Eicosapentaenoic Acid (EPA) Inhibits Low-Density Lipoprotein Oxidation in a Concentration- and Time-dependent Manner at Pharmacologic Doses In Vitro

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Introduction

Recent cardiovascular (CV) outcome trials have failed to show a benefit with low doses (1 g/day) of omega-3 mixtures (O3FA) containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on top of contemporary medical therapy.^{1,2} Recently, the ASCEND trial tested the effect of O3FA supplementation in 15,480 patients with diabetes but without evidence of atherosclerotic vascular disease (ASCVD).³ The results failed to demonstrate a reduction in first serious vascular events in the O3FA arm. A similar failure was reported in the VITAL study among more than 25,000 patients with low CV risk using again a low dose O3FA.⁴ In contrast to these low-dose O3FA trials, a high dose of highly purified and stable EPA ethyl ester was tested in patients with high CV risk in REDUCE-IT (Reduction of Cardiovascular Events with Icosapent Ethyl – Intervention Trial).

The REDUCE-IT trial was a randomized, double-blinded, placebo-controlled trial designed to examine the benefits of icosapent ethyl, a prescription-grade, highly-purified ethyl ester of EPA.⁵ The objective of this study was to determine if robust EPA treatment (4 g/d) reduces ischemic events in statin-treated patients with elevated and high baseline fasting TG levels (150-499 mg/dL) and elevated risk for clinical cardiovascular events such as diabetes. The primary composite endpoint of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization, or unstable angina reported a highly statistically significant ($p<0.001$) 25% relative risk reduction (RRR) and 4.8% absolute risk reduction (ARR).

While its mechanism of action is not fully understood, EPA has been shown to have various biological effects, including improved endothelial function, reduced inflammation, reduced levels of oxidized LDL (oxLDL), decreased platelet aggregation, and lipid-lowering actions.⁶

Elevated plasma levels of oxLDL are associated with an increased risk for myocardial infarction, metabolic syndrome, and the severity of acute coronary syndromes.^{2,7} EPA has potent antioxidant activity in apoB-containing lipoproteins, including low-density lipoprotein (LDL), that is sustained over time as compared to DHA.⁸ The space dimensions of EPA may allow it to insert efficiently into, and function effectively within, lipoprotein particles (Figure 1).^{8,9} However, the concentration-dependent effects of EPA on LDL oxidation have not been fully elucidated.

Figure 1. Schematic illustration of the protective effects of EPA on LDL lipid oxidation.

Methods

Materials

Eicosapentaenoic acid (EPA; 20:5, n-3) was purchased from Sigma-Aldrich (Saint Louis, MO) and solubilized in ethanol to 1.0 mM under nitrogen atmosphere. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA) and basic reagents were also purchased from Sigma-Aldrich and stored at 4°C.

LDL Isolation and Oxidation

Venous blood was collected from healthy volunteers into vacutainer tubes. Iodixanol density gradient ultracentrifugation was used to isolate LDL from the plasma of healthy volunteers and to adjust to a final apolipoprotein concentration of 2 mg/mL.

An aliquot of LDL (50 μ L) was incubated with either 10 μ L vehicle (ethanol) or 10 μ L of various EPA concentrations (1.0, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5, and 10.0 μ M) for 30 min at 37°C. Oxidation was initiated with addition of CuSO_4 (10.0 μ M) and the extent of oxidation was measured by levels of malondialdehyde (MDA), a reactive aldehyde produced by LDL oxidation.^{10,11}

Calculations and Statistical Analyses

Data are presented as mean $\pm$ standard deviation (SD) for (n) separate samples or experiments. Differences between groups were analyzed using 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

Figure 2. Concentration and Time-dependent Effects of EPA on LDL Oxidation Over a 6 hr Time Course. Results shown as mean $\pm$ SD (N=3).

Figure 3. EPA Inhibits LDL Oxidation in a Nonlinear, Concentration-dependent Manner at 3 hr. Statistical indicators: *** $p<0.001$ vs vehicle; * $p<0.05$ vs vehicle; $\dagger p<0.05$ vs 1.0 μ M; $\ddagger p<0.001$ vs 1.0 and 2.0 μ M; $\S p<0.001$ vs 1.0, 2.0, and 3.0 μ M; $\& p<0.001$ vs 1.0, 2.0, 3.0, and 3.5 μ M; $\# p<0.01$ versus 4.0 μ M; $\& p<0.001$ versus 1.0, 2.0, 3.0, 3.5, and 4.0 μ M (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p<0.0001$, $F = 474.20$). Results are mean $\pm$ SD (N = 3). Results shown as mean $\pm$ SD (N = 3).

Figure 4. At Concentrations ≥ 4.5 μ M, EPA Inhibits LDL Oxidation Over 6 hr. Statistical indicators: *** $p<0.001$ vs vehicle; $\dagger p<0.001$ versus 4.0, 4.5, and 5.0 μ M; $\ddagger p<0.001$ versus 4.0 and 4.5 μ M; $\S p<0.001$ versus 4.0 μ M (Student-Newman-Keuls Multiple Comparisons Test; overall ANOVA: $p<0.0001$, $F = 394.31$). Results are mean $\pm$ SD (N = 3).

Key Findings

These results showed a concentration- and time-dependent antioxidant effect for EPA across a range of micromolar concentrations (1.0 – 10.0 μ M) over 6 hours as shown in **Figures 2 and 3**. Large effect shifts were observed with relatively small concentration shifts, with the critical concentration shifts being time dependent.

Sustained significant antioxidant activity in LDL was only achieved at EPA concentrations ≥ 4.5 μ M through 6 hours (**Figure 4**). Above 7.5 μ M, EPA inhibited LDL oxidation by $>88\%$ ($p<0.001$) at 6 hr, and the half-maximum concentration of LDL inhibition for EPA was 5 μ M (50%, $p<0.001$) at 6 hr.

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Figure 5. LDL oxidation leads to reduced clearance, endothelial dysfunction and plaque instability. This *in vitro* study shows that EPA inhibits LDL oxidation over an extended time at a pharmacologic dose.

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Conclusion

EPA exhibited concentration- and time-dependent antioxidant activity in human LDL that were non-linear and consistent with a “threshold” of activity. The antioxidant benefits were sustained over time only at pharmacologic levels. The antioxidant activity of EPA is attributed to its ability to efficiently intercalate into the monolayer of LDL particles due to its molecular structure. At this location, the ideal combination of hydrocarbon chain length, number of double bonds, and double-bond omega-3 localizations facilitate scavenging of oxygen free radicals, thus preserving the structure and integrity of LDL particles. This benefit of EPA reduces the atherogenicity of LDL while enhancing its clearance (**Figure 5**).