Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice.

Anticoagulant Effect of Dietary Fish Oil in Hyperlipidemia
A Study of Hepatic Gene Expression in APOE2 Knock-in Mice

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Objective—In hyperlipidemia, dietary fish oil containing n-3 polyunsaturated fatty acids (PUFA) provokes plasma triacylglycerol lowering and hypocoagulant activity. Using APOE2 knock-in mice, the relation of these fish-oil effects with altered gene expression was investigated.

Methods and Results—Male APOE2 knock-in mice, fed regular low-fat diet, had elevated plasma levels of triacylglycerol and coagulation factors. Plasma lipids and (anti)coagulant factors reduced on feeding the mice with fish oil (n-3 PUFA) or, to a lesser degree, with sunflowerseed oil (n-6 PUFA). The fish-oil diet provoked a 40% reduction in thrombin generation. Microarray (Affymetrix) and single-gene expression analysis of mouse livers showed that fish oil induced: (1) upregulation of genes contributing to lipid degradation and oxidation; (2) downregulation of genes of γ-glutamyl carboxylase and of transcription factors implicated in lipid synthesis; (3) unchanged expression of coagulation factor genes. After fish-oil diet, vitamin K–dependent coagulation factors accumulated in periportal areas of the liver; prothrombin was partly retained in uncarboxylated form. Only part of the changes in gene expression were different from the effects of sunflowerseed oil diet.

Conclusions—The hypocoagulant effect of n-3 PUFA is not caused by reduced hepatic synthesis of coagulation factors, but rather results from retention of uncarboxylated coagulation factors. In contrast, the lipid-lowering effect of n-3 PUFA links to altered expression of genes that regulate transcription and fatty acid metabolism. (***Arterioscler Thromb Vasc Biol*. 2008;28:2023-2029**)

Key Words: APOE2 mice ■ coagulation ■ fish oil ■ gene expression profiles ■ thrombin

Both observational studies and clinical trials point to a close association between the consumption of fatty fish and a reduction in the risk of coronary heart disease. Considering that the main active components of fish oil are the n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic and docosahexaenoic acid, this has led to the recommendation of a daily intake of n-3 PUFA to prevent cardiovascular disease, particularly in the case of hypertriglyceridemia.[1] Although reduction in plasma level of triacylglycerol is one of the most consistent effects of regular fish-oil intake, in many studies this effect is accompanied by a diminished coagulant activity. The latter was observed as a lowering of several coagulation factors as well as a reduced capability of plasma to support thrombin generation.[2–4] How these seemingly different ways of action of n-3 PUFA are connected is presently unknown.

Studies using plasmas from healthy subjects have pointed to an unexpected correlation between the levels of triacylglycerol and those of a number of coagulation factors.[5–7] Because this all concerns factors which are produced by the liver, it is possible that the levels in plasma are controlled by a common hepatic factor or pathway. An attractive hypothesis is that fish oil modulates an hepatic transcriptional or translational process, which controls the production or release of triacylglycerol as well as coagulation factors.

In experimental animals, dietary n-3 PUFA similarly evoke a decrease in triacylglycerol and coagulation factor levels. From studies with rats, it has been suggested that dietary fish oil reduces vitamin K uptake in the liver and, hence, restricts the extent of vitamin K–dependent γ-glutamyl carboxylation of Gla-containing coagulation factors.[8,9] However, this proposal was challenged by the observation that fish oil also reduces the plasma levels of vitamin K–*in*dependent factors, such as fibrinogen and factor V.[10,11] Also because the normal ingestion of vitamin K is far above rate-limiting, it is unlikely that the anticoagulant effect of fish oil is attributable to insufficient vitamin K delivery.

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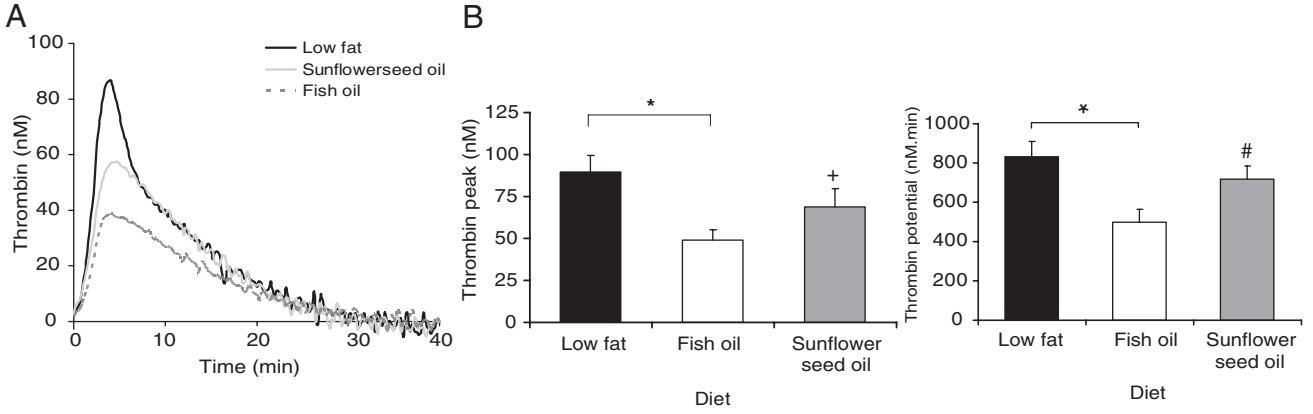

Figure 1. Hypocoagulant effect of fish oil diet in APOE2 ki mice. Mice were fed with low-fat, fish oil or sunflowerseed oil diet, as indicated. A, Representative thrombin generation traces of platelet-free plasma triggered with tissue factor. B, Averaged thrombin peak height and endogenous thrombin potential, as obtained from thrombin generation traces. Mean±SEM (n=7 to 10); *$P<0.05$ vs low-fat; +$P<0.1$, #$P<0.05$ vs fish oil.

In the present article, we investigated whether the lipid-lowering and anticoagulant effects of dietary n-3 PUFA are linked by a joint (post)transcriptional mechanism. We used a murine model of type III hyperlipidemia, ie, apolipoprotein E2 (APOE2) knock-in mice, where the endogenous murine *apoe* gene has been replaced by the human APOE2 gene.[12] In man, the APOE2 polymorphism encodes for an ApoE protein form with greatly reduced binding affinity to the low-density lipoprotein receptors, when compared to the common APOE3 polymorphism.[13] By using APOE2 knock-in mice, we first established the effect of fish oil (n-3 PUFA) on plasma lipid and coagulation parameters. Using microarray technology, we then evaluated the diet-induced changes in hepatic gene expression profile. In this study, sunflowerseed oil, containing only n-6 PUFA, served as reference diet.

Materials and Methods

Male APOE2 knock-in mice (C57BL/6 background)[12] were fed with either a low-fat chow diet (low-fat group), a diet rich in n-3 PUFA (fish oil group), or a reference diet consisting of n-6 PUFA (sunflowerseed oil group). Blood and livers collected from the mice were used for analysis of lipid and coagulation parameters. mRNA isolated from livers was used for microarray expression analysis (Affymetrix Gene Chips) and real-time quantitative polymerase chain reaction (PCR). Experiments were approved by the local Animal Ethics Committee. For additional information, see the online supplement, available at http://atvb.ahajournals.org.

Results

Effects of Fish Oil and Sunflowerseed Oil Diets on Plasma Triacylglycerol and Coagulant Activity in APOE2 Knock-In Mice

On feeding with standard low-fat diet, male APOE2 knock-in mice at a C57BL/6 genetic background had relatively high fasting levels of plasma triacylglycerol, ie, 143±19 mg/dL (mean±SEM, n=7). In comparison, male wild-type C57BL/6 mice fed with the same diet had substantially lower triacylglycerol levels, ie, 82.6±5.7 mg/dL (n=10, $P=0.01$ versus APOE2 knock-in). Similar differences have been reported before.[12–14] This confirms the suitability of transgenic APOE2 mice as a model for hypertriglyceridemia.

Strikingly, in comparison to wild types, APOE2 mice also had 1.6- to 1.9-fold increased levels of (anti)coagulant plasma proteins, including the vitamin K–dependent γ-glutamyl carboxylated coagulation factors (prothrombin and factors VII, X), the vitamin K–independent factor V, and the anticoagulant protein antithrombin (supplemental Figure I). Fibrinogen in plasma was not increased. Platelet-free plasma from these mice was used to determine thrombin generation, as an integrated measure of coagulant activity.[15–17] At optimal concentrations of tissue factor and phospholipids, thrombin generation curves were about twice as high for APOE2 mice as for wild-type mice. Together, this indicates that, also in the knock-in mice, hyperlipidemia associates with hypercoagulant activity.

Earlier studies with rats have shown that dietary n-3 PUFA provoke a coagulation-lowering effect.[11,18] This was tested for APO2E knock-in mice held on standard, low-fat diet. One group of the mice was kept on the same diet (low-fat group), while another group of mice received a diet enriched in fish oil with n-3 PUFA (fish oil group), and a third, reference group received sunflowerseed oil, containing only n-6 PUFA (supplemental Table I). After 21 days of feeding, the fish oil but not sunflowerseed oil diet provoked a 30% reduction in plasma triacylglycerol, when compared to the mice kept on low fat (supplemental Table II). Fish oil diet further caused a 29% to 47% reduction in vitamin K–dependent (prothrombin, factors VII, X) and vitamin K-independent (factor V) coagulation factors; it had a borderline reducing effect on fibrinogen. Fish oil furthermore reduced the levels of the anticoagulant factors, antithrombin (−38%) and protein C (−21%). Hence, after the dietary period, most factor levels approached those of wild-type C57BL/6 plasma. Also feeding with sunflowerseed oil diet reduced coagulation factors levels, but at a lesser degree (−9 to −19%). Plasma concentrations of prothrombin and factors VII and X remained higher than in the fish-oil group (supplemental Table II). Sunflowerseed oil diet though strongly reduced the antithrombin concentration (−38%).

Measurement of thrombin generation in plasma showed, after fish-oil diet, a 40% to 45% reduction in thrombin peak height and endogenous thrombin potential (Figure 1). This

Table. Overview of Effects of Diets on Hepatic mRNA Expression by Microarray Analysis

Metabolic Pathway	ΔFO (Fold Change)	ΔSSO (Fold Change)
Lipid degradation and oxidation (n=15)	1.30±0.06*	1.00±0.06#
Electron transport (n=26–28)	1.32±0.11*	1.06±0.07#
Lipid synthesis and transport (n=13–15)	0.62±0.10*	0.70±0.09*
Coagulation and anticoagulation (n=18)	1.04±0.06	1.00±0.05

Genes are grouped according to 4 characteristic metabolic pathways. Indicated are expression changes between fish oil (ΔFO) and sunflowerseed oil (ΔSSO) groups in comparison to low-fat group. For individual genes, see supplemental Table III. Data are average fold-changes of gene expression. Mean±SEM (n genes); *$P<0.01$ vs low-fat, #$P<0.05$ vs fish oil.

was different from the insignificant effect of sunflowerseed oil. For all dietary groups together, the thrombin peak height closely correlated with the prothrombin level ($R^2=0.24$, $P<0.05$), whereas the levels of other coagulation factors covaried. Apparently, the n-3 PUFA diet reversed the hypercoagulant phenotype of APOE2 knock-in mice.

Effects of Fish Oil and Sunflowerseed Oil Diets on Gene Expression in Mouse Liver

Considering that the liver is a common source of plasma triacylglycerol and (anti)coagulation factors, expression patterns of mRNA were determined in liver lobes that were collected after the dietary intervention, using microarray chip technology. Pools were made of total RNA isolated from 4 livers per diet group to limit effects of individual variation. Thus, 2 pools were created from livers of mice fed low-fat (baseline pools LF1 and LF2), 2 pools of mice fed fish oil (pools FO1 and FO2), and 1 pool of the reference sunflowerseed oil group (pool SSO1). Pair-wise comparison of the expression signals of all 22 690 sequences on the Affymetrix chips pointed to good reproducibility for the LF1/LF2 ($R=0.99$) and FO1/FO2 ($R=0.98$) pools (supplemental Figure I). Per microarray, 7891 to 8610 sequences (35% to 38%) were expressed at significant signal intensity ($P<0.05$), and these were further analyzed. When comparing the two FO and LF pools, 537 to 737 (5%) of the sequences showed a difference in expression, whereas the comparison of SSO1 and LF pools gave 398 to 425 (3.1%) different sequences (supplemental Figure II).

Using Affymetrix software, 1596 of differentially expressed sequences were related to specific metabolic pathways or biological functions. Comparison of the signal ratios of FO/LF and SSO/LF pools suggested that the fish oil diet had a positive effect on the expression of genes involved in lipid degradation/oxidation and electron transport, whereas the fish oil and sunflowerseed oil diets negatively influenced the group of genes involved in lipid synthesis and transport (Table; for details, see supplemental Table III). The semiquantitative microarray analysis did not point to marked diet-induced changes in expression of genes of (anti)coagulation factors. With respect to transcription factors linked to lipid metabolism, the gene of nuclear factor I/X tended to be higher expressed in response to fish oil and sunflowerseed oil, whereas the genes of retinoblastoma binding protein 4 and sterol regulatory element binding factor 1 might be upregulated by sunflowerseed oil only (supplemental Table III). Fish oil diet may negatively affect the genes of peroxisome proliferator-activated receptor (PPAR) α and of nuclear factor E2 p45-related factor 2 (Nrf2, produced by Nfe2l2 gene).

Guided by the microarray analysis, the mRNA samples from individual mouse livers were analyzed by real-time PCR to obtain stronger quantitative data (supplemental Table IV). Concerning transcription factors, real-time PCR demonstrated a reduced expression of PPAR α, δ and γ after fish oil diet, but this effect was not different from the effect of sunflowerseed oil (Figure 2). In agreement with the microarray data, fish oil diet more potently reduced the expression of NFe2l2 (Figure 2, black bar).

Quantitative PCR further pointed to a reduction by fish oil in mRNA of the anticoagulant factors, tissue factor pathway inhibitor, antithrombin, protein C and protein Z, in comparison to the low-fed group (Figure 2). Here, data of the fish oil and sunflowerseed oil groups did not differ significantly. A key observation was that only fish oil suppressed the gene expression of γ-glutamyl carboxylase, which enzyme is responsible for the formation of Gla residues of vitamin K–dependent factors. On the other hand, with the exception of fibrinogen β-chain, neither fish oil nor sunflowerseed oil diet affected the messenger levels of coagulation factors (prothrombin and factors V, VII, X).

Two genes regulating fatty acid synthesis were significantly downregulated by the PUFA diets, eg, stearoyl-coenzyme A (CoA) desaturase and fatty acid synthase (Figure 2). The latter gene was not influenced by sunflowerseed oil. On the other hand, mRNA levels of key enzymes of lipid degradation and oxidation were upregulated by the fish oil diet (black bars). These included: long-chain fatty acid CoA ligase 2, carnitine palmitoyltransferase, acyl-CoA oxidase, very long chain acyl-CoA dehydrogenase, and acetyl-CoA acyltransferase. Overall, the quantitative PCR data correlated well with the microarray results (supplemental Figure III).

Effects of Fish and Sunflowerseed Oil Diets on Coagulation Factor Proteins in Liver

The results so far suggested that fish oil reduced the plasma concentrations of vitamin K–dependent coagulation factors without affecting mRNA of these factors in the liver. To study this in more detail, coagulation factor proteins were determined in lysates from all mouse livers. Chromogenic kinetic assays were used that measure the total amounts of prothrombin and factor X (ie, uncarboxylated plus carboxylated Gla-containing forms). As shown in Figure 3A, total levels of prothrombin and factor Xa were similar in the livers from all three diet groups (Figure 3A), such in agreement with the mRNA data.

Pooled liver lysates were subjected to a concentration step by barium chloride precipitation, after which the precipitates were resolved by gel electrophoresis and Western blotting. Staining of the blots for prothrombin resulted in two bands with molecular weights around 75 kDa in case of the fish oil

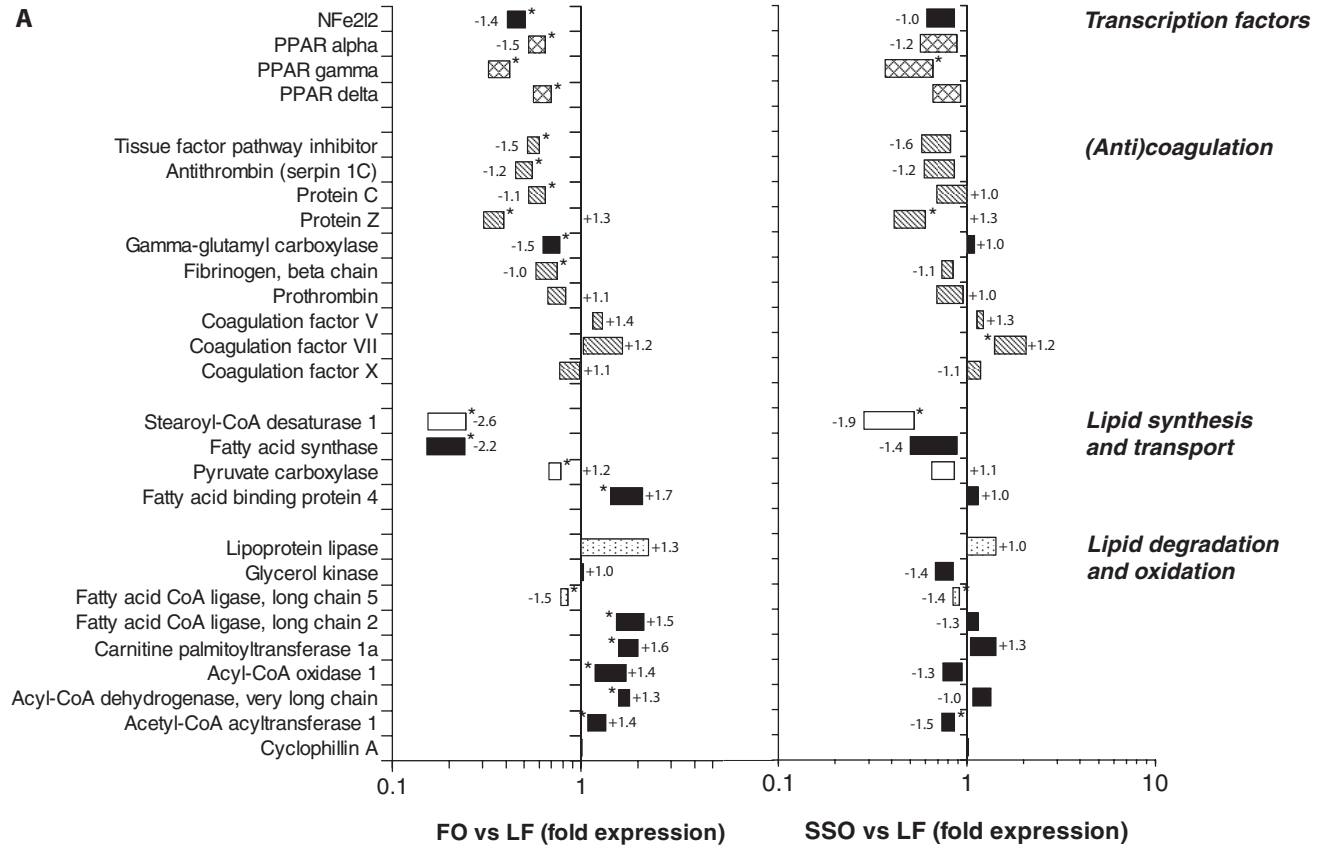

Figure 2. Diet effects on mRNA expression of individual genes in liver. Gene expression was determined by real-time quantitative PCR, and normalized to expression of cyclophyllin A mRNA. A, Fold expression changes of fish oil (FO) and sunflowerseed oil (SSO) groups relative to low fat (LF) group per indicated gene. Mean±SEM (n=10 to 12); *$P<0.01$ vs LF group. Black bars indicate significant differences between FO and SSO groups ($P<0.05$). Numbers are mean fold-expression changes as obtained by microarray analysis.

group (Figure 3B). Only the upper but not the lower band stained positively for Gla residues. In contrast, liver lysates from the low-fat and sunflowerseed oil groups gave only one Gla-positive band at 75 kDa. These results suggested accumulation of uncarboxylated coagulation factors in the livers from fish oil–fed mice.

To confirm this, liver slices were immunohistochemically stained for prothrombin and other vitamin K–dependent coagulation factors. Slices derived from mice fed fish oil displayed many areas of increased staining for prothrombin and factor VII, especially around veins in the periportal areas of the liver lobulus (Figure 3C). Perivenous areas of the livers stained normally. Slices from mice fed low fat or sunflowerseed oil showed less, if any, of such areas of increased staining (Figure 3D). The surface areas with high-staining for prothrombin, factor VII, or factor X were quantified by image analysis. They were more frequent in livers from the fish oil group, when compared to the low fat and sunflowerseed oil groups (Figure 3E). Liver slices were also stained with an anti-Gla antibody, which informs on the overall content of vitamin K-dependent Gla-containing proteins.[19] In this case, focal areas of increased staining were absent, and no difference was seen between the diet groups (Figure 3E). Consequently, the locally increased accumulation of vitamin K–dependent coagulation factors in livers after fish-oil feeding was not accompanied by an increase in Gla residues. This again pointed to (local) undercarboxylation of vitamin K–dependent coagulation factors.

Discussion

In this article, we compared the lipid-lowering and anticoagulant effects of dietary fish oil and sunflowerseed oil, using the hyperlipidemic and proatherogenic APOE2 mice, in which the endogenous murine apoE gene has been replaced by the human APOE2 gene. In man, the APOE2 genotype (frequency of 7%) carries an $Arg^{158}\rightarrow Cys$ mutation, which impedes the binding of ApoE to the low-density lipoprotein receptors.[13,20] This polymorphism is strongly associated with type III hyperlipoproteinemia; it leads to reduced clearance of chylomicrons and very low-density lipoproteins from the circulation and, hence, to increased plasma lipid levels. The transgenic APOE2 mice also have elevated plasma triacylglycerol levels. Typically, the dyslipidemia is accompanied by up to two-fold increased levels of (anti)coagulation factors, when compared to wild-type mice of the same genetic background (C57BL/6), sex (male), and dietary regime (low-fat). Furthermore, the APOE2 knock-in mice have a procoagulant phenotype, which is apparent from the greatly increased thrombin generation potential of plasma, ie, a parameter that measures the net effect of procoagulant and anticoagulant activity.

Feeding of APOE2 knock-in mice with fish oil (rich in n-3 PUFA) markedly reduced—and almost normalized—the lev-

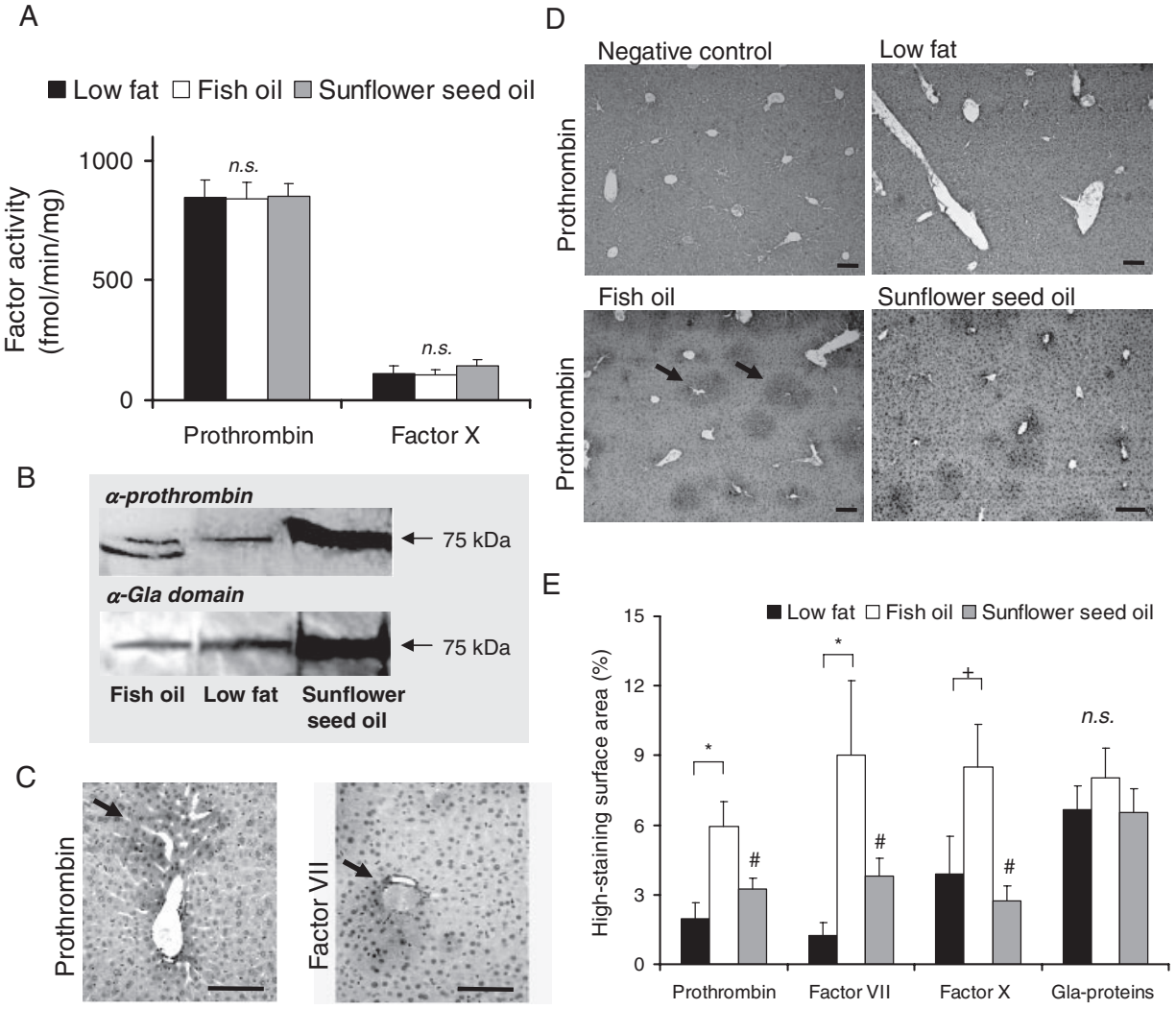

Figure 3. Diet effects on expression of coagulation factors in liver. A, Total levels of prothrombin and factor X (±Gla-domains) in liver lysates. B, Representative Western blots, stained for prothrombin or Gla-domains, from pooled liver lysates precipitated with $BaCl_2$. Arrow points to band with expected size of carboxylated prothrombin. C, High-magnification images of liver from a mouse fed with fish oil, showing focal staining (dark gray) of prothrombin and factor VII in periportal areas. D, Representative images of livers from various diet groups, stained for prothrombin. Primary antiprothrombin antibody was omitted in negative control (bars, 100 μm). Arrows indicate staining in periportal areas. E, Quantification of areas with high staining for prothrombin, factor VII, X, or Gla-proteins. Mean±SEM (n=10 to 12), +P<0.10 and *P<0.05 vs low-fat #P<0.05 vs sunflowerseed oil.

els of triacylglycerol and (anti)coagulant factors. The lipid-lowering effect is in agreement with other nutrition studies using different murine APOE models.[21–23] In the APOE2 mice, fish oil feeding lowered all measured vitamin K–dependent factors in plasma, ie, the coagulant factors prothrombin, factors VII, X, and the anticoagulant factor protein C. Similarly, this diet reduced vitamin K–independent factor V and the anticoagulant antithrombin. The reference diet, sunflowerseed oil diet (rich in n-6 PUFA), also provoked an overall reduction in coagulant factor levels, but this effect was less drastic in case of prothrombin and factors VII and X. Antithrombin reduced to a similar extent as with fish oil, which together provide an explanation for the less prominent effect of sunflowerseed oil on thrombin generation potential. Jointly these data indicate that, in the dyslipidemic mice, particularly fish oil corrects the triacylglycerol state and suppresses the coagulant activity. Others have shown that fish oil has an antiatherosclerotic effect in hyperlipidemic mice.[22,23] By extension, this suggests that (n-3) PUFA may reduce atherosclerosis by a combined action on lipids and coagulation factors.

In man, hyperlipidemia can associate with increased levels of coagulation factors.[5,24,25] Thus, in several patient groups, plasma levels of triacylglycerol were found to correlate with those of prothrombin and factors VII and X.[7] This may point to a mechanistic link, eg, in the form of a retro-signal from plasma triglycerides (PUFA?) to control the synthesis or secretion of lipids and coagulation factors in the liver (via PUFA-sensitive transcription?). The microarray expression analysis points to marked changes in hepatic mRNA expression, amounting to 5% of the detected gene sequences, in response to fish oil diet. Confirmed by quantitative PCR, the data further show increased expression of several key genes of lipid degradation and oxidation, and decreased expression

of genes implicated in lipid synthesis. Notably for the genes regulating lipid degradation, fish oil diet (n-3 PUFA) seems to have a stronger effect than sunflowerseed oil diet (n-6 PUFA). Collectively, these results point to a net reduction in lipid biosynthesis in the livers from fish oil–fed animals. Support for this conclusion comes from proteomics analysis of the livers from APOE3-Leiden mice, where fish oil appeared to increase the protein expression of several enzymes of fatty acid degradation.[21] Other data demonstrate that n-3 PUFA increase the catalytic activity of acyl-CoA oxidase and of other enzymes controlling fatty acid oxidation.[26]

We find that fish oil and sunflowerseed oil diets had similar reducing effects on the mRNA levels of nuclear receptors implicated in fat-modified gene regulation, ie, PPARα, δ and γ. This may point to a common action mechanism of n-3 and n-6 PUFA. Not much is known of the expression regulation of these transcription factors. It is described that PPARα is a positive sensor of dietary n-3 as well as n-6 PUFA.[27] Another report shows that binding of PUFA to PPARα promotes the oxidation of fatty acids by regulating target enzymes.[28] The isoform PPARγ is upregulated in patients with overweight or diabetes,[29] who respond well to fish oil diet.[7] Taken together, it is conceivable that increased interaction of (n-3) PUFA with these transcription factors, as during regular fish-oil intake, leads to secondary upregulation of genes implicated in lipid degradation.

Fish oil differed from sunflowerseed oil diet in affecting gene expression of the transcription factor Nrf2 (NFe2l2 gene). Recently, the βzipper Nrf2 has been recognized as a control element for the expression of antioxidant and cytoprotective enzymes.[30] Because dietary n-3 PUFA can alter the oxidative state of a cell, Nrf2 might be an interesting target of transcriptional control by fish oil.

Surprisingly, in spite of the effect on plasma coagulation factor levels, neither fish oil nor sunflowerseed oil diet modified the transcriptional regulation of genes of vitamin K–dependent coagulation factors (prothrombin, factors VII, and X). However, fish oil did reduce the expression of γ-glutamyl carboxylase (where vitamin K acts as a cofactor). The diets tended to decrease the expression of several genes encoding for anticoagulant proteins (tissue factor pathway inhibitor, antithrombin, proteins C and Z). By itself, this would be compatible with a procoagulant rather than a hypocoagulant phenotype.

This discrepancy was resolved by the identification of an uncarboxylated (non-Gla domain) form of prothrombin only in livers from mice fed with fish oil. Furthermore, immunostaining of liver sections demonstrated a focal accumulation of prothrombin, factor VII, and factor X proteins in periportal areas of the liver lobulus. Together, this points to accumulation of uncarboxylated prothrombin—and likely also other vitamin K–dependent factors—in the periportal areas of livers from mice fed with fish oil. In combination with the reduced expression of vitamin K–dependent γ-glutamyl carboxylase, it is concluded that incomplete carboxylation of these coagulation factors leads to retention in and, thus, impaired secretion by the liver.

In summary, our findings point to a complex effect of n-3 PUFA on hepatic synthesis of lipids and coagulation factors, operating at the levels of gene expression, posttranslational protein modification, and control of secretion from the liver. The hypocoagulant effect likely results from retention of uncarboxylated coagulation factors. In contrast, the lipid-lowering effect links to altered expression of genes that regulate transcription and fatty acid metabolism. How Nrf2 and PPAR isoforms are implicated in the dietary PUFA effects needs further investigation.

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Disclosures

None.

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