model membranes treated with high cholesterol levels to simulate atherosclerotic-like conditions.

Methods: Membranes were prepared as binary mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol at a cholesterol-to-phospholipid mole ratio of 1.5:1 and treated with EPA, DHA, equimolar EPA + DHA, nicotinic acid, fenofibrate, or gemfibrozil to achieve a total drug-to-phospholipid mole ratio of 1:30. Controls were treated with vehicle alone. Changes in membrane lipid structural organization were measured using small angle x-ray scattering. Cholesterol domains were identified in experimental samples by the presence of characteristic diffraction peaks with a unit cell periodicity of 34 Å. Cholesterol domain peak intensities were measured, normalized to total phospholipid peak area, and used to quantitate differences between the various treatments. As a positive control, we also tested glycyrrhizin, a glycosylated sterol that has been shown to reduce cholesterol domain levels in this membrane system.

Results: All samples yielded diffraction patterns consistent with a biphasic membrane structure containing prominent cholesterol and phospholipid-enriched membrane bilayer domains, with unit cell periodicities of 34 Å and 56 Å, respectively. EPA reduced relative cholesterol domain peak intensity by 65% (p<0.05) as compared to vehicle treated controls. Similar reductions were observed with glycyrrhizin (p<0.05). By contrast, DHA and the other TG-lowering agents had no significant effect on relative cholesterol domain peak intensity. The EPA + DHA combination treatment reduced cholesterol domain levels by ~30% but was not statistically significant as compared to controls.

Conclusion: EPA significantly reduced membrane cholesterol domain levels in a manner that was not reproduced with other TG-lowering agents or DHA. These data indicate physico-chemical differences among these agents and support a potential benefit for EPA in reducing atherosclerotic cholesterol domain formation and associated pathology.

Background

- The unstable atherosclerotic lesion is characterized by extracellular lipid deposits containing cholesterol (both free and esterified), phospholipids, and triacylglycerol.[1,2] Membrane-associated cholesterol crystals have been characterized in cell culture systems and tissue explants using electron microscopy.[3,4] Cholesterol crystalline domains are believed to contribute to mechanisms of cell death and inflammation during atherosclerosis (**Fig. 1**).[5]

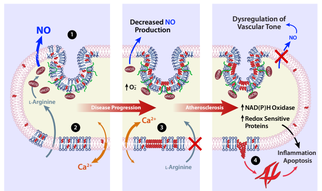

Figure 1. Schematic diagram of changes in lipid raft structure and cell function during cholesterol enrichment and atherosclerosis. Subtypes of lipid rafts enriched with sphingolipid (blue) and cholesterol (red) include caveolae (❶) that contain caveolin protein (green) and detergent-resistant membrane domains (❷). With progressive cholesterol accumulation, separate cholesterol crystalline membrane domains (❸) form and precede the development of extracellular cholesterol crystals (❹), which contribute to mechanisms of cell injury and death, , including apoptosis. Cholesterol enrichment also increases the number of membrane caveolae, leading to inhibition of endothelial nitric oxide (eNOS) following by a reduction in nitric oxide (NO) production and associated vascular benefits. Loss of normal membrane structure and function with cholesterol enrichment is also associated with disruptions in calcium regulation and redox potential.

- Eicosapentaenoic acid (EPA) is a long-chain omega-3 fatty PUFA (20:5; n-3) that has been shown to have various biological effects, including anti-inflammatory, decreased platelet aggregation, and lipid-lowering actions. In the *Japan EPA Lipid Intervention Study* (JELIS), purified EPA (1.8 g/day) was effective in preventing coronary artery disease (CAD) in hypercholesterolemic patients also receiving statin treatment.[6]
- Vascepa® is a high-purity prescription form of EPA ethyl ester (icosapent ethyl) indicated as an adjunct to diet to reduce triglyceride (TG) levels in adult patients with severe hypertriglyceridemia (TG levels ≥ 500 mg/dL). In the pivotal Phase 3 MARINE and ANCHOR trials, Vascepa® was shown to significantly reduce TG levels without increasing LDL-C, while also reducing various markers of inflammation (hsCRP, Lp-PLA$_2$, oxLDL) as compared to placebo.[7-9]
- In this study, we tested the hypothesis that EPA directly blocks the formation of cholesterol crystalline domains in biological membranes prepared under hypercholesterolemic conditions. The basis for this benefit with EPA is its direct physicochemical interactions with lipid molecules associated with the cell membrane. This work is an extension of a recent study in which EPA was shown to inhibit the formation of cholesterol domains following conditions of oxidative stress.[10] We do not expect these benefits to be reproduced by other TG-lowering agents or DHA given differences in their membrane lipid interaction properties.

Purpose

The goal of this study was to test the effects of EPA, docosahexaenoic acid (DHA), equimolar EPA and DHA treatment (EPA + DHA), and other TG-lowering agents (fenofibrate, nicotinic acid, gemfibrozil) on cholesterol domain structural organization in model membranes exposed to high cholesterol levels to simulate atherosclerotic-like conditions. As an additional control, we also tested the domain disruptive effects of glycyrrhizin, a bioactive compound found in licorice that has been shown to disrupt constituent domains in lipid raft models.[11]

Methods

Materials

- 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and dissolved in HPLC-grade chloroform.
- *cis*-5,8,11,14,17-Eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) were purchased from Sigma-Aldrich (Saint Louis, MO) and solubilized in ethanol to 1.0 mM under nitrogen atmosphere.
- Fenofibrate, nicotinic acid (niacin), and gemfibrozil were purchased from Sigma-Aldrich and solubilized in ethanol to 1.0 mM.
- Glycyrrhizic acid ammonium salt (glycyrrhizin) was purchased from Sigma-Aldrich and solubilized in methanol to 1.0 mM.
- All test compounds were further diluted in ethanol or aqueous buffer as needed.

Preparation of Membrane Lipid Vesicles

- Multilamellar vesicles (MLVs) were prepared from binary mixtures of POPC and cholesterol, at a cholesterol-to-phospholipid (C/P) mole ratio of 1.5:1, as previously described.[12] Component lipids (in chloroform) were transferred to borosilicate culture tubes and combined with vehicle (ethanol) or an equal volume of EPA, DHA, fenofibrate, niacin, gemfibrozil, or glycyrrhizin stock solution(s), adjusted to achieve a drug-to- phospholipid (D/P) mole ratio of 1:30. Samples were then shell-dried under nitrogen gas and placed under vacuum for 3 hr to remove residual solvent.
- After drying, each sample was resuspended in saline buffer (0.5 mM HEPES, 154 mM NaCl, pH 7.3) 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.[13]

X-ray Diffraction Analysis

- Membrane samples were oriented for x-ray diffraction analysis as previously described.[12] Briefly, 100 µL aliquots of each MLV preparation (containing 250 µg of phospholipid) were transferred to Lucite® sedimentation cells fitted with a removable aluminum foil substrate designed to collect MLVs into a single membrane pellet upon centrifugation. Samples were then loaded into a Sorvall AH-629 swinging bucket ultracentrifuge rotor (Dupont Corp., Wilmington, DE) and centrifuged at 35,000 *g*, 5°C for 1.5 hr.
- Following membrane orientation, sample supernatants were aspirated and aluminum foil substrates mounted onto curved glass slides. The membrane samples were then placed in hermetically sealed containers in which temperature and relative humidity were controlled during x-ray diffraction analysis. Data reported in this study were collected at 20°C and 74 % relative humidity.
- Each oriented membrane sample was subjected to small angle x-ray diffraction analysis as shown in **Fig. 2**.

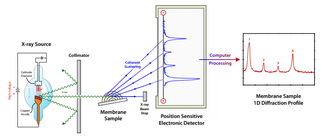

Figure 2. Membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic x-ray beam produced by a Rigaku Rotaflex RU-200, high-brilliance microfocus generator (Rigaku-MSC, The Woodlands, TX). Analytical x-rays were generated by electron bombardment of a rotating copper anode and filtered through a thin nickel foil to provide monochromatic radiation (λ = 1.54 Å). 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.

- 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 presence of cholesterol domains in a given membrane sample results in the production of distinct Bragg (diffraction) peaks that have singular periodicity values of 34 and 17 Å. These peaks are typically referred to as first- and second-order cholesterol domain peaks (**Fig. 3**).

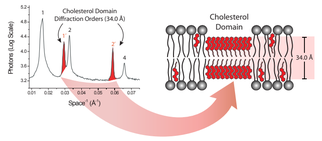

Figure 3. Schematic illustration of a typical diffraction pattern collected from a biphasic membrane sample, exhibiting sterol-poor, phospholipid bilayer domains (peaks 1, 2, and 4) and cholesterol crystalline domains (peaks 1' and 2'), and its relationship to the spatial arrangement of these domains in a representative membrane bilayer.

Calculations and Statistical Analyses

- Data are presented as mean ± SEM for (n) separate samples or experiments. Differences between groups were analyzed using the unpaired, two-tailed Student's t-test (for comparisons between only two groups) or ANOVA followed by Student-Newman-Keuls multiple comparisons *post hoc* analysis (for comparisons between three or more groups). Only differences with probability values less than 0.05 were considered significant.

Results

Representative X-Ray Diffraction Patterns Collected from Cholesterol- Enriched Model Membranes Treated with EPA, DHA, EPA + DHA (Combination Treatment), Fenofibrate, Niacin, Gemfibrozil, or Glycyrrhizin versus Vehicle-Treated Control

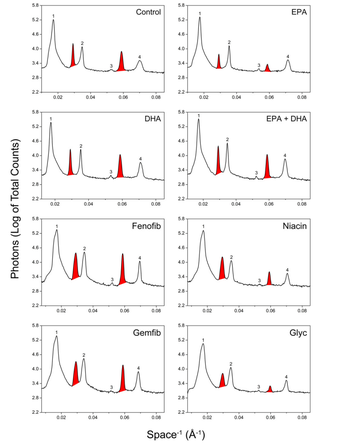

Figure 4. Membranes were reconstituted from POPC and cholesterol at a cholesterol-to-phospholipid (C/P) mole ratio of 1.5:1 and treated with each of the various agents to achieve a total drug-to-phospholipid (D/P) mole ratio of 1:30 (EPA and DHA were tested together at equimolar concentrations). In each 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. Abbreviations are: EPA, *cis*-5,8,11,14,17-eicosapentaenoic acid; DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; Fenofib, fenofibrate; Gemfib, gemfibrozil; Glyc, glycyrrhizin.

Quantitative Assessment of the Cholesterol Domain Disruptive Effects of EPA and Other Agents Examined in this Study

Treatment	Relative Cholesterol Domain Peak Intensity	% Change (vs. Control)	*P* Values*
Vehicle	42.3 ± 4.3	---	---
EPA	14.6 ± 5.0	-65.5 ± 17.1	0.0139
DHA	37.6 ± 2.4	-11.1 ± 11.7	0.3975
EPA + DHA	29.8 ± 7.6	-29.6 ± 20.9	0.2279
Fenofib	41.7 ± 6.8	-1.4 ± 19.1	0.9451
Niacin	27.5 ± 12.2	-35.0 ± 30.8	0.3157
Gemfib	46.9 ± 12.6	10.9 ± 43.3	0.8122
Glyc	19.1 ± 1.2	-54.9 ± 12.3	0.0002

Table 1. Values are reported as mean ± SEM (N = 3-6). Each agent was tested at a total D/P mole ratio of 1:30; EPA and DHA, in combination, were tested at equimolar concentrations. Relative cholesterol domain peak intensity values were derived by integrating the second-order cholesterol domain peak and normalizing to total phospholipid peak area associated with a give diffraction pattern. *P values were calculated against vehicle-treated controls using an unpaired, two-tailed Student's t-test.

Comparative Effects of EPA, DHA, EPA + DHA, and Fenofibrate on Membrane Cholesterol Domain Structural Integrity

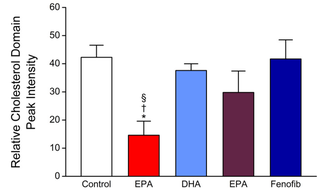

Figure 5. Each agent was tested at a total D/P mole ratio of 1:30; EPA and DHA, in combination, were tested at equimolar concentrations. Values are mean ± SEM (N=3). *p<0.05 versus control; †p<0.05 versus DHA; ‡p<0.05 versus Fenofib (Student-Newman-Keuls multiple comparisons test; overall ANOVA: p=0.0275, F=4.326).

Comparative Effects of EPA, DHA, EPA + DHA, Fenofibrate, and Glycyrrhizin on Membrane Cholesterol Domain Structural Integrity

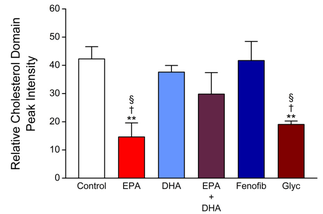

Figure 6. Each agent was tested at a 1:30 total D/P mole ratio. EPA and DHA, in combination, were tested at equimolar concentrations. Values are mean ± SEM (N=3-6). **p<0.01 versus control; †p<0.05 versus DHA; ‡p<0.05 versus Fenofib (Student-Newman-Keuls multiple comparisons test; overall ANOVA: p=0.0010, F=7.624).

Summary

- Cholesterol crystalline domains were observed in cholesterol-enriched, atherosclerotic-like model membranes, consistent with effects observed previously in membrane models of atherosclerosis.[14-16]
- EPA reduced cholesterol crystalline domain levels in cholesterol-enriched model membranes by 65% (p<0.05) as compared to vehicle-treated controls. Similar reductions were observed with glycyrrhizin (p<0.05).
- No significant changes in membrane cholesterol domain levels were observed with DHA or any of the other TG-lowering agents tested in this study as compared to controls.
- The EPA + DHA combination treatment had an intermediate effect, reducing cholesterol domain levels by ~30% (*n.s.*) compared to controls, a result likely due to the 50% reduction in EPA concentration in these samples since DHA alone had little to no effect in this study.

Interpretation

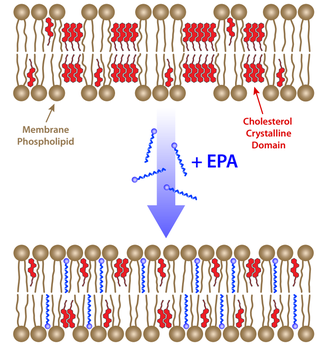

Figure 7. Schematic summary of the membrane lipid structural effects of EPA as determined in this study. Membrane cholesterol domains, typically observed under conditions of elevated cholesterol, were highly diminished in the presence of EPA.

Conclusion

In model membranes prepared under hypercholesterolemic conditions, EPA significantly reduced cholesterol domain levels in a manner that was not reproduced with DHA or any of the other TG-lowering agents examined in this study. These data indicate physico-chemical differences among these agents and support a potential benefit for EPA in reducing atherosclerotic cholesterol domain formation and associated pathology.

References

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Acknowledgements

This study was conducted with financial support from Amarin Pharma, Inc.