Biochimica et Biophysica Acta 1848 (2015) 502–509

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Eicosapentaenoic acid inhibits glucose-induced membrane cholesterol crystalline domain formation through a potent antioxidant mechanism

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ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form 2 October 2014
Accepted 14 October 2014
Available online 22 October 2014

Keywords:
Omega-3 fatty acid
Eicosapentaenoic acid
Glucose
Lipid peroxidation
Cholesterol domains
Atorvastatin

ABSTRACT

Lipid oxidation leads to endothelial dysfunction, inflammation, and foam cell formation during atherogenesis. Glucose also contributes to lipid oxidation and promotes pathologic changes in membrane structural organization, including the development of cholesterol crystalline domains. In this study, we tested the comparative effects of eicosapentaenoic acid (EPA), an omega-3 fatty acid indicated for the treatment of very high triglyceride (TG) levels, and other TG-lowering agents (fenofibrate, niacin, and gemfibrozil) on lipid oxidation in human low-density lipoprotein (LDL) as well as membrane lipid vesicles prepared in the presence of glucose (200 mg/dL). We also examined the antioxidant effects of EPA in combination with atorvastatin *o*-hydroxy (active) metabolite (ATM). Glucose-induced changes in membrane structural organization were measured using small angle x-ray scattering approaches and correlated with changes in lipid hydroperoxide (LOOH) levels. EPA was found to inhibit LDL oxidation in a dose-dependent manner (1.0–10.0 μM) and was distinguished from the other TG-lowering agents, which had no significant effect as compared to vehicle treatment alone. Similar effects were observed in membrane lipid vesicles exposed to hyperglycemic conditions. The antioxidant activity of EPA, as observed in glucose-treated vesicles, was significantly enhanced in combination with ATM. Glucose treatment produced highly-ordered, membrane-restricted, cholesterol crystalline domains, which correlated with increased LOOH levels. Of the agents tested in this study, only EPA inhibited glucose-induced cholesterol domain formation. These data demonstrate that EPA, at pharmacologic levels, inhibits hyperglycemia-induced changes in membrane lipid structural organization through a potent antioxidant mechanism associated with its distinct, physicochemical interactions with the membrane bilayer.

1. Introduction

At elevated levels, the aldose sugar glucose produces non-enzymatic chemical modifications to membrane proteins and phospholipids, leading to advanced glycation endproducts (AGEs) and cell injury [1]. Oxidative stress and AGEs have been implicated in both microvascular and macrovascular complications of diabetes and other metabolic disorders [1,2]. In membranes enriched with polyunsaturated fatty acids (PUFAs), hyperglycemia promotes the formation of free radicals and cholesterol crystalline domains associated with atherosclerosis [3–6]. The non-enzymatic effects of glucose on cholesterol crystalline domain formation were shown to be enhanced under conditions of high cholesterol and could not be reproduced by mannitol [3]. Oxidative damage to PUFAs with glucose is of particular interest given its role in the propagation of free radicals during vascular injury and insulin resistance [1,7,8]. In addition to cell membrane-mediated effects, oxidation of PUFAs in low-density lipoprotein (LDL) contributes to endothelial dysfunction, inflammation, and atherosclerotic foam cell formation [9,10].

Available clinical evidence indicates that consumption of long-chain, omega-3 fatty acids correlates with reduced risk for cardiovascular mortality and morbidity [11,12]. By incorporating into cellular membranes associated with the atherosclerotic plaque, omega-3 fatty acids interfere with various signal transduction pathways linked to inflammation and endothelial dysfunction during atherogenesis [12,13]. Eicosapentaenoic acid (EPA; 20:5, n-3) is an omega-3 fatty acid indicated for the treatment of very high triglyceride levels. In the Japan EPA Lipid Intervention Study (JELIS), highly-concentrated, purified EPA was effective in preventing coronary artery disease (CAD) in hypercholesterolemic patients also receiving statin treatment [14]. In other clinical studies, treatment with purified EPA slowed atherosclerotic disease progression in patients with type 2 diabetes and documented CAD [15–17]. Purified EPA treatment has also been reported to reduce levels of triglycerides, high-sensitivity C-reactive protein (hsCRP), lipoprotein-associated phospholipase A_2 (Lp-PLA_2), arachidonic acid/EPA (AA/EPA) ratio, and oxidized LDL levels as compared to placebo [18–23]. This is in contrast

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http://dx.doi.org/10.1016/j.bbamem.2014.10.016

to clinical findings with other TG-lowering agents, including fenofibrate and niacin, which failed to reduce cardiovascular events as compared to statin treatment alone (HPS2-THRIVE, FIRST, ACCORD, AIM-HIGH, FIELD) [24–28]. The basis for these differences may be, in part, the result of direct effects of EPA on plaque formation and stability due to its distinct physicochemical properties. In particular, its lipophilic structure and molecular space dimensions allow EPA to insert efficiently into lipoprotein particles and cell lipid membranes as compared to other TG-lowering agents.

In this study, we used small angle x-ray scattering (SAXS) to examine the ability of EPA to interfere with the effects of high glucose on membrane lipid peroxidation and structural organization in vesicles enriched in PUFAs. Pronounced changes in membrane lipid organization were observed with hyperglycemia, including the formation of cholesterol crystalline domains, which correlated with increased formation of lipid hydroperoxide (LOOH), an intermediate product of oxidative lipid damage. Treatment with EPA, but not vitamin E or other TG-lowering agents (fenofibrate, niacin, and gemfibrozil), inhibited changes in membrane structural organization due to its potent, chain-breaking antioxidant actions. EPA also inhibited human LDL oxidation in a manner distinct from the other TG-lowering agents. The membrane antioxidant effects of EPA were enhanced in combination with atorvastatin *o*-hydroxy (active) metabolite (ATM).

2. Materials and methods

2.1. Materials

1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine [18:2 (Cis) PC or DLPC] and monomeric cholesterol (isolated from ovine wool) were purchased from Avanti Polar Lipids (Alabaster, AL) and solubilized at 25 and 10 mg/mL, respectively. EPA was purchased from Sigma-Aldrich (Saint Louis, MO) and solubilized in ethanol to 1 mM under nitrogen atmosphere. Vitamin E (α-tocopherol) was also purchased from Sigma-Aldrich and prepared in ethanol at 1.0 mM ($\varepsilon = 3.06 \times 10^4\ M^{-1}\ cm^{-1}$ at 294 nm) just prior to experimental use. Atorvastatin *ortho*- (*o*-) hydroxy (active) metabolite was purchased from Toronto Research Chemicals (North York, Ontario, Canada) and solubilized in methanol to 1.0 mM. Fenofibrate, nicotinic acid (niacin), and gemfibrozil were purchased from Sigma-Aldrich and solubilized in ethanol to 1.0 mM. All test compounds were further diluted in ethanol or aqueous buffer as needed. Glucose was prepared in saline buffer (0.5 mM HEPES, 154 mM NaCl, pH 7.3) at 11.0 mM (200 mg/dL).

CHOD-iodide color reagent (stock) was prepared, with slight modification, as described by from El-Saadani et al. [29] and consisted of 0.2 M K_2HPO_4, 0.12 M KI, 0.15 mM NaN_3, 10 μM ammonium molybdate, and 0.1 g/L benzalkonium chloride. Prior to experimental use, the CHOD reagent was activated by adding 24 μM ethylenediaminetetraacetic acid (EDTA), 20 μM butylated hydroxytoluene (BHT), and 0.2% Triton X-100.

2.2. LDL isolation and oxidation

LDL was isolated from the plasma of healthy volunteers by density gradient ultracentrifugation and adjusted to a final apolipoprotein (apoB100) concentration of 10 mg/mL. Samples were prepared at 100 μg/mL apoB100 and incubated with test agents for 30 min at 37 °C in a shaking water bath. Oxidation was initiated by adding 10 μM $CuSO_4$. After 1 hr, 100 μL aliquots were removed from each sample and combined with 1.0 mL thiobarbituric acid (0.5%), 10 μL trichloroacetic acid (10%), 10 μL BHT (35 mM in methanol), and 10 μL EDTA (5 mM). Sample aliquots were incubated at 100 °C for 30 min and then assayed for the formation of thiobarbituric acid-reactive substances (TBARS), which have a molar absorptivity (ε) value of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm and are derived principally from the reaction of thiobarbituric acid with malondialdehyde (MDA), a reactive aldehyde produced by LDL oxidation [30,31]. Sample TBARS concentrations were determined spectrophotometrically and expressed as molar equivalents of MDA.

2.3. Preparation of membrane lipid vesicles

Multilamellar vesicles (MLVs) were prepared as binary mixtures of DLPC (1.0 or 2.5 mg total phospholipid per sample) and cholesterol at a fixed cholesterol-to-phospholipid (C/P) mole ratio of 0.6:1. Component lipids (in chloroform) were transferred to 13 × 100 mm borosilicate culture tubes and combined with vehicle (ethanol) or an equal volume of omega-3 fatty acid, vitamin E, or ATM stock solution, each adjusted to achieve desired treatment concentrations. Samples were shell-dried under nitrogen gas and placed under vacuum for 1 h to remove residual solvent. After desiccation, each sample was resuspended in 1.0 mL glucose-containing saline to yield final phospholipid concentrations of 1.0 or 2.5 mg/mL (for lipid peroxidation or x-ray diffraction analysis, respectively). Lipid suspensions were then vortexed for 3 min at ambient temperature to form MLVs [32].

2.4. Lipid peroxidation analysis

All MLV samples were subjected to time-dependent autoxidation by incubating at 37 °C in an uncovered, shaking water bath. This method allows lipid peroxidation to occur gradually without requiring the use of exogenous initiators. Small aliquots (5–100 μL) of each sample were removed, immediately following MLV preparation (0 hr) and after exposing samples to oxidative conditions for 72 or 96 hr, and combined with 1.0 mL of activated CHOD-iodide color reagent. Aliquot volume

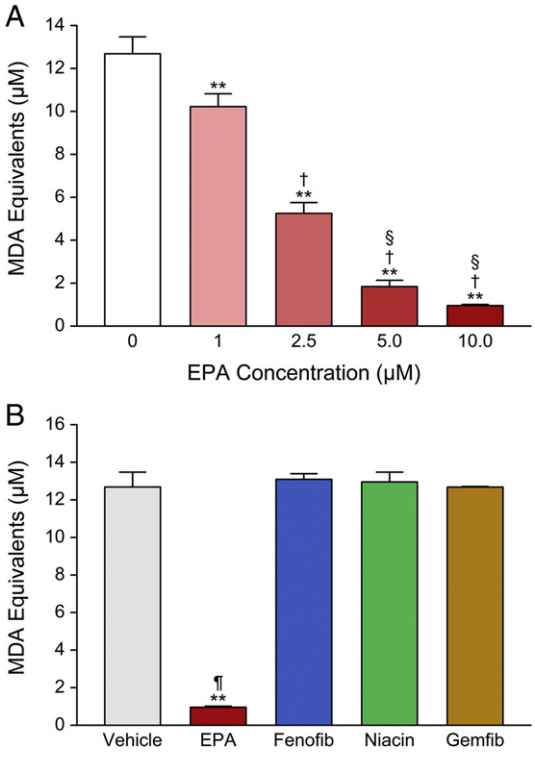


Fig. 1. Effects of EPA on human LDL oxidation examined (A) at various doses and (B) in comparison to fenofibrate (Fenofib), nicotinic acid (Niacin), and gemfibrozil (Gemfib), each at 10.0 μM. Samples were prepared at 100 μg/mL LDL (based on apoB100 content) and incubated with test agents (at doses indicated) for 30 min prior to initiating lipid oxidation using 10 μM $CuSO_4$. Samples were maintained at 37 °C in a shaking water bath for 1 hr. Lipid oxidation was measured by colorimetric assay of TBARS formation and expressed as molar equivalents of malondialdehyde (MDA). Values are mean ± S.D. (N = 3). $^{**}p < 0.001$ versus vehicle-treated control; $^{\dagger}p < 0.001$ versus 1.0 μM EPA; $^{\S}p < 0.001$ versus 2.5 μM EPA; $^{\P}p < 0.001$ versus all other TG-lowering agents (Student–Newman–Keuls multiple comparisons test; overall ANOVA—panel A: $p < 0.0001$, $F = 298.14$; panel B: $p < 0.0001$, $F = 132.37$).

was reduced with each successive time point to ensure that spectrophotometric readings were within the optimal absorbance range. Test samples were covered and incubated in darkness at room temperature for at least 4 hr. Sample absorbances were then measured against a CHOD blank at 365 nm using a Beckman DU-640 spectrophotometer. The CHOD colorimetric assay is based on the oxidation of iodide (I^-) by lipid hydroperoxide (LOOH) to form triiodide (I_3^-), the quantity of which is directly proportional to the amount of LOOH present in the lipid sample. The molar absorptivity (ε) of I_3^- is $2.46 \times 10^4\ M^{-1}\ cm^{-1}$ at 365 nm [32].

2.5. X-ray diffraction analysis

The membrane structural effects of glucose and the various compounds examined in this study were measured at 0, 72, and 96 hr intervals. Membrane lipid vesicles were oriented for x-ray diffraction analysis as described previously [33]. Briefly, a 100 μL aliquot (containing 250 μg MLV) was aspirated from each sample and transferred to a Lucite® sedimentation cell fitted with an aluminum foil substrate upon which a given sample could be collected by centrifugation. Samples were then loaded into a Sorvall AH-629 swinging bucket rotor (DuPont Corp., Wilmington, DE) and centrifuged at 35,000 *g*, 5 °C, for 90 min.

After centrifugal orientation, sample supernatants were aspirated, and aluminum foil substrates, each supporting a single membrane pellet, were removed from the sedimentation cells. Sample pellets were dried for 5–10 min at ambient conditions, mounted onto curved glass supports, and placed in hermetically-sealed, brass or glass containers (for immediate analysis or temporary storage, respectively). All x-ray diffraction experiments were conducted at 20 °C, 74% relative humidity. The latter was established by exposing membrane samples to saturated solutions of L-(+) tartaric acid ($K_2C_4H_4O_6 \cdot ½H_2O$). Samples were incubated at these conditions for at least 1 hr prior to experimental analysis.

Oriented membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic CuK_α x-ray beam ($K_{\alpha1}$ and $K_{\alpha2}$ unresolved; λ = 1.54 Å) produced by a Rigaku Rotaflex RU-200, high-

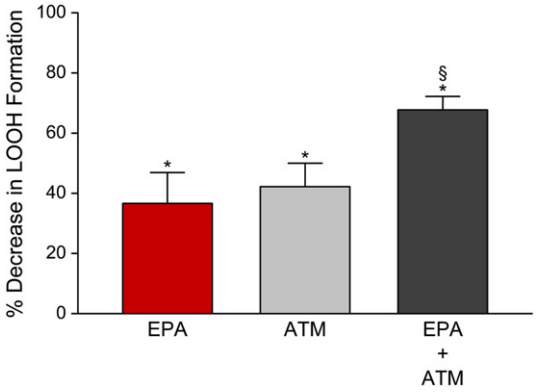


Fig. 3. Separate and combined effects of EPA and atorvastatin *o*-hydroxy (active) metabolite (ATM) on glucose-induced membrane lipid peroxidation. Samples were prepared as previously described and tested for LOOH formation following exposure to oxidative conditions for 96 hr. Values are mean ± S.D. (N = 6) and represent % difference between treatment and glucose-treated controls. $^*p < 0.001$ versus glucose-treated control (Student–Newman–Keuls multiple comparisons test; overall ANOVA: $p < 0.0001$, F = 111.69). $^§p < 0.001$ versus separate EPA or ATM treatments (Student–Newman–Keuls multiple comparisons test; overall ANOVA: $p < 0.0001$, F = 26.635).

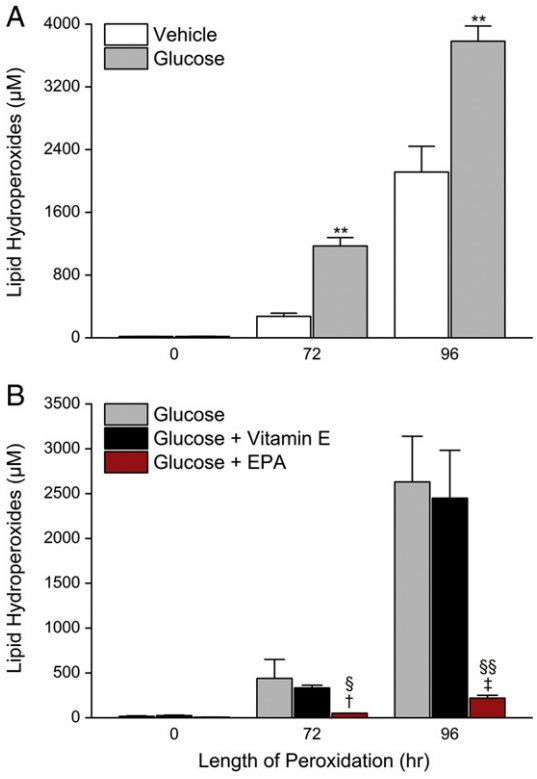


Fig. 2. (A) Effect of glucose on membrane lipid peroxidation. Model membranes were reconstituted from DLPC and cholesterol at a C/P mole ratio of 0.6:1 and prepared in the absence or presence of glucose (200 mg/dL). Values are mean ± S.D. (N = 6). $^{**}p < 0.001$ versus vehicle-treated control (unpaired, two-tailed Student's t-test). (B) Comparative effects of vitamin E and EPA (each at 1:30 drug-to-phospholipid mole ratio) on glucose-induced membrane lipid peroxidation. Values are mean ± S.D. (N = 3). $^†p < 0.05$ and $^‡p < 0.001$ versus glucose treatment alone; $^§p < 0.05$ and $^{§§}p < 0.001$ versus vitamin E treatment (Student–Newman–Keuls multiple comparisons test; overall ANOVA—0 hr data: $p = 0.0073$, F = 12.474; 72 hr data: $p = 0.0204$, F = 7.986; 96 hr data: $p = 0.0008$, F = 29.764).

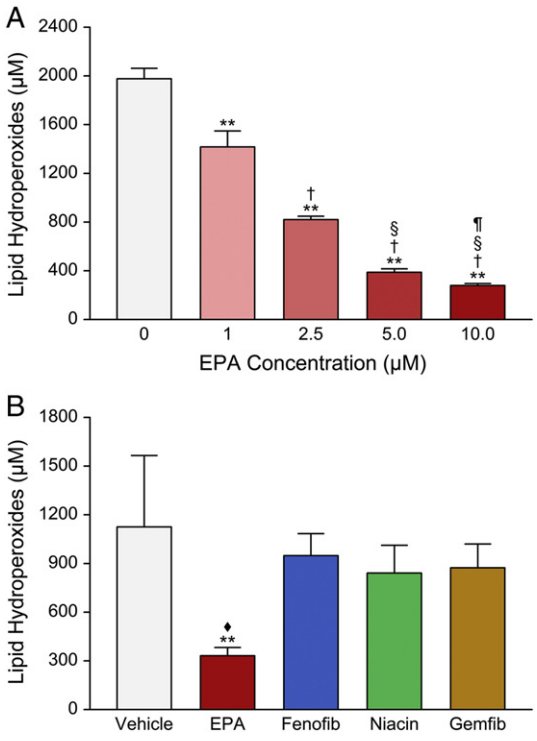


Fig. 4. Effects of EPA on glucose-induced membrane lipid peroxidation examined (A) at various doses and (B) in comparison to Fenofib, Niacin, and Gemfib, each at 10.0 μM. Model membrane samples were reconstituted from DLPC and cholesterol at a C/P mole ratio of 0.6:1, treated with glucose (200 mg/dL), and tested for LOOH formation following exposure to oxidative conditions for 48 hr. Values are mean ± S.D. (N = 6). $^{**}p < 0.001$ versus vehicle-treated control; $^†p < 0.001$ versus 1.0 μM EPA; $^§p < 0.001$ versus 2.5 μM EPA; $^¶p < 0.05$ versus 5.0 μM EPA; $^♦p < 0.001$ versus all other TG-lowering agents (Student–Newman–Keuls multiple comparisons test; overall ANOVA—panel A: $p < 0.0001$, F = 561.62; panel B: $p < 0.0001$, F = 9.940).

brilliance microfocus generator (Rigaku-MSC, The Woodlands, TX) as previously described [34]. Diffraction data were collected on a one-dimensional, position-sensitive electron detector (Hecus X-ray Systems, Graz, Austria) at a sample-to-detector distance of 150 mm. Detector calibration was performed by the manufacturer and verified using crystalline cholesterol monohydrate.

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, $h\lambda = 2\,d\,\sin\theta$, 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 (typically referred to as first- and second-order cholesterol domain peaks) [35]. Under the specific temperature and relative humidity conditions established for these experiments, the second-order, 17 Å cholesterol domain peak was well-delineated from other, neighboring cholesterol and phospholipid diffraction peaks and was thus used to quantitate relative cholesterol domain peak intensity. Routines written in Origin 8.6 (OriginLab Corporation, Northampton, MA) were used to determine total phospholipid peak area against which the second-order cholesterol domain peak was normalized.

2.6. Statistical analyses

Data are presented as mean ± S.D. for (N) separate samples or treatment groups. LDL oxidation analyses were conducted in triplicate. Lipid peroxidation and cholesterol domain peak intensity measurements were conducted in sextuplicate and triplicate, respectively. 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. Effects of EPA versus other TG-lowering agents on LDL oxidation

We tested the antioxidant effects of EPA and the other TG-lowering agents in human LDL as it is an important, lipid-enriched particle that undergoes oxidation in atherosclerosis and other forms of cardiovascular disease. We first examined EPA at 1.0, 2.5, 5.0, and 10.0 μM in human LDL followed by comparison to other TG-lowering agents (fenofibrate, niacin, gemfibrozil) at 10.0 μM (Fig. 1). EPA was observed to inhibit lipid oxidation by 19 ± 8% ($p < 0.001$) at the lowest dose tested (1.0 μM) and by 92 ± 8% ($p < 0.001$) at the highest dose tested (10.0 μM). The IC_{50} for EPA, calculated from these data, is approximately 3.0 μM. The antioxidant effects of EPA were not reproduced by the other TG-lowering agents.

3.2. Comparative effects of EPA and vitamin E on glucose-induced lipid peroxidation

We also tested the comparative effects of EPA and vitamin E (each at a 1:30 drug-to-phospholipid mole ratio) on glucose-induced lipid peroxidation. As shown in Fig. 2, glucose significantly increased LOOH formation in a time-dependent manner as compared to vehicle treatment alone. EPA inhibited the peroxidative effects of glucose by 85% and 91% at 72 and 96 hr, respectively, which was highly significant ($p < 0.001$) as compared to glucose treatment alone. By contrast, vitamin E had no significant effect on lipid peroxidation under identical conditions.

3.3. Separate and combined effects of EPA and ATM on glucose-induced membrane lipid peroxidation

The active metabolite of atorvastatin or ATM has been shown in previous studies to have potent antioxidant properties, as observed in human low-density lipoprotein as well as model liposomes [36,37]. In this study, we re-examined the antioxidant effects of ATM, separately and in combination with EPA (each at 1.0 μM), in membrane lipid vesicles treated with glucose at 200 mg/dL and exposed to oxidative conditions for 96 hr (Fig. 3). Both EPA and ATM were observed to have separate and potent antioxidant effects under these conditions; however, their combination was even more effective, inhibiting LOOH formation by >60% ($p < 0.001$) as compared to either treatment alone.

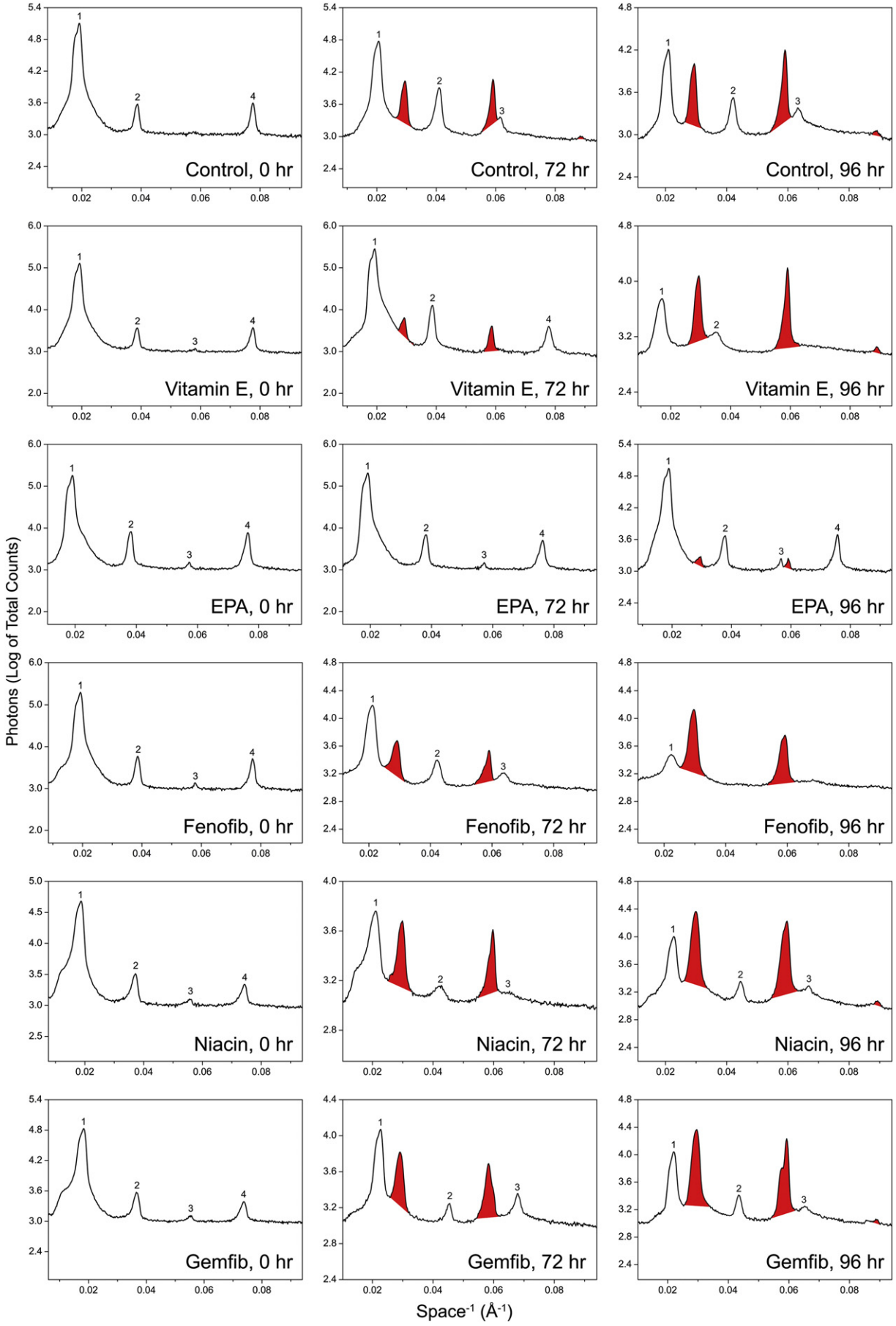
3.4. Effects of EPA versus other TG-lowering agents on glucose-induced lipid peroxidation

Using glucose-treated lipid vesicle preparations, we tested the antioxidant effects of EPA at 1.0, 2.5, 5.0, and 10.0 μM following exposure to oxidative conditions for 48 hr and in comparison to the fenofibrate, niacin, and gemfibrozil, each at 10.0 μM (Fig. 4). As observed in similar LDL experiments, EPA was shown to inhibit lipid oxidation in a highly significant and dose-dependent manner. At the lowest dose tested (1.0 μM), EPA reduced LOOH levels by 28 ± 8% ($p < 0.001$) as compared to vehicle-treated controls; at the highest dose tested (10.0 μM), EPA inhibited lipid oxidation by 86 ± 6% ($p < 0.001$). The IC_{50} for EPA was calculated to be approximately 3.5 μM. The antioxidant effects of EPA were not reproduced by the other TG-lowering agents in this system.

3.5. Effects of EPA, vitamin E, and other TG-lowering agents on glucose- and peroxidation-induced changes in membrane lipid structural organization

Lipid peroxidation is highly disruptive to the structural organization of biological membranes and has been shown, in previous studies, to contribute directly to the formation of cholesterol crystalline domains [4,35]. We have also reported that glucose promotes similar changes in membrane structural organization by increasing lipid peroxidation [3]. In this study, we used small angle x-ray diffraction to characterize the structural properties of model membranes treated with glucose and prepared in the absence or presence of the various test agents (each at 1:30 drug-to-phospholipid mole ratio), before and after exposure to oxidative conditions (Fig. 5). At the start of this experiment, EPA, vitamin E and other TG-lowering agents were observed to have no appreciable effect on membrane structure as compared to control samples. Scattering data collected from each membrane preparation yielded up to four diffraction orders having an average unit cell periodicity (*d*-space) of 51.5 Å, and consistent with a homogenously-distributed, lipid bilayer phase. Following exposure to oxidative conditions for 72 hr, additional peaks, with an average *d*-space value of 34 Å and consistent with a cholesterol crystalline domain phase, were observed in control and vitamin E-treated membrane samples. At 96 hr, cholesterol domains peaks were observed in all experimental samples; however, these peaks were disproportionately greater in control, vitamin E, fibrate, or niacin-treated samples as compared to those treated with EPA.

Quantitative assessment of cholesterol domain peak intensity (expressed as the quotient of cholesterol- to total phospholipid-peak area) indicated that vitamin E and other TG-lowering agents had no significant effect on cholesterol domain formation as compared to control at any experimental time point (Fig. 6). In contrast, EPA inhibited relative cholesterol domain peak intensity by more than 99% at the 96 hr time point, as compared to either vehicle or vitamin E treatments. This inhibitory effect was preserved at EPA concentrations as low as 1:120 drug-to-phospholipid mole ratio, which is equivalent to 10 μM in aqueous suspension (data not shown).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Control, 72 hr
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Vitamin E, 0 hr
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EPA, 0 hr
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Fenofib, 0 hr
Fenofib, 72 hr
Fenofib, 96 hr
Niacin, 0 hr
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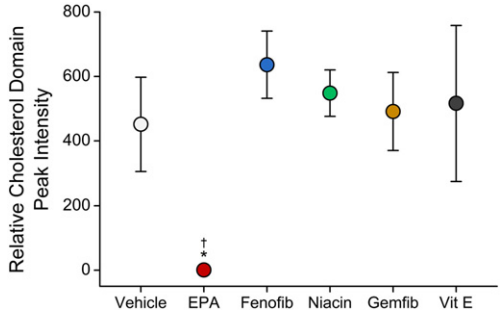

Fig. 6. Quantitative assessment of the comparative effects of vitamin E (Vit E), EPA, Fenofib, Niacin, and Gemfib on cholesterol domain formation in model membranes subjected to glucose-induced lipid peroxidation for 96 hr as shown in Fig. 5. Relative cholesterol peak intensity values were derived by integrating the second-order cholesterol domain peak and normalizing to total phospholipid peak area associated with a given diffraction pattern. Values are mean ± S.D. (N = 3). $^{*}p < 0.01$ versus vehicle-treated control; $^{\dagger}p < 0.01$ versus all other treatments (Student–Newman–Keuls multiple comparisons test; overall ANOVA: $p = 0.0014$, F = 8.229).

4. Discussion

The essential finding from this study is that EPA, at pharmacologically-relevant treatment levels [38], significantly inhibited glucose-induced lipid peroxidation and cholesterol crystalline domain formation in model membrane lipid vesicles. EPA also potently inhibited lipid oxidation in isolated human LDL. These antioxidant effects are attributed to the ability of EPA to quench reactive oxygen species (ROS) associated with the phospholipid membrane, thereby preserving normal lipid structure and organization. Following intercalation into the membrane lipid bilayer, the conjugated double bonds associated with EPA facilitate electron stabilization mechanisms that interfere with free radical propagation (Fig. 7). The effects of EPA could not be reproduced with vitamin E or other FDA-approved, TG–lowering agents. These findings indicate a preferred intercalation of the EPA molecule into the membrane where it can trap free radicals. The absence of activity for vitamin E under these conditions is attributed to its limited lipophilicity and scavenging potential, as previously observed in membranes enriched with cholesterol [35]. Vitamin E was also unable to interfere with cholesterol crystalline domain development with hyperglycemia.

At high levels, glucose promoted the formation of LOOH, prominent intermediates of peroxidative reactions, that lead to changes in the organization of membrane lipid components [39]. The biophysical consequences of lipid peroxidation have been well characterized and include changes in membrane fluidity, increased membrane permeability, and changes in membrane protein activity [40–44]. Oxidative modification of PUFAs also causes a marked reduction in membrane *d*-space (i.e., bilayer width plus surface hydration) associated with interdigitation of the phospholipid acyl chain terminal methyl segments [3,45]. These alterations in the intermolecular packing characteristics of membrane phospholipids promote the displacement of cholesterol into discrete domains (*d*-space of 34 Å) within the phospholipid bilayer environment [4,46]. Cholesterol crystalline domains were previously shown to be induced in model membranes by increasing membrane cholesterol to very high levels (>50 mol%) [46]. Similar changes in cholesterol domain formation have been observed in models of atherosclerosis [6,47,48]. We have also observed the formation of these domains in membranes prepared at constant cholesterol levels but exposed to glucose and glucose-induced lipid peroxidation [3]. Thus, agents that slow or block the aggregation of cholesterol into discrete crystalline domains may interfere with mechanisms of atherogenesis associated with hyperglycemia without otherwise reducing cholesterol levels.

As a reducing monosaccharide, glucose is susceptible to reaction at its anomeric carbon with singlet oxygen or other radical initiators [49]. This redox reaction can generate glucose radicals or other reactive oxygen species that have a pro-oxidant effect in biological membranes. Several reaction mechanisms are responsible for the formation of glycoxidation and lipoxidation products resulting from the reaction of glucose radicals with proteins or lipids to form sugar-amine adducts [50,51].

The presence of cholesterol in the membrane also contributes to rates of LOOH formation, allowing more efficient radical penetration and propagation through the bilayer. The steroid nucleus of cholesterol has an ordering effect on adjacent phospholipid molecules, thus reducing the intermolecular distance between adjacent PUFA chains of the lipids and facilitating the exchange of free radicals within the hydrocarbon core [4]. We previously demonstrated, in similar model membrane preparations, a cholesterol-dependent increase in LOOH formation, which was enhanced by glucose treatment [3]. Evidence suggests that even minor physicochemical modifications to the cell membrane may lead to the disruption of caveolae (cholesterol-enriched membrane domains that are critical to many cellular processes) leading to loss in insulin receptor activity and endothelial nitric oxide synthase (eNOS) function [8,52].

Glucose-mediated oxidative stress contributes to inflammatory pathways associated with diabetes and atherosclerosis pathophysiology [53]. Elevated glucose levels, obesity, and oxidative stress reduce intracellular antioxidant defense mechanisms while activating inflammatory responses from transcription factors and kinases, such as c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and inhibitor of kappa B kinase-β (IKKβ) [54,55]. Some inflammatory pathways, such as activation of IKKβ, have a causative role in the deleterious effects of hyperglycemia on endothelial cell function [56]. Hyperglycemia also stimulates NF-kB, which in turn promotes the overexpression of NADPH, a primary source of cellular superoxide. Overproduction of superoxide, accompanied by increased nitric oxide generation, leads to formation of the highly reactive peroxynitrite molecule.

The results of our findings suggest a novel role for EPA in ameliorating the effects of hyperglycemia through its potent antioxidant properties. In clinical studies, purified EPA reduced CAD-related events in hypercholesterolemic patients receiving statin treatment [14]. EPA treatment has also been shown to reduce triglycerides and other atherogenic lipids, as well as oxidized LDL levels, as compared to placebo [18–20]. These antioxidant effects are consistent with our findings, which demonstrate EPA to be a potent and direct scavenger of free radicals. ROS and related oxidative damage have been implicated in the pathogenesis of various human chronic diseases. Due to its multiple conjugated double bonds, EPA has higher singlet oxygen quenching ability as compared to vitamin E. EPA is expected to fully and efficiently incorporate into the membrane bilayer where its free radical scavenging properties can provide greatest benefit.

The antioxidant effects of EPA were enhanced in combination with the active metabolite of atorvastatin. According to primary pharmacokinetic studies, atorvastatin (parent) is extensively metabolized by hepatic cytochrome P450 to yield active, hydroxylated metabolites, which account for approximately 70% of circulating HMG-CoA reductase inhibitory activity. This is in contrast to other statins like pravastatin and rosuvastatin that are not metabolized into active forms [37]. Beyond their enzymatic effects on serum LDL-C levels, the active metabolites of atorvastatin may provide benefit by interfering with oxidative stress pathways due to the scavenging activity of its phenoxy group [35,57]. In

Fig. 5. Representative x-ray diffraction patterns collected from model membranes prepared in the presence of glucose, treated with vehicle (control), vitamin E, EPA, or other TG-lowering agents (Fenofib, Niacin, Gemfib) and subjected to oxidative conditions for 96 hr. Membranes were reconstituted from DLPC and cholesterol at a C/P mole ratio of 0.6:1 and treated with the various agents to achieve a total drug-to-phospholipid mole ratio of 1:30. At 0 hr, each sample exhibited a single lipid bilayer phase with an average periodicity (*d*-space value) of 51.5 Å, represented by diffraction peaks 1 through 4. At 72 hr, cholesterol crystalline domains, having a characteristic *d*-space value of 34 Å and represented by a set of distinct diffraction peaks (shown in red fill), were also observed in membrane samples treated with vehicle (control), vitamin E, and other TG-lowering agents. At 96 hr, cholesterol domains peaks were observed in all experimental samples; however, these peaks were disproportionately greater in samples treated with vehicle (control), vitamin E, and other TG-lowering agents as compared to EPA.

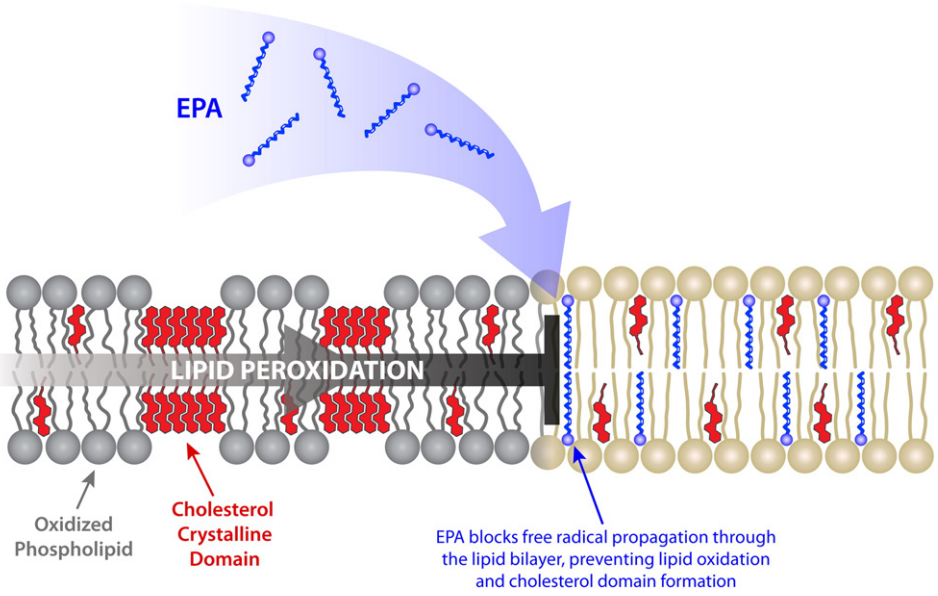

Fig. 7. Schematic illustration of the proposed antioxidant and membrane structural effects of EPA as determined by biochemical and biophysical analysis in this study. EPA is shown to intercalate into the membrane lipid bilayer where it interferes with the propagation of free radicals. The conjugated double bonds associated with EPA are believed to facilitate electron stabilization mechanisms that quench free radical reactions and preserve membrane lipid structural organization.

a small study designed to evaluate the effects of atorvastatin therapy on markers of protein oxidation and inflammation, atorvastatin was found to significantly reduce circulating levels of chlorotyrosine, nitrotyrosine, and dityrosine, all of which act as surrogate markers for specific oxidative pathways upregulated in the atheroma. Interestingly, these effects were observed at a relatively low treatment dose (10 mg, administered for just 12 weeks) and were more significant than reductions in other inflammatory markers, including C-reactive protein [58]. In a larger study involving 2,341 patients, treatment with a high dose of atorvastatin (80 mg) for 16 weeks caused a significant reduction in levels of oxidized lipids associated with all apoB100-containing lipid particles [59].

The ability of EPA to interfere with oxidative stress under conditions of hyperglycemia has important clinical implications. Levels of oxidized lipid, measured using monoclonal antibodies against oxLDL, correlate with the severity of acute coronary syndromes and plaque instability [60]. In a longitudinal investigation of 634 patients, we found that patients with baseline levels of TBARS in the highest quartile had significantly increased relative risk for major vascular events and procedures [61]. The predictive effect of TBARS was observed in a multivariate model adjusted for inflammatory markers (C-reactive protein, sICAM-1, IL-6) and other risk factors (age, LDL-C, HDL-C, total cholesterol, triglycerides, BMI, and blood pressure). These analyses indicated that TBARS had an independent effect on major vascular events and procedures. Similar predictive value was observed for LOOH in these same subjects [62]. The potent antioxidant effects of EPA may account, in part, for the reduction in CV events reported for hypercholesterolemic patients receiving combined EPA and statin treatment as observed in JELIS [14]. In contrast to EPA, the other FDA-approved, TG-lowering agents failed to interfere with oxidative damage to the membrane or cholesterol crystal domain development. As shown in Fig. 6, the addition of the different agents (fenofibrate, gemfibrozil, niacin) was actually associated with an increase in cholesterol domain size. The adverse effects of other approved TG-lowering agents on cholesterol crystal formation may account, in part, for the absence of clinical benefits in randomized trials despite their ability to reduce TG levels (*HPS2-THRIVE*, *FIRST*, *ACCORD*, *AIM-HIGH*, *FIELD*) [24–28]. Its lipophilic structure and molecular space dimensions may allow EPA to insert efficiently into lipoprotein particles and cell lipid membranes as compared to other TG-lowering agents. Additional prospective studies are underway to assess the broader clinical benefits of EPA among high-risk CV patients. One study, the Reduction of Cardiovascular Events with EPA-Intervention Trial (REDUCE-IT; NCT01492361), is investigating the effects of icosapent ethyl (ethyl-EPA) on cardiovascular outcomes in statin-treated, high-risk patients with hypertriglyceridemia.

Acknowledgements

This study was conducted with financial support from Amarin Pharma, Inc. Experimental and technical support was provided by Seth A. Gerard and Samuel C.R. Sherratt.

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