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Authors

Harris, Todd R
Kodani, Sean
Yang, Jun
et al.

Publication Date

2016-12-01

DOI

10.1016/j.jnutbio.2016.08.010

Peer reviewed

An ω-3 enriched diet alone does not attenuate CCl$_4$-induced hepatic fibrosis

Todd R. Harris[*], **Sean Kodani**[*], **Jun Yang**[*], **Denise M. Imai**[†], and **Bruce D. Hammock**[*],[§]

[*]Department of Entomology and Comprehensive Cancer Center, University of California, Davis, CA 95616, USA

[†]Comparative Pathology Laboratory, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

Abstract

Exposure to the halogenated hydrocarbon carbon tetrachloride (CCl$_4$) leads to hepatic lipid peroxidation, inflammation, and fibrosis. Dietary supplementation of ω-3 fatty acids has been increasingly advocated as being generally anti-inflammatory, though its effect in models of liver fibrosis is mixed. This raises the question of whether diets high in ω-3 fatty acids will result in a greater sensitivity or resistance to liver fibrosis as a result of environmental toxicants like CCl$_4$. In this study we fed CCl$_4$-treated mice a high ω-3 diet (using a mix of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) ethyl esters). We also co-administered an inhibitor of soluble epoxide hydrolase, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), which has been shown to boost anti-inflammatory epoxy fatty acids that are produced from both ω-3 and ω-6 dietary lipids. In this study, we showed that soluble epoxide inhibitors reduced CCl$_4$-induced liver fibrosis. Three major results were obtained. First, the ω-3 enriched diet did not attenuate CCl$_4$-induced liver fibrosis as judged by collagen deposition and collagen mRNA expression. Second, the ω-3 enriched diet raised hepatic tissue levels of several inflammatory lipoxygenase metabolites and prostaglandins, including PGE2. Third, treatment with TPPU in drinking water in conjunction with the ω-3 enriched diet resulted in a reduction in liver fibrosis compared to all other groups. Taken together, these results indicate that dietary ω-3 supplementation alone did not attenuate CCl$_4$-induced liver fibrosis. Additionally, oxylipin signaling molecules may play role in the CCl$_4$-induced liver fibrosis in the high ω-3 diet groups.

1. Introduction

The health benefits of fish oil and dietary supplementation of ω-3 fatty acids has been supported by a number of human trials, mostly focusing on the cardiovascular effects, such as reduction of coronary disease and sudden cardiac death [1–4]. In addition, animal models

of liver damage have reported ω-3 fatty acids to be organ protective and anti-inflammatory [5–7]. These reports raise the question of how dietary supplementation of high concentrations of ω-3 fatty acids might influence chronic inflammatory liver diseases such as fibrosis caused by exposure to environmental chemicals.

Hepatic fibrosis is the result of repair processes that are initiated in response to damage caused by factors such as alcohol consumption, viral infection and exposure to xenobiotics like carbon tetrachloride and arsenic [8, 9]. After injury, hepatic inflammation triggers hepatocyte apoptosis and necrosis and activates hepatic stellate cells [10]. Inflammatory cytokines, chemokines and growth factors are produced both by infiltrating and resident cells to form a positive feedback loop that leads to proliferation of activated hepatic stellate cells, greater deposition of collagen, an increase in tissue inflammation and the remodeling of the extracellular matrix [11].

The ω-3 fatty acid docosahexaenoic acid (DHA) has been shown to possess anti-inflammatory effects in several animal models, decreasing the expression of inflammatory cytokines, inhibiting leukocyte chemotaxis and reducing T-cell reactivity [12]. It has been hypothesized that some of these effects may be due the interaction of DHA with enzymes of the arachidonic acid pathway. DHA can compete with arachidonic acid as a substrate for the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (P450) enzymes, reducing the highly inflammatory metabolites of arachidonic acid, as well as form compounds such as the protectins and resolvins, which have anti-inflammatory effects [13]. We have also presented evidence that the anti-hypertensive, anti-inflammatory and analgesic effects of ω-3 lipids are due in part to their epoxide metabolites [14, 15]. DHA has been found to attenuate fibrosis is several animal models.

DHA has been shown to reduce paraquat-induced pulmonary fibrosis in mouse models [16]. In rodent models of non-alcoholic steatohepatitis (NASH), dietary supplementation of DHA reduced both hepatic inflammation and fibrosis [17, 18]. Similarly, in a high fat diet rat model of metabolic syndrome, DHA attenuated heart fibrosis and liver steatosis [19]. However, the dietary supplementation of ω-3 lipids has produced mixed results in other models of liver fibrosis. In a bile duct ligation model, fish oil caused an increase in hepatic fibrosis and decreased hepatic function,[20] while supplementation with DHA reduced hepatic fibrosis [21]. Conflicting results have also been obtained in ethanol models of liver damage and fibrosis. In two studies performed in the 1990s, a fish oil diet worsened the inflammation and liver fibrosis,[22, 23] while more recently it has been reported that a 1:1 arachidonic acid:DHA diet reduces liver dysfunction [24]. Some of this variation may be due to the fact that there are different and often unspecified levels of EPA, DHA and other components in the fish oil and the lipid enriched diets may have been oxidized.

The carbon tetrachloride (CCl_4)-treated rodent is a long-established model of lipid peroxidation and hepatic fibrosis following halogenated hydrocarbon exposure [25]. After activation by cytochrome P450 2E1, CCl_4 forms a radical that initiates lipid peroxidation, which causes damage to plasma membranes and eventual fibrosis [25]. In this study we report the effect of dietary supplementation with a ω-3 enriched oil on CCl_4-induced liver damage and fibrosis.

2. Materials and methods

2.1. Chemicals

All commercial chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. TPPU (1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea) was synthesized and analyzed for purity as reported [26]. Prior to administration in drinking water, TPPU was solubilized in PEG-400 at a concentration of 1 mg/mL, then added dropwise to 1 L of room temperature drinking water with periodic agitation to obtain the final concentration of either 10 mg/L or 3 mg/L. Blood levels of TPPU in mice following such exposure at a variety of doses have been reported [27].

2.2. Animals

All animal studies were approved by the University of California Davis Animal Use and Care Committee and were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Either female or male C57BL/6 mice (20g) were purchased from Charles River (Wilmington, MA). ω-3 enriched oil was obtained under nitrogen from Solutex (Sterling, VA) and shipped to Research Diets, Inc. (New Brunswick, NJ) where the diets were formulated and pelleted. Solutex0365 Oil is an enriched form of ω-3 fatty ethyl esters consisting of at least 65% docosahexanoic ethyl ester (by weight) and 10% eicosapentaenoic ethyl ester (Solutex, Inc., Sterling, VA). Lot# 1245-1113 was used for the dose-response and lot# 1114-1301 for the TPPU co-administration experiment. The corn oil primarily consists of 11% palmitate, 25% oleate and 60% linoleate as analyzed and provided by Research Diets. Analysis of the oil was performed by Solutex and validated by OmegaQuant, LLC (Sioux Falls, SD). The compositions of all dietary oils are shown in Supplementary Materials (Table S1.) Diets were formulated by Research Diets with 0%, 1.625%, and 6.5% of Solutex0365 with the balance made up from corn oil (weight of oil/ total weight of diet.) Because polyunsaturated fatty acids and particularly ω-3 fatty acids are easily oxidized upon exposure to air, the diet was stored in vacuum sealed pouches at −20 °C until used. Unused portions of an open bag were stored under nitrogen at −20 °C, however, Research Diets cooks their diet to form pellets. For the dose response experiment, diet containing 6.5% Solutex0365 and diet containing 6.5% corn oil was ground and mixed to obtain powdered diet with 6.5%, 3.25%, and 0.65% Solutex0365 (hereafter called ω-3 enriched oil), maintaining a constant of 6.5% total fat. A TBARs assay (Cayman Chemical, Ann Arbor MI) revealed a low level of peroxidation in the Solutex oils as well as the diets used in this experiment (Table S2), compared to a recent study on food oxidation [28].

2.3 Experimental Design

The CCl_4 mouse model employed is described in detail in [29]. Briefly, CCl_4 was diluted 1:7 in Neobee M-5 immediately before injection. Mice were injected i.p. with 197 mg/kg CCl_4 every five days for a total of 7 injections and sacrificed 3 days after the final injection. Mice were placed on either the control diet or ω-3 enriched diets 1 week prior to the first injection. TPPU was administered at the time of the first injection. For the dose response experiment, the animals were divided into eight treatment groups of 6 mice, each administered either control diet, or diet containing 6.5%, 3.25%, or 0.65% ω-3 enriched oil and either injected with CCl_4 or vehicle. For the TPPU co-treatment experiments, the mice

were divided into six groups of six: control diet, 1.625% ω-3 diet, control diet+CCl_4, 1.625% ω-3 diet+CCl_4, 1.625% ω-3 diet+CCl_4+10 mg/L TPPU, 1.625% ω-3 diet+CCl_4+3.0 mg/L TPPU.

2.4 Histopathology

Liver samples were immersion-fixed in 10% neutral-buffered formalin for 48 hours. Samples were stored in 70% ethanol prior to routine processing, paraffin-embedding and sectioning for histopathology. Tissue sections were stained with hematoxylin and eosin or Picrosirius red. Liver samples were evaluated for hepatocellular vacuolation, hepatocellular necrosis, Kupffer lipofuscinosis, fibrosis or inflammation. Histopathologic parameters evaluated were scored on a scale of 0 (no histologic evidence of a specific parameter) to 3 (most abundant evidence of a specific parameter). A Nikon Diaphot inverted microscope was used to image the Picrosirius red-stained slides. The slides were quantified using ImageJ (NIH, Bethesda, MD).

2.5 LC-MS/MS

Total liver tissue hydroxyproline, oxylipin levels and TPPU blood levels were determined by LC-MS/MS as previously described [30]. Retention time, selected reaction monitoring (SRM) and internal standard information are reported in Supplementary Materials Table S3.

2.6 Statistics

P-values for student T-tests were generated using Excel (Microsoft, Redmond, WA).

3. Results

With the aim of boosting the levels of ω-3 EpFA, we first performed a dose-response to determine the hepatic effects of dietary supplementation with the ω-3 enriched oil. The animals were divided into eight experimental groups and treated as described in Materials and Methods. The mice gained weight over the course experiment (Supplementary Materials Figure S1), although the ω-3 enriched diet and CCl_4-treated groups gained less weight than the control group, with the 6.5% ω-3 enriched diet groups having the lowest weights.

The livers were first assessed for collagen deposition. The CCl_4 treatment resulted in a roughly 2.5-fold increase in collagen deposition, as assessed by picrosirius red staining (Figure 1A and B). Interestingly, supplementation with the ω-3 enriched oil resulted in a slight increase in collagen deposition compared to control as judged by this stain, resulting in statistically significant changes in the CCl_4-treated animals on the ω-3 enriched diet compared to the CCl_4-treated animals on the control diet for the 0.65% diet and 6.5% diet groups. Similarly, hydroxyproline levels were found to be elevated the control diet+CCl_4 treated group, as well as all the ω-3 enriched diet groups (Figure 1C), though the differences did not achieve statistical significance judged by Student's t-test (P-value < 0.05.)

We next assessed mRNA expression of markers of fibrosis and inflammation (Supplementary Materials Figure S2). Agreeing with the picrosirius red and hydroxyproline results, we found that collagen 1a1 mRNA levels were elevated 15 fold in the control diet

+CCl$_4$ treated group, and this expression further increased in the 3.25% diet+CCl$_4$ group. Examination of other indicators of liver fibrosis revealed that our treatment increased mRNA expression of MMP-2, MMP-9, and Tissue Inhibitor of Metalloprotease 1 (TIMP-1). The mRNA expression was not significantly altered by the ω-3 diet.

In order to further characterize the changes in the tissue, we also examined hepatic MMP activity using gelatin zymography as well as an activity assay involving antibody capture and a general MMP-2 substrate (Supplementary Materials Figure S3). Here the increase in MMP activity as a result of CCl$_4$ treatment was not attenuated by the ω-3 enriched diet (Supplementary Materials Figure S3A and B). We followed the zymography with an MMP2 activity assay which measures only active MMP-2 and confirmed the increase in MMP-2 activity in the CCl$_4$-treated group was not attenuated by the ω-3 enriched diet, and even increased with increasing amounts of ω-3 in the diet, though the difference between these groups and the control corn oil group did not attain statistical significance judged by Student's t-test requiring a p-value of less than 0.5 (Supplementary Materials Figure S3C). We next determined hepatic soluble epoxide hydrolase and microsomal epoxide hydrolase activity and found the neither CCl$_4$ or the ω-3 enriched diet altered enzyme activity significantly (Supplementary Materials Figure S4).

We then scored the H&E stained slides for various indications of inflammation and liver damage (Supplementary Materials Tables S4 and S5). The CCl$_4$ treated groups exhibited higher lesion scores than other groups. The lipofuscin parameter appears to be primarily affected by the higher % ω-3 diet. Lipofuscin represents end-products of lipid oxidation, and may reflect increased cell membrane turnover or damage or excess dietary unsaturated fatty acids. Because of this histopathology result as well and the increased collagen expression and deposition and the slight elevation in MMP activity, plasma markers of liver damage were assessed to see if the mice fed the enriched ω-3 diet experienced liver damage. As expected in this moderate model of liver fibrosis, we found that the CCl$_4$ treatment did not alter commonly used plasma markers of severe hepatotoxicity in the treated groups, or in the groups with the ω-3 enriched diet (Supplementary Materials Figure S5).

Finally, in order to understand the changes in EpFA signaling that occurred after dietary supplementation, we examined the EpFA in liver tissue (Figure 2). The epoxide-to-diol ratios of EpFA derived from DHA and EPA were elevated in the ω-3 enriched diet groups (Figure 2B and D), peaking in the 3.25% group (for a description of these pathways, see Supplementary Materials Figure S6.) The hepatic tissue epoxide-to-diol ratios of the epoxyeicosatrienoic acids (EETs), derived from arachidonic acid, and the epoxyoctadecamonoenoic acids (EpOMEs), derived from linoleic acid, did not display statistically significant changes after CCl$_4$ treatment and/or administration of the ω-3 enriched diet. All oxylipin data are shown in Tables S6 and S7 in Supplementary Materials.

We previously reported that modulation of oxylipin levels in CCl$_4$-treated animals with sEH inhibitors resulted in an attenuation in liver fibrosis [30]. Given the changes in EpFA levels in the ω-3 enriched diet groups, we wondered how perturbation of the oxylipin homeostasis would alter the hepatic response to CCl$_4$ exposure. Because of the drop in weight and the histological scores of the 6.25% ω-3 enriched group, the peak in epoxides in the 3.25%

group compared to the 0.62% and 6.25% groups, as well as the increase in the MMP activity in the higher percentage groups, we chose a 1.625% ω-3 enriched diet for a second experiment in which we co-treated with a soluble epoxide hydrolase inhibitor, TPPU, in water, to boost the levels of EpFA signaling molecules in the liver. This amount approximates a dose of 2 g/kg DHA and 0.5 g/kg EPA assuming each mouse eats 5 g per day and each mouse weighs 25 g. Converting to human doses, this diet corresponds to 170 mg/kg DHA and 39 mg/kg EPA (or 11 g DHA and 2.4 g EPA for a 62 kg person). This represents a 29-fold and 5-fold higher doses of DHA and EPA, respectively, compared to the recent WELCOME studies investigating the effects of purified fatty acids on NAFLD. We divided the male mice into six groups: animals on a 1.625% ω-3 enriched or control diet were exposed to CCl_4 and administered either 10 mg/L or 3 mg/L TPPU in drinking water. The blood concentrations of sEHI in the high and low TPPU groups were 575±91 and 121±41nM (average ± S.D.), respectively. A study showing the blood levels of TPPU given at a range of concentrations has been published [27]. Continuous exposure to TPPU results in steady state levels [31]. All groups gained weight during the experiment, with the ω-3 only group gaining the most (Supplementary Materials Figure S7).

As previously reported in rodent models of hypertension and angiogenesis, [15, 32] the lower dose of TPPU was found to be more efficacious than the higher dose. The 3 mg/L TPPU-treated group displayed attenuated collagen deposition compared to the other CCl_4 treated groups, as judged by picrosirius red staining and hepatic hydroxyproline levels (Figure 3). Interestingly, the 1.625% ω-3 enriched diet alone slightly increased the collagen deposition compared to the control diet+CCl_4 group as judged by picrosirius red staining (Figure 3A and B). Examination of hepatic mRNA expression again showed a robust fibrotic response, with elevation in the mRNA levels of collagen 1a1, MMP-2. MMP-9, TGFβ, and TIMP-1 (Supplementary Materials Figure S8). We found no significant change in any of the markers when compared to the control diet+CCl_4 group except for MMP-2, which was significantly lowered in the 3 mg/L TPPU-treated group, although not back to the levels seen in the control diet group (Supplementary Materials Figure S8).

Next hepatic oxylipin levels were determined. When we calculated the epoxide-to-diol ratios, the 3 mg/L TPPU-treated group had elevated levels of EETs and the EpOMEs, compared to both the control diet group and the 10 mg/L TPPU treated group (Figure 4A and B). The EPA metabolites were elevated in all groups compared to the control diet group except for the control diet+CCl_4 group (Figure 4C). The 3 mg/L TPPU-treated group had elevated levels of the EPA metabolites compared to the 10 mg/L TPPU-treated group (Figure 4C). The results for DHA metabolites were the most inconsistent (Figure 4D). For the most part, the increases in epoxide-to-diol ratios occurred for the 19,20- and 16,17-EpDPE isomers, which have the epoxide moiety furthest from the carboxylic acid group. The 19,20 epoxide (ω epoxide) is turned over much more slowly than the internal EpDPEs, and terminal ω-3 epoxides are turned over far slower than ω-6 epoxides [33].

Looking more broadly at selected metabolites in the COX and LOX pathways of the arachidonic acid cascade, we found that both ω-3 dietary supplementation and CCl_4 treatment raised the levels of COX (Fig 5A, B, and D) and LOX (Figure 5C and D) metabolites and the P450 metabolite 20-HETE (Fig 5D), all suggesting inflammation (for a

simplified diagram of these pathways, see Supplementary Materials Figure S9.) This increase in COX and LOX metabolites of arachidonic acid in animals on an ω-3 rich diet was contrary to our expectations based on the literature [34, 35]. In general, the highest levels of these arachidonic acid metabolites were obtained in the ω-3 enriched diet CCl_4-treated mice, though even on the ω-3 enriched diet alone, the levels were higher than the control diet. For all metabolites, the 3 mg/L TPPU group returned the metabolites back to or below controls diet levels. These results are contrary to previous studies from this laboratory where increased levels of ω-3 reduced levels of 20-HETE [15].

3. Discussion

Given the reported anti-inflammatory and anti-fibrotic properties of DHA, we expected the ω-3 enriched diet to reduce the collagen deposition and other fibrotic markers in our CCl_4-induced model of liver fibrosis. We instead detected no change in these markers or even a slight elevation in some cases, such as in the collagen deposition in the 1.625% ω-3 diet group. In fact, when oxylipins in the arachidonic acid cascade were analyzed, we found that many compounds shown to be pro-inflammatory in other animal models were elevated in the mice on the ω-3 enriched diet.

Our result lies midway between the Japanese studies in which a DHA diet made ethanol-induced liver fibrosis significantly worse [22, 23], and the BDL model in which DHA decreased fibrosis [21]. Placing the differences between fish oil and DHA aside for a moment, one reason for these differing results may be due to important differences between the CCl_4 model and these other models of hepatic fibrosis. In CCl_4 exposure, the CCl_3 radical produced after CYP2E1 metabolism instigates a lipid peroxidation cycle, and this damage is the primary cause of the fibrosis. In the case of the BDL model, ligation of the bile duct results in a highly inflammatory model of fibrosis. It is possible in the case of the severe insult caused by ongoing lipid peroxidation, DHA has a limited effect compared to models in which oxidative stress and inflammation play a more defined role. These data support a hypothesis that ω-3 lipids, due to their ease of oxidation and peroxidation, are linked to toxicity in models with enhanced reactive oxygen species (ROS) and peroxide formation. When reviewing these results, one should keep in mind that blood markers of liver disease and damage were not elevated in the ω-3 enriched diet groups, and that histological analysis did not reveal a substantial increase in tissue damage or inflammation in these groups. Given these caveats, our results still raise concerns for people that may be exposed to pro-fibrotic environmental chemicals or are more susceptible to liver fibrosis, such as diabetics. As with all changes in diet, the cost benefit analysis must be made, weighing benefits for the cardiovascular system, for example, against the probable increased risks to the liver given one's environment or medical condition.

We employed a sEH inhibitor (sEHI) in this study as a tool to boost endogenous epoxy fatty acids (EpFA) by blocking their major route of metabolism, treating the mice with two different concentrations of a highly potent and bioavailable inhibitor, TPPU. In a previous study, we found that administration of sEHI reduced collagen deposition, MMP activity and ER stress due to CCl_4 treatment [30]. In both the current and previous studies, we have found that sEHI alter the expression of extracellular matrix enzymes but do not have a

significant impact on markers of inflammation such as IL-6 and IL-1β. This is an ongoing area of research, and raises the possibility that combination therapy with anti-inflammatory drugs such as NSAIDs with sEHI may have a beneficial effect in models of fibrosis. In the ω-3 background, the low dose but not the high dose of TPPU also had the effect of reducing collagen expression and deposition, as well as tissue hydroxyproline.

The low dose of sEHI was also more efficacious when we determined the levels of ω-3 and ω-6 derived EpFA. When we calculated the epoxide-to-diol ratios of EpFA, we found that the expected increase in this ratio was highest for the low dose of sEHI, rather than the high dose. Although we used only two concentrations of sEHI, this increase in effect given a lower dose is an indication of hormesis, which can be modeled by a J-shaped or U-shaped therapeutic curve [36, 37]. Recent studies in angiogenesis and hypertension have reported a hormetic dose-response when employing sEH inhibitors [15, 32]. These results raise the caution that more is not always better when it comes to sEHI treatment, and that experimental groups that vary the sEHI dose should be included whenever possible.

When we examined the metabolites of COX and LOX derived from arachidonic acid, we were surprised to find that metabolites in both the COX and LOX pathways were increased in the ω-3 enriched diet and CCl_4-treated groups when compared to the control diet. This increase was brought back to control levels in the group receiving the low dose of TPPU. In general, these COX and LOX metabolites are pro-inflammatory and have been implicated in several pathological conditions, including liver fibrosis [38]. Our result runs counter to liver damage and tumorigenesis studies in which dietary supplementation with DHA or mixtures of ω-3 fatty acids results in a lowering of these COX and LOX metabolites [39, 40], although a recent study has reported that in humans, dietary supplementation with DHA resulted in variation in prostaglandin levels between individuals, with some individuals displaying no decrease or a slight increase in certain COX and LOX metabolites [41]. Before interpreting these results, a number of differences between previous studies and the present study must be taken into account. First, most studies utilize plasma or urine samples for analysis of these oxylipins, whereas we measured hepatic levels directly, which may not reflect systematic changes. Second, both the length of time of this fibrosis model (40 days) compared to other shorter studies, and the difference in the composition of the ω-3 oils between studies make it hard to compare results, which will be discussed in the next paragraph. Third, the degree of elevation in these markers is relatively low when compared to established rodent models of inflammation, such as LPS models, where these markers will rise a hundred fold [42]. Lastly, eicosanoids will continue to be metabolized until the tissue has been frozen, so care must be taken to rapidly process or freeze the tissue. Substantial differences in organ harvesting time will result in inter-lab variation. Of all of these factors, perhaps the most variable component of experimental design is the composition of the diet.

There are multiple studies on the effects of ω-3 supplementation on liver disease; however, the quality of oil and concentrations of dietary lipids has varied dramatically. In this study, an enriched form of ω-3 fatty acid consisting of approximately 70% DHA and 15% EPA (see Supplementary Table S1) was used to study the effects of ω-3 supplementation on toxicant-induced liver fibrosis. The Solutex oil, in particular, is purified by supercritical liquid chromatography and thus lacks many fatty acids and non fatty acid components

present in many commercial products. Our analysis showed it to be very low in lipid peroxides. In comparison, most other studies use either purified (>95%) ω-3 oil [43, 44] or marine-derived oil such as menhaden oil, which typically contains >25% combined DHA and EPA [20, 45]. Among these studies, the ratio of EPA to DHA varies dramatically. While this study used DHA at a 5:1 ratio to EPA, a recent study that used oil with higher percentages of EPA has shown a decrease in toxicant-induced fibrotic scores through multiple markers [46]. It is unclear whether this difference is due to differences in the oil or other experimental factors including model species or length of toxicant exposure. Outside of toxicant-induced fibrosis, ω-3 supplementation has been beneficial in a number of diet-related liver diseases including steatosis [47], fibrosis [43, 45] and nonalcoholic steatohepatitis [5, 44]. As mentioned above, toxicant induced fibrosis is mediated by generation of radical species. In addition to the differences in concentrations of ω-3 oils, there are significant differences in the molecular form of fatty acids (as ethyl esters, triacyl glycerides, etc.) depending on source [48], which may result in different bioavailabilities. Because ω-3 lipids are sensitive to oxidizing environments, it is possible the ω-3 supplementation in toxicant-induced fibrosis, and particularly in a free radicle driven model, may not have the same response as these other models. Finally, in evaluating the effects of ω-3 supplementation there is a balance between DHA and EPA reducing inflammation and ER stress by a number of mechanisms including the formation of their corresponding epoxides and their ease of peroxidation and propagation of inflammatory peroxidative reactions. Since our fibrosis model is driven by a free radical peroxidative mechanism, the negative effects of easily oxidized ω-3 supplementation could outweigh positive effects in which one would expect ω-3 lipids in tissues to help propagate free radicle oxidation. These effects could result from their propagation of free radical reactions, the resulting lipid peroxides or the resulting rearrangement and oxidized products.

In conclusion, recent studies have implicated the COX and LOX pathways in liver fibrosis. 5-LOX inhibitors have been shown to increase the activation of cultured hepatic cells and increase Kupffer cell apoptosis [49, 50]. The treatment with the COX inhibitor celecoxib results in a significant reduction in fibrosis in rat models [51, 52]. Taken together with our results, these studies indicate that combination therapy involving both COX and sEH inhibitors might constitute a possible clinical tool for the reduction of liver fibrosis as a result of toxicant exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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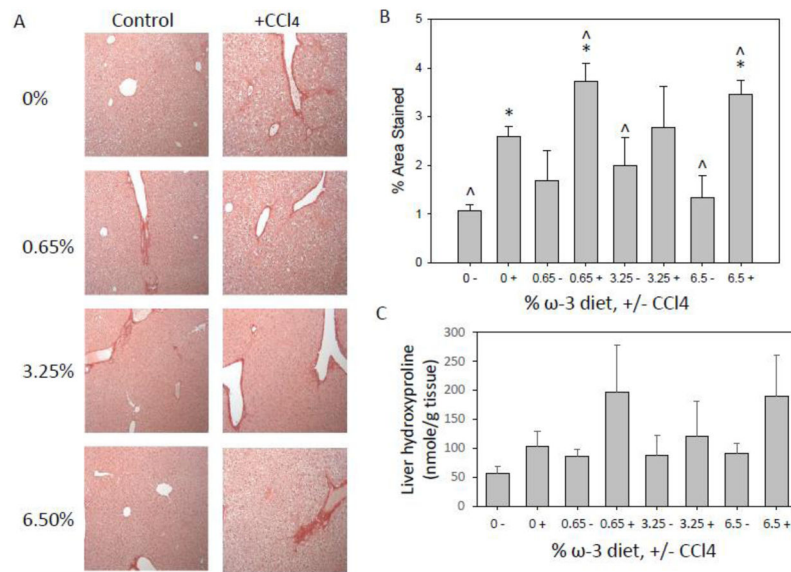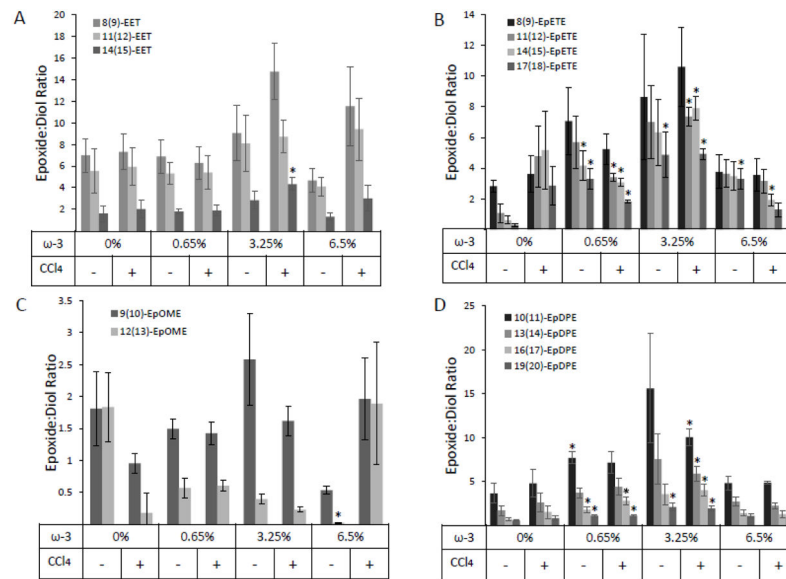**Figure 1.**

ω-3 enriched diet does not attenuate collagen deposition due to CCl$_4$ treatment. Mice were injected (i.p.) with CCl$_4$ for 5 weeks. Diets were formulated by Research Diets with 0%, 0.65%, 3.25%, and 6.25% of Solutex0365 (by weight) with the balance made up from corn oil. A) Representative slides of liver sections stained with picrosirius red (40x). B) Quantification of staining expressed in percent area. C) Quantification of hydroxyproline levels in liver tissue. Error bars represent standard error. *P-value vs. control diet group 0.05. ^P-value vs. control diet+CCl$_4$ 0.05. N = 3–4 animals per group.

Figure 2.
ω-3 enriched diet raised the epoxide to diol ratios of hepatic epoxy fatty acids. Tissue was collected and analyzed by LC-MS/MS after solid phase extraction as described in Materials and Methods. Diets were formulated by Research Diets with 0%, 0.65%, 3.25%, and 6.25% of Solutex0365 (by weight) with the balance made up from corn oil. A) Hepatic epoxide to diol ratios of EETs, P450 metabolites of arachidonic acid. B) Hepatic epoxide to diol ratios of the epoxyeicosatetraenoic acids (EpETEs), P450 metabolites of EPA. C) Hepatic epoxide to diol ratios of the epoxyoctadecamonoenoic acids (EpOMEs), P450 metabolites of linoleic acid. D) Hepatic epoxide to diol ratios of the epoxydocosapentaenoic acids (EpDPEs), P450 metabolites of DHA. For a simplified diagram of the enzymes and metabolites involved in these pathways see Supplementary Materials Figure S6. Error bars represent standard error. *P-value vs. control diet group 0.05. N = 3–4 animals per group.
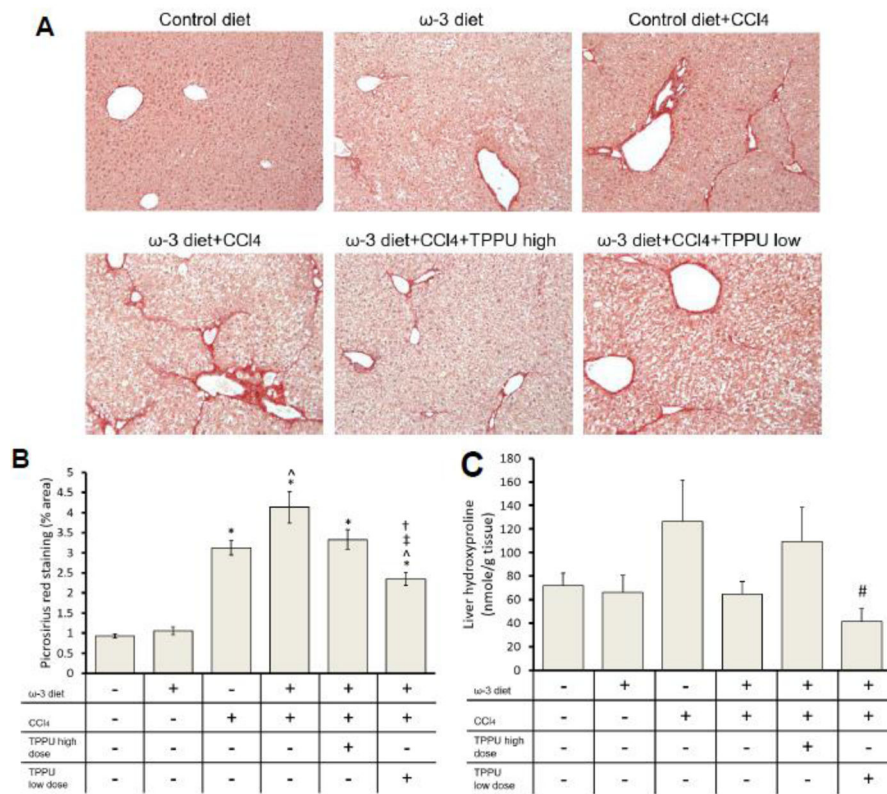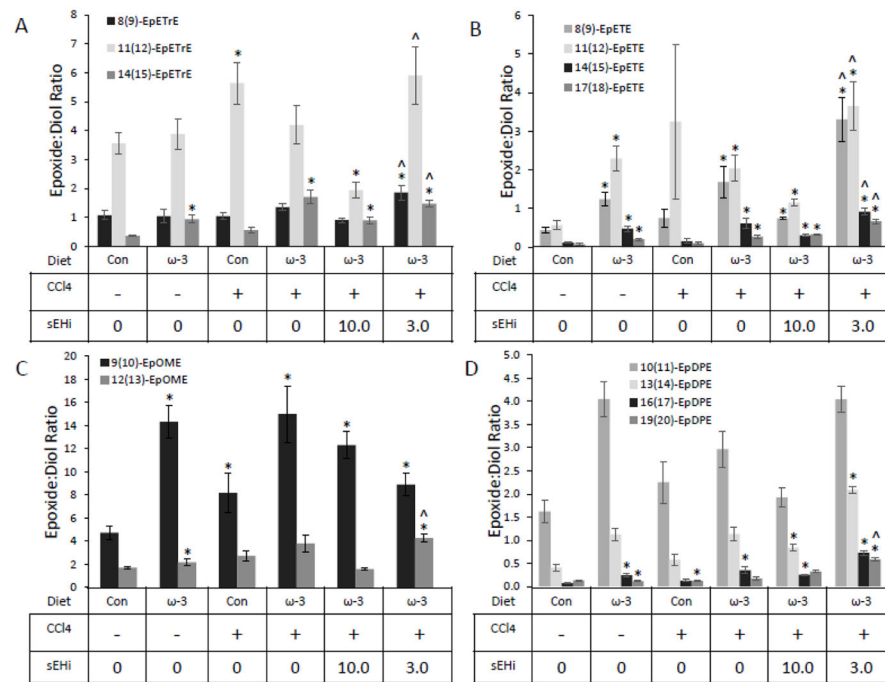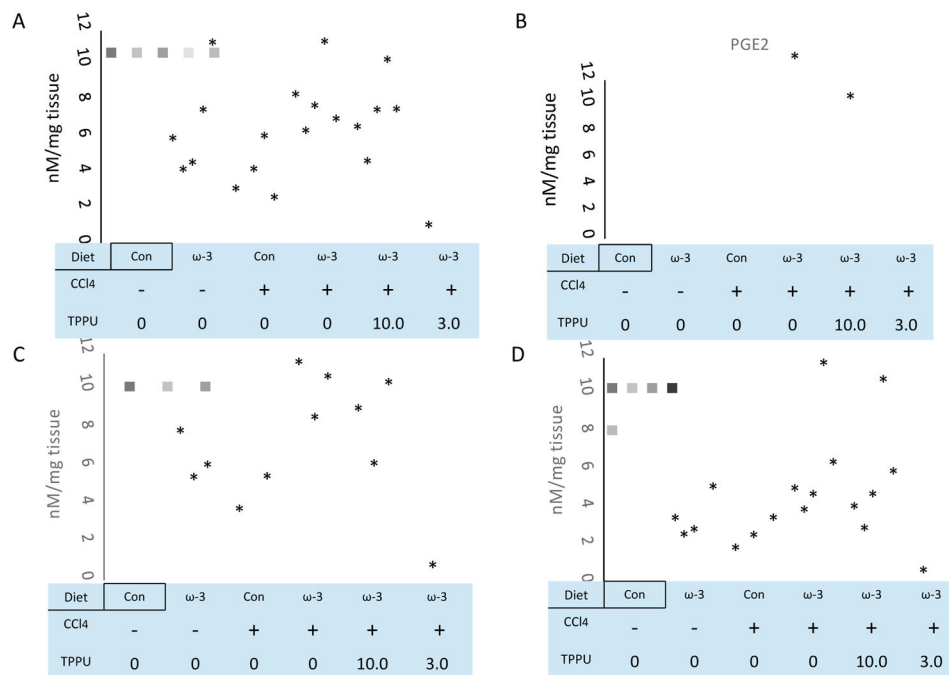
Figure 3.
ω-3 enriched diet combined with treatment with an sEH inhibitor attenuates collagen deposition due to CCl_4 treatment. Mice were injected (i.p.) with CCl_4 for 5 weeks. Diets were formulated by Research Diets with the ω-3 group receiving a diet of 1.625% of Solutex0365 (by weight) with the balance made up from corn oil. The sEH inhibitor TPPU was administered in drinking water at 3.0 and 10.0 mg/L. A) Representative slides of liver sections stained with picrosirius red (40x). B) Quantification of staining expressed in percent area. C) Quantification of hydroxyproline levels in liver tissue. Error bars represent standard error. *P-value vs. control diet group < 0.001. ‡P-value vs. ω-3 diet+$CCl_4 < 0.001$. #P-value vs. control diet+$CCl_4 < 0.05$. ^P-value vs. control diet+$CCl_4 < 0.05$. †P-value vs. ω-3 diet +CCl_4+TPPU high dose < 0.05. N = 5–6 animals per group.

Figure 4.
ω-3 enriched diet combined with treatment with an sEH inhibitor raised the epoxide to diol ratios of hepatic epoxy fatty acids. Tissue was collected and analyzed by LC-MS/MS after solid phase extraction as described in Materials and Methods. The sEH inhibitor TPPU was administered in drinking water at 3.0 and 10.0 mg/L. Diets were formulated by Research Diets with the ω-3 group receiving a diet of 1.625% of Solutex0365 (by weight) with the balance made up from corn oil. A) Hepatic epoxide to diol ratios of EETs, P450 metabolites of arachidonic acid. B) Hepatic epoxide to diol ratios of the epoxyeicosatetraenoic acids (EpETEs), P450 metabolites of EPA. C) Hepatic epoxide to diol ratios of epoxyoctadecamonoenoic acids (EpOMEs), P450 metabolites of linoleic acid. D) Hepatic epoxide to diol ratios of the epoxydocosapentaenoic acids (EpDPEs), P450 metabolites of DHA. For a description of these pathways, see Supplementary Materials Figure S6. Error bars represent standard error. *P-value vs. control diet group   0.05. ^P = value vs. the 10.0 mg/L TPPU group. N = 5–6 animals per group.

Figure 5.

The elevation in COX and LOX metabolites of arachidonic acid induced by the ω-3 enriched diet was lowered to control levels by treatment with an sEH inhibitor. Tissue was collected and analyzed by LC-MS/MS after solid phase extraction as described in Materials and Methods. The sEH inhibitor TPPU was administered in drinking water at 3.0 and 10.0 mg/L. Diets were formulated by Research Diets with the ω-3 group receiving a diet of 1.625% of Solutex0365 (by weight) with the balance made up from corn oil. A) and B) Hepatic levels of COX metabolites of arachidonic acid. C) Hepatic levels of LOX metabolites of arachidonic acid. D) Hepatic levels of LOX (5-, 8-, 11-, and 12-HETE) and P450 (20-HETE) metabolites of arachidonic acid. For a simplified diagram of these pathways see Supplementary Figure S9. Error bars represent standard error. *P-value vs. control diet group ⩽ 0.05. N = 5–6 animals per group.