A Wax Ester and Astaxanthin-Rich Extract from the Marine Copepod *Calanus finmarchicus* Attenuates Atherogenesis in Female Apolipoprotein E–Deficient Mice


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Abstract

The aim of this study was to investigate the effect of dietary supplementation with an oil extracted from the zooplankton copepod *Calanus finmarchicus* (calanus oil (CO)) on atherosclerosis in apoE-deficient (apoE<sup>2−/2</sup>) mice. Thirty 6-wk-old female apoE<sup>2−/2</sup> mice (n = 10/group) were fed: 1) a Western-type, high-fat diet (HFD); 2) HFD supplemented with 1% (wt:wt) CO; or 3) HFD supplemented with 0.88% (wt:wt) corn oil + 0.12% (wt:wt) EPA+DHA ethyl esters (EPA+DHA) for 13 wk. Dietary CO supplementation lowered total aorta atherogenesis by 36.5% compared to the HFD (P < 0.01), whereas the reduction in the lesion prone aortic arch was 34.8% (P < 0.01). The degree of aortic atherogenesis was intermediate in mice fed EPA+DHA compared to those fed HFD and CO. The effect on atherogenesis was paralleled by reduced expression of hepatic genes for the proinflammatory cytokines, *Ccl2*, *Icam1*, *Il1b*, and *Nfkb1*, in mice fed CO compared to those fed HFD and CO. For mice fed EPA+DHA, gene expression did not differ compared to those fed CO or HFD. Plasma concentrations of total cholesterol, TG, and cytokines did not differ between the groups at the end of the study. However, mice fed CO gained more weight compared to those fed HFD but not compared to those fed EPA+DHA. In conclusion, dietary CO supplementation attenuated atherosclerotic lesion formation in female apoE<sup>2−/2</sup> mice and may be an effective and safe dietary intervention to reduce the development of atherosclerosis.

Introduction

Atherosclerosis is a disease with a multi-faceted etiology and diet is a major environmental factor influencing the progression of the disease. The effect of dietary long-chain (n-3) PUFA on cardiovascular disease morbidity and mortality has received considerable attention for decades and is generally found to be cardioprotective in humans. However, contrasting effects of marine (n-3) PUFA supplementation have been reported in murine atherosclerosis models and currently there is some controversy about the antiatherogenic effects of fish oil (1).

Marine lipid-rich organisms at lower trophic levels such as krill (*Euphausia superba*) and calanoid copepods (*Calanus spp.*) have recently attracted attention due to their remarkable lipid composition and low levels of pollutants (2). In the North Atlantic, the copepod *Calanus finmarchicus* is the most abundant herbivorous zooplankton (3), with an estimated annual production of 300 million tons in the Norwegian Sea (3). CO<sup>8</sup> is extracted from this resource and is commercially available.

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3 Supplemental Tables 1–4 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
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Traditionally, (n-3) long-chain PUFA have been considered responsible for the beneficial effects associated with dietary intake of seafood and marine oils (4). CO is, however, distinct from other marine oils in that long-chain fatty alcohols are attached to (n-3) PUFA as wax esters. A large proportion of fatty acids are found as monounsaturated 20:1(n-9) and 22:1 (n-11) alcohols (5), which may be oxidized to MUFA during
digestion (6). Beside its large amounts of EPA and DHA, CO is an abundant source of 18:4(n-3) (SDA), which may function as a precursor for biosynthesis of EPA and DHA in mammals (7,8). These (n-3) PUFAs exhibit antiinflammatory effects and prevent blood platelet aggregation (9–11); however, all possible bioactivities associated with SDA remain unclear (8). The antioxidant, antiinflammatory, and antiatherogenic potential of astaxanthin has been extensively investigated in both humans and animal models (12–15). Astaxanthin and the astaxanthin esters, which give CO its characteristic deep red color, provide antioxidant potency and stability to the oil.

The apoE−/− mouse model has proven valuable for determining the role of dietary risk factors in in vivo atherogenesis (16,17). In this study, we evaluated the atheroprotective effects of CO and equal amounts of EPA and DHA ethyl esters (EPA +DHA) from fish oil and compared their putative antiinflammatory and antiatherogenic effects in apoE−/− mice. To our knowledge, this is the first study investigating the effects of dietary CO supplementation in mammals.

Materials and Methods

Animals and housing. Thirty female, 5-wk-old apoE−/− mice (B6.129P2-Apoe−/−m1UncN11) were purchased from Taconic. After 1 wk of acclimation, the mice were ear-marked and randomly allotted to three experimental groups, with equal numbers of cages per treatment. This study was approved by the Norwegian National Animal Research Committee and all experiments were performed following Federation of European Laboratory Animal Science Associations recommendations and according to the Norwegian legislation on the care and use of experimental animals. A health certificate stated that the mice were pathogen free. All mice were housed in the same room at 21°C and 55% relative humidity, on a 12-h-day/night cycle (light on at 0600 h) in a conventional laboratory animal unit. The mice consumed feed ad libitum for 13 wk and cages and bedding were changed once per week.

Diets. The mice were fed a high-fat, Western-type diet (TestDiet) containing 17.4% protein, 20.0% anhydrous milk fat, 1.0% corn oil, 0.2% cholesterol, and no cholic acid. They were divided into 3 groups (10 females/group) that were fed a HFD or a HFD enriched with either 10 g/kg CO (Calanus) or 8.8 g/kg corn oil + 1.2 g/kg Omacor (Pronova BioPharma). The diets are referred to as HFD, CO, and EPA+DHA, respectively (Supplemental Tables 1–3). The amount of Omacor was adjusted to supply the same concentration of (n-3) PUFAs (EPA+DHA) as the CO diet. The diets provided similar amounts of carbohydrates, proteins, cholesterol, and vitamins, whereas the CO and EPA+DHA diets both contained 1% (wt/wt) more fat than the HFD. The amounts of SFA were similar among all diets. The EPA+DHA diet contained more 18:1(n-9) and 18:2(n-6) compared to the other 2 diets, whereas the CO diet contained more 18:4(n-3) than the other diets, and finally, the CO and the EPA+DHA diets both contained 0.5% (wt/wt) EPA+DHA compared to 0.1% EPA+DHA in the HFD. The experimental diets were stored at −20°C and the feed was changed each week.

Protein, fat, moisture, and ash. The contents of protein, water, and ash were determined using the AOAC methods 981.10, 925.04, and 938.08, respectively (18). The fat content of the diets was determined by the AOCs method Ba 3–38 (19) using a Soxhlet extractor (Soxtec System HT6, Tecator).

FA. The FA composition of the diets was determined as previously described (20).

Analysis of total amino acids. For determination of total amino acids, 0.2 g of sample was mixed with 0.2 mL of the internal standard norleucine (20 mmol/L), 0.8 mL distilled water, and 1.2 mL of concentrated HCl and hydrolyzed at 110°C for 24 h (21). Hydrolysates were dried under N2 and redissolved in lithium citrate buffer, pH 2.2, prior to quantification of total amino acids using a Biochrom B30 amino acid analyzer (Biochrom) running the Chromelone software (Dionex). A physiological amino acid standard solution (A9906; Sigma Chemical) was used for calibration.

Analysis of atherosclerosis. After consuming the HFD for 13 wk, the mice were subjected to 3 h daytime feed deprivation and anesthetized by i.p. pentobarbital (80 mg/kg pentobarbital; Nembutal, Abbott Laboratories). Blood was drawn by heart puncture and the whole mouse was perfused through the left ventricle with sterile saline (0.9%) until no residual blood was left in the circulation/major organs. The entire aorta (and its major branches: left and right distal carotid, brachiopheliac, left and right iliac, left and right subclavian arteries) from the proximal ascending aorta to the bifurcation of the iliac arteries was cleaned in situ from the periadventitial tissue, dissected from the aortic arch down to the iliac bifurcation, opened longitudinally, fixed, and prepared as previously described (22). Aorta images were taken using a MOTICAM 1000 camera attached to a MOTIC SMZ-168 TL microscope and evaluated for lesion area using the ImageJ software (23) and calculated as previously described (22).

Plasma cholesterol and TG concentrations. Plasma samples were obtained for total cholesterol and TG determinations at baseline (wk 0) and at the end of the study (wk 13). After 3-h feed deprivation, venous blood was collected from the lateral saphenous vein into Microvette CB 300 LH tubes (Sarstedt). Plasma was isolated by centrifugation at 2000 × g for 5 min at 20°C and stored at −70°C. When thawed, the samples were centrifuged at 1800 × g for 10 min and diluted 1:10 in PBS/BSA (1%), pH 7.4. Enzymatic colorimetric methods on a Cobas-Mira analyzer (Roche Products) were used to measure total cholesterol (Cholesterol PAP Uni-kit; Roche Products) and TG (TAG PAP Uni-kit; Roche Products).

Whole blood FA. Whole blood FA were determined as previously described (24), based on direct collection of a drop of venous blood from a puncture on the lateral saphenous vein, by using the Blood collection kit for the evaluation of FA status in blood (Sigma-Aldrich Chemie).

RNA isolation, cDNA synthesis, and real-time PCR. Total RNA from liver samples was isolated and DNase treated using the PerfectPure RNA Cell and Tissue kit (5 PRIME) according to the manufacturer’s protocol. cDNA was generated using the high capacity cDNA RT kit (Applied Biosystems). Primers and probes were designed using the Beacon design software (Supplemental Table 4). Primer pairs were selected to span exon junctions to avoid coamplification of genomic DNA and the oligonucleotides were purchased from Eurogentec. TaqMan qPCR (40 cycles of 95°C for 15 s and 60°C for 1 min) was performed using TaqMan Universal Fast PCR Master mix (Applied Biosystems) in 96-well plates with the ABI Prism 7900 Sequence Detection system (Applied Biosystems). A dilution series of all samples was used to determine the PCR efficiency of all genes and all assays included no template controls. Relative expression ratios of the target genes were normalized to the stably expressed Gapdh gene and calculations were performed using the relative expression software tool (25).

Plasma cytokines and vascular growth factors. Quantitative measurements of cytokines and vascular growth factors were performed using Luminex xMAP technology. The levels of IL-4, IL-6, IL-8, IL-1β, IL-10, chemokine (C-X-C motif) ligand 1 (mouse IL-8 homolog), monocyte chemoattractant protein-1, macrophage colony-stimulating factor, TNFα, and vascular endothelial growth factor were analyzed using a mouse cytokine multiplex assay (Bio-Rad, Hercules). Plasma samples were thawed on ice, diluted 1:4, and measured in duplicate using the Bio-Plex 200 analytical platform (Bio-Plex 200, Bio-Rad) according to the assay instructions.

Statistical analyses. The results are presented as means ± SEM. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine the distribution of the variables, and non-normally distributed variables were log transformed before statistical analysis. Data were analyzed by ANOVA followed by Tukey’s post hoc test or the Kruskal-Wallis test (SPSS 17.0; SPSS). P < 0.05 was considered significant.
Results

General outcome and changes in body weight. Throughout the experiment, all mice appeared healthy and gained weight regardless of which diet they received. Mice fed CO gained more weight (20.5 ± 1.10 g) compared to mice fed the HFD (16.0 ± 0.76 g) (P < 0.05) but not compared to those fed EPA+DHA (19.1 ± 1.40 g). There were no differences in tissue weights at the end of the study (white adipose tissue, liver, spleen, kidney, or heart) and by visual inspection, all tissues appeared normal.

Plasma total cholesterol and TG concentrations. Plasma total cholesterol (overall 14.9 ± 0.77 mmol/L) and TG (overall 0.88 ± 0.05 mmol/L) concentrations did not differ between the groups at the end of the study.

Whole blood FA composition. The mice fed both the CO and EPA+DHA diets received equal amounts of EPA and DHA, but PUFA incorporation into whole blood differed between all three diet groups (Table 1). Both EPA and DHA were significantly higher in whole blood from the CO-fed mice compared to EPA+DHA-fed mice, whereas whole blood from the EPA+DHA-fed mice had higher levels of EPA and DHA than HFD-fed mice. Whole blood docosapentaenoic acid levels did not differ among the groups. Whole blood levels of linoleic acid [18:2(n-6)] were higher in both the CO and EPA+DHA groups compared to HFD mice. The other major physiologically important (n-6) PUFA, arachidonic acid [20:4(n-6)], did not differ between the groups. Mice fed CO received higher levels of SDA compared to the other two groups, and a GC peak representing SDA was detected in their blood. It was, however, too small to be quantified. No peak representing SDA was identified in whole blood from mice fed EPA+DHA or HFD.

Atherosclerotic lesions. Atherosclerotic lesions were distributed mainly in the aortic arch and the areas surrounding the branching points of the major arteries (Fig. 1). For the complete aortic area, en face-quantified atherosclerotic lesions in the mice receiving CO compared with those fed the HFD, whereas lesion areas in mice fed EPA+DHA were moderately smaller compared to those in HFD-fed mice. Lesion formation was also less in the thoraco-abdominal aorta in CO-fed mice than in HFD-fed mice, whereas mice fed EPA+DHA had an intermediate degree of lesion formation.

Plasma cytokine and vascular growth factors. There were no differences between the groups for any of the quantified cytokines and vascular growth factors [IL-4, IL-6, IL-18, IL-1β, IL-10, chemokine (C-X-C motif) ligand 1 (mouse IL-8 homolog), MCP-1, macrophage-colony stimulating factor, TNFα, or vascular endothelial growth factor] (results not shown). Plasma concentrations of IL-4, IL-6, IL-18, and TNF were all below the detection limits of the chosen assay (results not shown).

Liver gene expression. In mice fed CO, the expression of the inflammatory genes Cc1, Nfkbi, Icam1, and Il1b was lower compared to that in mice fed the HFD (Table 2). Gene expressions in mice fed EPA + DHA were intermediate and did not differ from either group.

Discussion

The present study was designed to compare the effects of dietary supplementation with CO or equal amounts of long-chain (n-3) PUFA from fish oil ethyl esters on atherosclerosis using female apoE−/− mice fed a Western-type HFD. Indeed, dietary CO supplementation for 13 wk significantly reduced atherosclerotic lesion formation. Plasma concentrations of inflammatory cytokines did not change after CO supplementation, but hepatic gene expression of inflammatory cytokines was moderately reduced. Despite the similar intake of EPA and DHA, blood levels of both EPA and DHA were significantly higher in the CO-fed mice compared to those fed EPA+DHA. This indicates that in apoE−/− mice, plasma uptake of (n-3) PUFA may be more efficient from the CO-enriched diet compared with the EPA+DHA ethyl ester-enriched diet.
TABLE 2  Inflammatory response-associated gene expression in livers of female apoE−/− mice fed HFD, CO, or EPA+DHA diets for 13 wk1

<table>
<thead>
<tr>
<th>Gene</th>
<th>HFD</th>
<th>CO</th>
<th>EPA + DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>1.0 ± 0.25a</td>
<td>0.51 ± 0.34a</td>
<td>0.68 ± 0.49a</td>
</tr>
<tr>
<td>Icam1</td>
<td>1.0 ± 0.28a</td>
<td>0.76 ± 0.35a</td>
<td>0.68 ± 0.40a</td>
</tr>
<tr>
<td>Il1b</td>
<td>1.0 ± 0.23a</td>
<td>0.47 ± 0.18a</td>
<td>0.75 ± 0.45a</td>
</tr>
<tr>
<td>Nfkb1</td>
<td>1.0 ± 0.24a</td>
<td>0.64 ± 0.63a</td>
<td>0.72 ± 0.45a</td>
</tr>
<tr>
<td>Pldgbfa</td>
<td>1.0 ± 0.38</td>
<td>0.91 ± 0.33</td>
<td>0.67 ± 0.55</td>
</tr>
<tr>
<td>Selp</td>
<td>1.0 ± 0.34</td>
<td>0.91 ± 0.50</td>
<td>0.69 ± 0.75</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 6–6. Means in a row with superscripts without a common letter differ, P < 0.05. apoE−/−, apoE-deficient; CO, calanus oil; HFD, high-fat diet.
2 The relative amount of each transcript was normalized to Gapdh.

axanthin. Antioxidants have been suggested as potential therapeutic agents against oxidative stress and inflammation, and they have been demonstrated to slow the process of atherosclerosis (26,27). Whereas investigation of the impact of axanthin on atherosclerosis in a rabbit hyperlipidemic atherosclerosis model did not demonstrate any antiatherosclerotic activity or protection against LDL oxidation (14), a recent study demonstrated that an axanthin-rich extract from green algae lowers plasma lipids in mice (15). Recent studies lend further support to a beneficial role of antioxidants in combination with (n-3) PUFA supplementation. Dietary supplementation of a marine (n-3) FA-containing oil in combination with extra virgin olive oil reduced atherogenesis in apoE−/− mice (22), and a mixture of fish oil in combination with the antioxidants lycopene, resveratrol, catechins, and vitamins E and C was recently reported to reduce atherosclerosis in apoE−/− Leiden transgenic mice and to reduce dyslipidemia, oxidative stress, and inflammatory markers in healthy, overweight men (28,29).

Due to their contents of long-chain PUFA, diets rich in seafood are generally recommended to lower the development of atherosclerosis in humans. However, crustaceans contain considerable amounts of cholesterol, and in atherogenesis-prone rabbits fed a diet comprised of ~50% shrimp meal, it was demonstrated that the cholesterol content of the shrimp meal was sufficient to induce cholesterol deposition in the aorta and subsequent development of atherosclerosis (30). CO also contains a notable amount of cholesterol, which was adjusted for in making the diets used in the present study.

It is noteworthy that the mice fed CO and EPA+DHA received 1% (wt:wt) more dietary fat and correspondingly more dietary energy compared to mice fed the HFD. This may explain why the mice supplemented with PUFA in general gained more weight throughout the experiment. This enhanced weight gain was only significant for mice fed CO, which paradoxically was the group with the least aorta lesion formation. However, the major causative factor for atherogenesis in the apoE−/− mice is cholesterol, and cholesterol levels were balanced in the three experimental diets.

Recently, dietary supplementation with crustacean oil has been investigated in humans and animal models (31–36). Krill oil supplementation has been indicated to protect against experimental rheumatoid arthritis in a murine model (34). The herbivorous zooplankton C. finmarchicus is principally free of environmental pollutants due to its position at the bottom of the food chain in the marine environment. Because there is no need for harsh refinement of the CO to remove pollutants, CO includes several other components such as phytosterols and axanthin. These are derived from phytoplankton, which comprises the diet of C. finmarchicus, and these components may have beneficial bioactivities previously not characterized (37). Unlike the common Antarctic krill Euphausia superba, where the (n-3) PUFA are associated with phospholipids (2), most marine zooplanktonic species in the Arctic contain large amounts of wax esters comprised of long-chain unbranched FA and long-chain fatty acids (38). Thus, SDA, EPA, and DHA bound to the fatty alcohols 20:1(n-9) and 22:1(n-11) as monoesters are characteristic of CO. The dietary role of MUFA as protective agents against metabolic syndrome and cardiovascular disease risk factors is being explored (39) and the unique chemical composition of CO certainly contributed to the beneficial effects observed in the present experiment.

The observed development of atherosclerotic lesions in response to the HFD was not accompanied by a substantial production of circulating soluble, acute-phase cytokines such as IL-6 and TNFα, and the plasma concentration of these cytokines did not differ between the groups. We used a relatively low concentration of the dietary supplements corresponding to levels obtainable through dietary supplementation in humans [daily intake of up to 0.1 g (n-3) PUFA/kg body weight]. Higher concentrations of the investigated supplementary marine oils or including more mice may have produced stronger effects.

No adverse effects were observed following 1% dietary enrichment with CO. The CO-supplemented group gained weight as expected and displayed no signs of irritation to the gastrointestinal tract (visual observation when dissecting the mice). The feces seemed normal. As discussed, mice fed a diet enriched with CO and gained more weight compared with those fed the HFD. Although few terrestrial organisms are considered adapted to dietary use of wax esters, several digestive enzymes in terrestrial mammals are capable of hydrolyzing wax esters, and the relative activities of these enzymes may explain adaptive differences in the ability to assimilate energy from wax esters (40,41). The elevated blood EPA and DHA levels found in mice fed CO strongly suggest that mice are able to utilize wax ester both as a dietary substrate and as a pool for the (n-3) FA metabolic pathways. These findings corroborate the growing body of evidence indicating that wax esters from plant-derived food have both nutritional and regulatory effects in humans (42).

In conclusion, the results of the present study demonstrate potent antiatherogenic and antiinflammatory properties of CO. Dietary CO supplementation appears to be an effective and safe treatment to prevent the development of atherosclerosis. Considering the scarce knowledge about the digestion and metabolism of wax esters and fatty acids derived from marine zooplankton such as C. finmarchicus, additional investigations are warranted to further elucidate the biological and molecular mechanisms responsible for the observed effects.

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extracted RNA from the liver samples, and carried out cDNA synthesis and real-time PCR analysis; J.O.O. performed lesson analysis and revised the manuscript; V.B. performed cytokine analyses, participated in data acquisition, and revision of the manuscript; J.B. participated in experimental design and revised the manuscript; E.O.E. conceived the study, participated in experimental design, and revised the manuscript; and B.O.C. conceived the study, participated in experimental design, and revised the manuscript. All authors read and approved the final manuscript.

Literature Cited


