Published in final edited form as:

J Nutr Biochem. 2008 October ; 19(10): 655–663. doi:10.1016/j.jnutbio.2007.08.005.

Echium oil reduces plasma lipids and hepatic lipogenic gene expression in apoB100-only LDL receptor knockout mice^{1,2}

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Abstract

We tested the hypothesis that dietary supplementation with Echium oil (EO), which is enriched in stearidonic acid (SDA; 18:4 n-3), the product of Δ -6 desaturation of 18:3 n-3, will decrease plasma triglyceride (TG) concentrations and result in conversion of SDA to eicosapentaenoic acid (EPA) in the liver. Mildly hypertriglyceridemic mice (apoB100-only LDLr KO) were fed a basal diet containing 10% calories as palm oil (PO) and 0.2% cholesterol for 4 wks, after which they were randomly assigned to experimental diets consisting of the basal diet plus supplementation of 10% of calories as PO, EO, or fish oil (FO) for 8 wks. The EO and FO experimental diets decreased plasma TG and VLDL lipid concentration, and hepatic TG content compared to PO and there was a significant correlation between hepatic TG content and plasma TG concentration among diet groups. EO fed mice had plasma and liver lipid EPA enrichment that was greater than PO fed mice but less than FO fed mice. Down regulation of several genes involved in hepatic TG bio-synthesis was similar for mice fed EO and FO and significantly lower compared to those fed PO. In conclusion, EO may provide a botanical alternative to FO for reduction of plasma TG concentrations.

Keywords

botanical oil; stearidonic acid; polyunsaturated fatty acids; cholesterol; triglyceride

Introduction

Cardiovascular disease (CVD³) is the leading cause of death in the United States and other Westernized societies. Elevated plasma LDL and low HDL concentrations have long been known as important risk factors for development of premature CVD in humans and animal models [1]. Recently, elevated plasma TG concentrations also have been recognized as an independent risk factor for CVD, suggesting that attention should be given to reducing elevated levels of plasma TG in addition to treatment of elevated plasma total cholesterol (TC) and LDL cholesterol [2].

¹This study was supported by NIH grant P50 AT0027820. No conflicts of interest to report.

²Abbreviations: ALA, alpha linolenic acid; CE, cholesteryl ester; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EC, esterified cholesterol; EO, Echium oil; EPA, eicosapentaenoic acid; FO, fish oil, FC, free cholesterol; PL, phospholipids; PO, palm oil; SDA, stearidonic acid; TC, total cholesterol; TG, triglyceride.

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Long chain (≥ 20 carbons) n-3 polyunsaturated (≥ 4 double bonds) fatty acids (PUFA) in fish oil (FO) are among the most effective and widely documented dietary components to reduce CVD risk in human and animal models. A wealth of evidence has accumulated for the efficacy of dietary FO in reducing CVD risk since the initial observation that Greenland Eskimos consuming a high fat diet, containing large amounts of FO, had lower death rates from CVD compared with their counterparts in Denmark [3]. Various animal models have produced similar results including pigs, monkeys, rats, dogs and mice [4–9]. Multiple mechanisms have been proposed for the cardioprotective benefits of FO including reduced blood pressure, decreased thrombosis, decreased arrhythmias, decreased inflammation, decreased endothelial activation, and decreased plasma TG concentration [10]. Indeed, one of the most consistent observations associated with the consumption of FO or purified n-3 PUFA is a reduction in plasma TG levels [11,12]. Thus, increased consumption of n-3 PUFA in FO seems to be an effective way to reduce plasma TG and, presumably, the risk of developing premature CVD.

Despite the well-documented health benefits of n-3 PUFA, the intake of n-6 to n-3 PUFA in the United States is approximately 10:1, with the principle n-3 PUFA consumed being ALA (90% of n-3 PUFA intake) [13]. The recommended intake ratio is 2.3:1 [14]. There are multiple barriers to achieving the recommended n-6 to n-3 PUFA ratio in the American diet. To achieve this by fish consumption alone would require a four-fold increased intake of fatty fish [15]. Given the relatively higher cost of fish to other sources of meat in the American diet and personal preferences in food, this option seems very unlikely. Supplementation of the diet with FO would be another means of increasing n-3 PUFA intake, but this option is unlikely to succeed due to the organoleptic aversion (i.e., fishy aftertaste) to FO supplements. Yet another possibility is to increase the consumption of foods and oils containing ALA, which can go through elongation and desaturation to EPA. However, studies have shown that ALA is poorly converted to EPA in humans and rodents (i.e., 4–15% conversion efficiency) and the degree of conversion depends on the amount of 18:2 in the diet, since 18:2 competes with ALA for $\Delta 6$ -desaturation and diminishes the conversion of ALA to EPA [16–20]. One suggested way to circumvent these problems is to enrich existing foods with EPA, DHA, and ALA using biotechnology, but the introduction of genetically engineered food sources has met stiff social resistance. Finally, an approach that holds promise is to use a botanical oil that is enriched in SDA (18:4 n-3), which is the immediate product of $\Delta 6$ -desaturation of ALA. Since $\Delta 6$ desaturase is the rate limiting step in the formation of EPA from ALA, dietary supplementation with SDA can enrich cellular membranes and plasma lipoproteins with EPA and may result in the beneficial cardiovascular effects of FO without the side effects mentioned above.

Echium oil (EO) from the seeds of *Echium plantagineum* has been identified as a natural source of SDA, which accounts for approximately 13% of total fatty acids in the oil [21,22]. In a recent human study, dietary supplementation of mild-to-moderate hypertriglyceridemic subjects with 15 g EO per day for 4 wk resulted in 21% reduction in serum TG concentrations compared with baseline values. Decreased serum TG levels were associated with a significant increase in long chain n-3 PUFA, including EPA, in plasma and neutrophils in these subjects [21]. Similar enrichment of EPA in plasma and red cells by SDA supplementation has also been reported in another human study [20]. However, the mechanisms for the plasma TG-lowering effects of EO are unknown. Considering the central role of the liver in TG synthesis and secretion, it is likely that dietary EO lowers plasma TG through effects on hepatic gene expression. However, how EO supplementation affects liver fatty acid composition, lipid content, and gene expression are not known and can only be examined in a suitable animal model.

To address the gaps in knowledge on EO supplementation, we performed supplementation experiments using apoB100-only LDLrKO mice. The B100-only LDLrKO mouse is an animal model of atherosclerosis that exhibits a mild elevation in plasma TG concentrations [23]. The

goal of this study was to determine whether EO supplementation would reduce plasma TG concentrations, enrich hepatic lipid fractions with EPA, and alter hepatic expression of genes involved in TG synthesis to a similar extent as that observed with FO supplementation.

MATERIALS AND METHODS

Animals and Diets

The apoB100-only LDLrKO mice in the C57B/L6 background (~93%) were housed in specific pathogen free facility at Wake Forest University School of Medicine. The mice were generated by crossing the apoB100-only mice, originally generated by Dr. Steve Young at the Gladstone Institute, San Francisco, CA [23], with LDLrKO mice in the C57BL/6 background. All protocols and procedures were approved by the Institutional Animal Care and Use committee.

Mice were maintained on a chow diet from weaning until 8 wks of age, at which time, the mice were fed a basal diet containing 10% calories as palm oil (PO) and 0.2% cholesterol (basal diet). A detailed description of similar diets has been previously published [24]. Each diet contains antioxidants to minimize lipid oxidation. After a four week lead in period on the basal diet, the animals were switched to experimental diets that consisted of the basal diet supplemented with an additional 10% of calories as PO, EO or FO for 8 additional weeks. Fatty acid composition of the experimental diets as well as EO is shown in Table 1.

At weeks 0, 2, 4, 6, and 8 of the experimental period, blood was taken from each mouse after a 4- to 6-h fast using a 75- μ l heparanized microhematocrit capillary tube (Fisher Scientific Co) and transferred to a tube containing EDTA at final concentration of 1 μ M. Plasma was promptly isolated by centrifugation at 12,000xg at 4 °C. At the end of the 8 wk experimental period, mice were anesthetized with 50 mg ketamine and 10 mg xylazine/kg body wt and blood was taken from the heart and plasma was isolated as described above. Livers were flushed with PBS to remove excess blood, flash frozen in liquid nitrogen, and stored at -80°C until lipid and mRNA analyses were performed.

Lipid and lipoprotein analysis

Plasma TC (Wako), free cholesterol (FC, Wako), phospholipid (PL, Wako), and TG (Roche) concentrations were determined by enzymatic assays as previously described [25]. Esterified cholesterol (EC) was calculated as the difference between TC and FC. Aliquots of liver were extracted with methanol and chloroform, the extract was dissolved in 1% Triton in chloroform, and TC, FC, TG, and PL mass was quantified by enzymatic assay as described previously [26].

Fresh plasma (200 μ l) was fractionated by fast phase liquid chromatography (FPLC) as described before except that two-30 cm Superose columns in series were used [25]. Fractions were collected and aliquots from each tube were assayed for TC mass to monitor elution position of the plasma lipoproteins. Fractions corresponding to the elution positions of VLDL, LDL and HDL were pooled and aliquots of the isolated lipoprotein pools were used for enzymatic measurement of TC, FC, TG, and PL mass as described for plasma. Cholesterol ester (CE) was calculated as (TC-FC) X 1.67 to account for the fatty acid mass removed during the assay.

Fatty acid analysis

Plasma and liver samples from each diet group were extracted using the Bligh-Dyer method [25]. The lipid extracts from plasma and liver were fractionated into CE, TG and PL bands by TLC using a neutral solvent system. The lipid fractions were methylated and analyzed for fatty acid content by gas-liquid chromatography as described previously [25].

RNA analysis

Total RNA was isolated from liver using the TRIzol™ reagent and reverse transcribed into cDNA as described previously [27]. One µg of total RNA from each liver was taken for analysis. Quantitative real time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) and the $2^{-\Delta\Delta CT}$ method. Primer sequences used for the study are available upon request.

Statistical analysis

The data are reported as mean $\pm$ SEM, unless indicated otherwise, and statistically significant differences among diets were detected by one way ANOVA ($p < 0.05$) using GraphPad prism software. When ANOVA results were statistically significant, individual paired diet comparisons were made using Tukey's post-test analysis.

Results

Plasma lipid response to Echium oil supplementation

Our initial studies were designed to determine how much EO supplementation was necessary to result in a 30–40% reduction in plasma TG in apoB100-only LDLrKO mice as a prelude to future atherosclerosis studies. We started with supplementing the basal diet (10% Cal from PO, 0.2% cholesterol) with an additional 10% Cal as PO, EO or FO for a total of 20% Cal from fat. Body weight and liver weight of mice fed the three experimental diets did not differ significantly at the end of 8 week supplementation period (data not shown). Time-related plasma lipids responses to the different diets are shown in Figure 1. At the end of the four week basal diet period, there was no difference in plasma lipids among three groups for any of the plasma lipid measurements. However, at 2 wk into the experimental phase, dietary supplementation with 10% EO or 10% FO resulted in a significant reduction in TPC, TG, EC, FC and PL compared with 10% PO supplementation. Despite a slight rebound of plasma TG values after 2 wk in the EO-fed mice, a significant 40% lower plasma TG concentration for EO-fed mice was observed at the end of the 8 wk supplementation period compared with PO-fed mice (146 ± 43 vs. 244 ± 61 mg/dl, respectively). FO-fed mice maintained a 58% lower TG level (102 ± 44 mg/dl) from 2–8 wks compared with PO-fed mice. TPC was significantly lower in EO (610 ± 203 mg/dl) and FO (477 ± 141 mg/dl) fed mice compared to those fed PO (1080 ± 443 mg/dl). Similar reductions in EO or FO-fed mice were observed for plasma EC, FC and PL relative to PO-fed mice (Figure 1), but there was no statistically significant difference in any of the lipid measurements between EO and FO-fed mice at the 8 wk time point.

Dietary EO lowers VLDL lipid

At the end of the eight week experimental period, blood was collected from each mouse and plasma was fractionated by FPLC to determine the TG, FC, PL and CE concentration of VLDL, LDL and HDL fractions (Figure 2). EO and FO compared with PO supplementation resulted in significantly lower VLDL TG (12.7 ± 8 and not detectable vs. 91.3 ± 28 mg/dl, respectively), FC (12.5 ± 6 and 1 ± 1 vs. 53.3 ± 15 mg/dl, respectively), PL (47.0 ± 6 and 46.0 ± 14 vs. 129.5 ± 39.1 mg/dl, respectively), and CE (55.5 ± 14.8 and 48.4 ± 13.5 vs. 181.8 ± 61.7 mg/dl, respectively) concentrations (Figure 2, top panel). Significant reductions in LDL FC and PL were also observed for EO and FO supplementation relative to PO (Figure 2, middle panel); CE concentrations followed a similar trend, but did not achieve statistical significance. The concentration of all lipid classes was similar in the HDL fraction for all three diet groups (Figure 2, bottom panel).

Dietary EO lowers liver lipid content

To determine whether EO supplementation affected hepatic lipid storage, livers were extracted at the end of the 8 wk experimental period to measure lipid content (Figure 3). PO-fed mice had the highest liver TG (168 mg/g wet weight) and TC (19.9 mg/g wet weight) content. FO-fed mice had significantly lower TG (63 mg/g wet weight, $p<0.05$) and TC (9.3 mg/g wet weight, $p<0.05$) content, representing a 62% reduction in TG and a 53% reduction in TC. EO-fed mice had a 29% lower liver TG (120 mg/g wet weight) and 36% lower TC (12.7 mg/g wet weight) compared with PO-fed mice, but these values did not reach statistical significance. Free cholesterol and phospholipid content was similar among the three diet groups of mice.

When the liver TG data from individual animals from the three diet groups were plotted against their respective plasma TG values, a significant linear relationship was observed ($r^2=0.56$; $p<0.0001$, Figure 4), suggesting that EO and FO may reduce plasma TG concentrations primarily through a reduction in hepatic synthesis of TG. Alternatively, EO and FO could reduce plasma TG concentrations by increased lipolysis of VLDL TG or increased VLDL particle clearance from plasma.

Dietary EO supplementation results in enrichment of EPA and DHA in plasma and liver lipids

The fatty acid composition of liver and plasma PL, TG and CE was measured at the end of the 8 wk experimental period for mice fed PO, EO and FO. There was little SDA observed in any of the plasma lipid fractions ($<1\%$) and no evidence for enrichment of SDA in plasma lipids of the EO group (Figure 5, top). There was, however, a >10 -fold enrichment of plasma neutral lipids (TG and CE), but not PL, with 18:3 n-3 in the EO group compared with the other two diet groups. This likely was due to the high content of 18:3 n-3 in the EO diet relative to the other diets (Table 1). There was also a relative enrichment of 20:3 n-6 in the plasma PL fraction, and to a lesser extent in TG and CE, in the EO group compared to PO and FO group. This was probably due to the two-fold higher amount of 18:2 and 18:3 n-6 in the EO diet that was available for elongation and/or de-saturation compared to the other two diets (Table 1).

Compared with the PO diet group, EO resulted in a significant enrichment of EPA in all three plasma lipid classes (Figure 5, top). The greatest enrichment was in the plasma TG fraction, in which there was a 23-fold increase in EPA (20:5 n-3), whereas there was an 8–9-fold enrichment in plasma PL and CE fractions in EO vs. PO diet groups. Although the enrichment in plasma TG EPA was quantitatively similar between the EO and FO diet groups (i.e., 23 and 29-fold increase relative to PO), enrichment of EPA in plasma PL and CE fractions in the EO-fed mice was approximately half that of mice fed FO. The EO diet also resulted in a four-fold enrichment of DHA (22:6 n-3) in the plasma TG fraction relative to the PO diet, but there was very little enrichment of DHA in the plasma PL or CE fractions of the EO-fed mice (Figure 5, top).

EO supplementation resulted in liver lipid fatty acid compositional changes that were qualitatively similar to those observed in plasma (Figure 5). Very little SDA was observed in any of the three liver lipid fractions and 18:3 n-3 accumulated in the liver neutral lipid fractions of EO-fed mice (Figure 5, bottom) as observed in plasma (Figure 5, top). Liver lipid EPA content was 4 to 16-fold greater in EO vs. PO-fed mice, but the extent of enrichment was still approximately half that of FO-fed mice (Figure 5, bottom). A six-fold enrichment of DHA was observed in the TG fraction of EO-fed mice compared to the PO-fed mice, similar to the trend observed in the plasma TG fraction.

Dietary EO reduces expression of genes involved in TG and fatty acid synthesis

One way in which EO may reduce plasma TG concentrations is by reducing hepatic TG synthesis and secretion. To test this possibility, we measured the hepatic mRNA abundance of

several genes known to be involved in fatty acid, TG and cholesterol biosynthesis using quantitative real time PCR (Figure 6). The mRNA abundance of SREBP1c, SCD-1, and FAS was significantly lower in livers of mice consuming the EO and FO diets compared to those consuming the PO diet, whereas expression of PPAR α , LXR α , and PGC1 α was similar among all three diet groups. ACC mRNA abundance for the EO and FO groups was lower, on average, than that for the PO group, but the values did not reach statistical significance. There was no difference in expression of HMGCoA synthase or LDL receptor mRNA among the three diet groups. The lack of change in these latter two genes likely resulted from maximum repression of these genes by cholesterol in the diet.

Discussion

The purpose of this study was to determine whether EO can serve as a botanical source of n-3 dietary PUFA to enrich tissues in EPA and result in some of the cardioprotective effects that have been documented for FO. One of the better documented effects of dietary FO is a reduction in plasma TG concentrations. Indeed, in our study using a mouse model of atherosclerosis with mildly elevated plasma TG concentrations [23,28], we observed a significant reduction in plasma TG values for EO-fed mice compared with PO-fed mice that was similar to the TG reduction obtained with FO. Plasma TC, EC, FC and PL were also reduced with EO compared to PO supplementation. Of the three lipoprotein classes, EO had the greatest effect on VLDL lipid concentration, with significant reductions in plasma concentration of all lipid classes, suggesting the hepatic secretion of VLDL lipid might be reduced with EO consumption. Supporting this notion, the average content of hepatic TG and TC was lower in EO vs. PO-fed mice and several hepatic genes involved in fatty acid and TG biosynthesis were significantly down regulated with EO and FO consumption. In addition, there was a significant positive association between hepatic TG content and plasma TG concentration, suggesting that down regulation of hepatic fatty acid and TG synthetic genes was responsible, in part, for the reduced plasma TG concentrations of EO and FO-fed mice through reduced hepatic TG synthesis and secretion in VLDL particles. Finally, EO supplementation resulted in significant enrichment of plasma and liver neutral lipid fractions with EPA and plasma and liver TG with DHA, though not to the extent achieved with FO. Thus, dietary EO resulted in a reduction of plasma lipids that was similar to that of FO with only half the enrichment of plasma and liver lipid fractions with EPA and DHA. These results suggest that dietary supplementation with EO may represent a botanical alternative to FO to reduce the risk of premature atherosclerosis.

Consumption of fatty fish or FO supplements is associated with many cardioprotective effects [11,29]. The cardioprotective ingredient in FO is believed to be highly unsaturated fatty acids (≥ 5 double bonds) with carbon chains ≥ 20 , such as EPA and DHA. Although the benefits of EPA and DHA are well-recognized, dietary consumption of these PUFAs in the US population is far below that needed for cardiovascular benefit [30]. As a result, attempts have been made to find dietary botanical sources enriched in n-3 PUFA that might function to increase cellular EPA and DHA content and provide cardioprotective benefit. There was initial hope that dietary botanical oils enriched in ALA might result in enrichment of tissues with EPA and DHA through fatty acid elongation and desaturation pathways. However, this approach proved to be disappointing because the Δ -6 desaturase reaction is rate limiting and results in only a small fraction of dietary ALA being converted to EPA (4 to 15 %, refs [17,18,31]). Several groups have used dietary supplementation with SDA or EO, which is relatively enriched in SDA, as an alternative to FO because SDA is the immediate product of Δ -6 desaturation of ALA and SDA supplementation results in significant enrichment of plasma and blood cells with EPA. In one study with mildly hypertriglyceridemic subjects, EO supplementation was shown to lower plasma TG concentrations, which is a consistently observed effect of FO, EPA and/or DHA supplementation in humans [21]. However, no study to date has examined the role of EO supplementation on hepatic lipid phenotype or gene expression. Given the central role of

the liver in TG production and energy partitioning in the body, this represents a fundamental gap in knowledge.

Several lines of evidence support the role of n-3 PUFAs in decreasing hepatic TG synthesis and secretion in VLDL. Several kinetic studies in humans demonstrated that dietary n-3 PUFAs decreased VLDL TG synthesis and secretion (reviewed in [29]). Liver perfusion studies in rats and non-human primates demonstrated that hepatic TG secretion, but not apoB secretion in the non-human primate study, was decreased with n-3 PUFA consumption compared to the control fat [32,33]. Several mechanisms for the decrease in hepatic TG synthesis and secretion are supported by experimental data. N-3 PUFA decreases expression of lipogenic genes, such as fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), through the decreased expression of the nuclear form of sterol regulatory element binding protein-1c (SREBP-1c) [34–38]. Part of the decreased expression appears related to accelerated SREBP-1c mRNA decay [39]. Another reported mechanism for decreased TG secretion with n-3 PUFA consumption is increased fatty acid oxidation, likely mediated by peroxisome proliferator activated receptor α (PPAR α), a nuclear transcription factor that is activated by fatty acid binding [34]. However, the latter mechanism is not rate limiting in vivo since FO reduced plasma TG concentrations in PPAR α knockout mice [40]. N-3 PUFA also downregulates fatty acid desaturation pathways in the liver (i.e., $\Delta 5$, $\Delta 6$, SCD-1) via SREBP-1c mediated transcriptional control [41,42]. Taken together, these studies suggest a multifaceted control of TG synthesis and secretion by n-3 PUFA in the liver and that dietary n-3 PUFA lowers plasma TG concentrations, in part, by decreased hepatic VLDL TG synthesis and secretion. Whether these mechanisms are responsible for the lower plasma TG concentrations in mice fed EO will require additional studies.

Another potential mechanism for plasma TG reduction by n-3 PUFA is increased TG-enriched lipoprotein catabolism. VLDL turnover studies in human subjects suggest a trend towards faster catabolic rates in those given n-3 PUFA [29]. Chylomicron lipid removal from plasma was also faster in n-3 PUFA-fed subjects compared with olive oil [43,44]. Post-heparin lipase activity, consisting of LPL and HL, in human subjects has not been particularly responsive to dietary n-3 PUFA [45,46], although increased LPL activity was observed several studies [47, 48]. However, the rate of hydrolysis of TG-enriched lipoproteins may be greater when TG is enriched with n-3 PUFA [49,50]. Another study demonstrated increased hepatic clearance of IDL/LDL in LDLrKO mice with a hepatic specific deletion of the LDL related protein (LRP) receptor, suggesting an increased removal of apoB lipoproteins by the liver by an LDLr and LRP-independent pathway [51]. The results from these studies collectively suggest that part of the plasma TG lowering effect of n-3 PUFA is related to increased apoB lipoprotein catabolism and this may also be the case with EO supplementation.

In summary, we have identified EO a dietary botanical oil that may function to reduce plasma TG concentrations and the development of cardiovascular disease by providing an enriched source of SDA that can be converted to EPA and DHA, providing similar health benefits as FO, but presumably without the side effects and low compliance of FO. A combination of EO with other sources of n-3 PUFAs, such as fatty fish, would aid in improving the intake of n-3 relative to n-6 PUFA in the general population. The results from this study suggest a relatively simple dietary modification that may have a high impact on reduction of premature cardiovascular disease in the American population.

Acknowledgments

The authors gratefully acknowledge Bioriginal Food and Science Corporation for supplying Echium oil of the study.

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Fig. 1. Plasma lipid response to dietary supplementation with PO, EO, or FO

Eight week old chow-fed apoB100-only LDLrKO mice were fed a basal 10% PO diet for four weeks, after which they were randomly assigned to an experimental diet consisting of the basal diet plus an additional 10% of calories as PO (20% PO; n=8), EO (10% PO + 10% EO; n=8) or FO (10% PO + 10% FO; n=9) for an additional 8 weeks. Mice were bled at the end of the basal diet period (0 wk of experimental phase) and every two weeks during the experimental diet period for measurement of total plasma cholesterol (TPC), triglyceride (TG), esterified cholesterol (EC), free cholesterol (FC), and phospholipid (PL) concentrations. Values are mean $\pm$ SEM.

Fig. 2. Plasma lipoprotein lipid concentration

Three groups of apoB100-only LDLr KO mice were fed diets containing PO, EO and FO as described in the Methods. At the end of the 8 week experimental diet period, plasma was fractionated by FPLC and lipoprotein elution was monitored by enzymatic cholesterol assay. Fractions corresponding to the elution position of VLDL, LDL, and HDL were pooled and assayed for TC, FC, PL and CE using enzymatic assays. Cholesterol ester content was calculated as total cholesterol – free cholesterol) $\times 1.67$. Data represent mean $\pm$ SEM for 6 mice in the PO and EO groups and 8 mice in the FO group. Bars with different letters denote significant differences among diet groups ($p < 0.05$).

Fig. 3. Liver lipid content

Mice were fed experimental diets containing PO, EO, or FO for 8 weeks before livers were harvested for extraction and measurement of lipid content by enzymatic assays. Bars represent mean $\pm$ SEM; $n=8$ for PO and EO, $n=9$ for FO. Values with different letters are significantly different for one another ($p<0.05$).

Fig. 4. Association between plasma TG and hepatic TG content

Mice were fed experimental diets containing PO, EO, or FO for 8 weeks before plasma and livers were harvested for TG mass measurement. The plot of plasma TG concentration vs. hepatic TG content shows the line of best fit ($r^2=0.56$) determined by linear regression analysis with GraphPad Prism software. Each data point represents an individual animal in one of the three designated diet groups.

Fig. 5. Liver and plasma lipid percentage fatty acid composition

Mice were fed experimental diets containing PO, EO, or FO for 8 weeks before plasma and livers were harvested for measurement of PL, TG and CE fatty composition. Lipids from plasma and liver were extracted and separated by TLC into PL, TG and CE bands that were visualized with premulin and scraped for fatty acid analysis as described in the Methods section. Data represent mean $\pm$ SEM; $n=5-8$ for each group. Values with different letters are significantly different ($p<0.05$) by ANOVA; data bars not marked with letters were not significantly different. Only the polyunsaturated fatty acids (≥ 2 double bonds) are shown in the figure.

Fig. 6. Hepatic gene expression

Mice were fed experimental diets containing PO, EO, or FO for 8 weeks before livers were harvested for measurement of gene expression by quantitative real time PCR. Liver RNA was isolated using TRIzol from individual mice and quantified for the indicated genes. Values represent mean $\pm$ SEM; $n=7-9$ per diet group. Values with different letters are significantly different for one another ($p<0.05$) for each gene. SREBP1c= sterol regulatory element binding protein 1c; ACC=acetyl CoA carboxylase; SCD-1=stearoyl CoA desaturase-1; FAS=fatty acid synthase; HMGCoA syn= hydroxymethylglutaryl CoA syntase; LDLr=low density lipoprotein receptor; LXR=live X receptor α ; PGC1 α =peroxisome proliferator-activated receptor γ , coactivator 1 and PPAR α =peroxisome proliferator-activated receptor α .

Table 1Fatty acid composition of experimental diets and Echium oil¹

Fatty acid	20% PO	10%PO+10%EO	10%PO+10%FO	EO
14:0	1.6	1.0	5.1	ND
16:0	43.8	25.1	34.4	6.7
16:1	ND	ND	6.0	ND
18:0	4.5	3.9	4.2	3.8
18:1	36.3	25.9	27.7	14.9
18:2	12.8	15.5	9.7	15.1
18:3 n-6	ND	5.4	ND	11
18:3 n-3	0.8	16.6	ND	32.3
18:4 n-3	ND	6.7	1.5	12.9
20:5 n-3	ND	ND	5.7	ND
22:6 n-3	ND	ND	4.4	ND

¹ Values represent averages for duplicate determinations of 2 diet preparations. All diets contain 20% Cal as fat. Only major fatty acids are listed. PO, palm oil; EO, Echium oil; FO, fish oil. ND, not detected.