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Docosahexaenoic Acid Attenuates Hepatic Inflammation, Oxidative Stress, and Fibrosis without Decreasing Hepatosteatosis in a *Ldlr*$^{-/-}$ Mouse Model of Western Diet-Induced Nonalcoholic Steatohepatitis[1–3]

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Abstract

The incidence of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) has increased in parallel with the incidence of obesity. While both NAFLD and NASH are characterized by hepatosteatosis, NASH is characterized by hepatic damage, inflammation, oxidative stress, and fibrosis. We previously reported that feeding *Ldlr*$^{-/-}$ mice a high-fat, high-cholesterol diet containing menhaden oil attenuated several markers of NASH, including hepatosteatosis, inflammation, and fibrosis. Herein, we test the hypothesis that DHA [22:6 (n-3)] is more effective than EPA [20:5 (n-3)] at preventing Western diet (WD)-induced NASH in *Ldlr*$^{-/-}$ mice. Mice were fed the WD supplemented with either olive oil (OO), EPA, DHA, or EPA + DHA for 16 wk. WD + OO feeding induced a severe NASH phenotype, characterized by robust hepatosteatosis, inflammation, oxidative stress, and fibrosis. Whereas none of the C20–22 (n-3) fatty acid treatments prevented WD-induced hepatosteatosis, all 3 (n-3) PUFA-containing diets significantly attenuated WD-induced inflammation, fibrosis, and hepatic damage. The capacity of dietary DHA to suppress hepatic markers of inflammation (*Clec4F*, *F4/80*, *Trl4*, *Trl9*, *CD14*, *Myd88*), fibrosis (*Procol1α1*, *Tgfβ1*), and oxidative stress (NADPH oxidase subunits *Nox2*, *p22phox*, *p40phox*, *p47phox*, *p67phox*) was significantly greater than dietary EPA. The effects of DHA on these markers paralleled DHA-mediated suppression of hepatic *Fads1* mRNA abundance and hepatic arachidonic acid content. Because DHA suppression of NASH markers does not require a reduction in hepatosteatosis, dietary DHA may be useful in combating NASH in obese humans. J. Nutr. 143: 315–323, 2013.

Introduction

The incidence of nonalcoholic fatty liver disease (NAFLD)[6] parallels the incidence of obesity in Western societies (1,2). NAFLD spans a spectrum of hepatic disorders ranging from simple fatty liver (hepatosteatosis) to nonalcoholic steatohepatitis (NASH) (3). Whereas simple hepatosteatosis is generally considered a benign condition, NASH is the progressive form of the disease that can lead to fibrosis, cirrhosis, and hepatocellular carcinoma (3,4). Progression of NAFLD to NASH has been modeled by a "2-hit" hypothesis (5). The first hit involves excess hepatic TG and cholesterol accumulation that is attributed to: *1*) elevated de novo lipogenesis; *2*) accumulation and reesterification of nonesterified fatty acids (NEFAs); *3*) decreased fatty acid oxidation; and *4*) reduced VLDL secretion (6). The first hit sensitizes the liver to the second hit, which consists of elevated hepatic oxidative stress, insulin resistance, and inflammation. Inflammation promotes hepatocellular damage and death. Furthermore, hepatic fibrosis is activated in stellate cells, which leads to excessive collagen deposition (7). Although management of lifestyle (diet and exercise) is one approach to control the onset and progression of NAFLD, the best strategy for managing NAFLD has yet to be defined (8).

Over the last 30 y, the overall consumption of fat in the United States has remained steady and even declined with respect to *trans* and SFAs (9,10). Total energy consumed,

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[3] Supplemental Figures 1–4 and Supplemental Tables 1–4 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[6] Abbreviations used: ALT, alanine aminotransferase; ARA, arachidonic acid, 20:4(n-6); HFHC, high-fat, high-cholesterol; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, nonesterified fatty acid; NP, nonpurified diet; OO, olive oil; WD, Western diet; WD + DHA, Western diet supplemented with DHA; WD + EPA, Western diet supplemented with EPA; WD + EPA + DHA, Western diet supplemented with EPA and DHA; WD + OO, Western diet supplemented with olive oil.
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however, has increased due to elevated carbohydrate consumption, mainly in the form of simple sugars (11,12). Thus, elevated carbohydrate and specifically fructose consumption has been linked to NAFLD and its progression to NASH (13,14).

We recently reported that menhaden oil has the capacity to attenuate several markers linked to NAFLD, including hepatosteatosis, inflammation, hepatic damage, and fibrosis (15). Menhaden oil is enriched in C20–22 (n-3) PUFAs. In that study, we used a high-fat, high-cholesterol (HFHC) diet supplemented with olive oil (OO) to induce NAFLD/NASH (15). The HFHC + OO diet contained starch as the main source of carbohydrate. In an effort to further define the strengths and limitations of C20–22 (n-3) PUFA in controlling NAFLD and NASH, we used a Western diet (WD) to induce NASH in *Ldlr*$^{-/-}$ mice in this study. The WD consisted of 41% total energy from fat and 43% total energy from carbohydrate; sucrose comprises 26.6% of total energy. The WD has been used extensively in atherosclerosis studies to model human disease (16). In this report, we test the hypothesis that DHA [22:6 (n-3)] is more effective than EPA [20:5 (n-3)] at preventing the onset and progression of NASH.

Twelve clinical trials (17) are investigating the use of (n-3) PUFAs in NAFLD and NASH therapy. Although some trials use either EPA or DHA as their source of dietary (n-3) PUFA, none are comparing the efficacy of EPA vs. DHA to manage fatty liver disease. Some animal studies have examined specific (n-3) PUFAs for the management of NAFLD and liver metabolism (18–22). One study focusing on insulin resistance directly compared EPA with DHA; both fatty acids equally lowered hepatic lipid content (22). The JELIS trial, a randomized clinical trial assessing the impact of dietary EPA on cardiovascular events, reported improvement in cardiovascular outcomes when patients received dietary EPA compared with placebo. Because these improvements correlated with increased blood concentrations of EPA, but not DHA (23), EPA alone may be sufficient to prevent diet-induced NASH. Our goal is to examine the capacity of EPA compared with DHA to prevent WD-induced NASH. The outcome of our study reveals a clear benefit in the use of DHA in attenuating the progression of NAFLD to NASH.

Methods and Materials

Animals and diets. All procedures for the use and care of animals for laboratory research were approved by the Institutional Animal Care and Use Committee at Oregon State University. Male *Ldlr*$^{-/-}$ mice (on the C57BL/6J background, Jackson Laboratories) at 2 mo of age consumed ad libitum 1 of the following 5 diets for 16 wk; each group consisted of 8 male mice. The control diet was Purina chow 5001 [nonpurified (NP)] consisting of 13.5% energy as fat and 58.0% energy as carbohydrates (**Supplemental Table 1**). The WD (D12709B, Research Diets) was used to induce NAFLD/NASH; it consisted of 17% energy as protein, 43% energy as carbohydrate, and 41% energy as fat; cholesterol was at 0.2% wt:wt. The WD was supplemented with OO (WD + OO), EPA (WD + EPA), DHA (WD + DHA), or EPA plus DHA (WD + EPA + DHA). Supplementation of the WD with OO, EPA, DHA, or EPA + DHA increased the total fat energy to 44.7% and reduced protein and carbohydrate energy to 15.8 and 39.5%, respectively. OO was added to the WD to have a uniform level of fat energy in all the WDs. Preliminary studies established that the addition of OO had no effect on diet-induced fatty liver disease in *Ldlr*$^{-/-}$ mice.

Dietary EPA was purchased from Futurebiotics as Newharvest EPA. This product, developed by DuPont, is a mix of TGs; 61 mole percent of the fatty acyl chains consists of EPA. Other fatty acids in this product include 16:0 (2.3%), 18:0 (2.3%), 18:1 (n-9) (4.7%), 18:2(n-6) (19.5%), 20:2 (n-6) (2.1%), 20:3 (n-3) (2.4%), and 22:5 (n-3) (2.8%). This product contains no arachidonic acid [ARA, 20:4 (n-6)], adrenic acid [22:4 (n-6)], or DHA. Dietary DHA was obtained as DHASCO and was a generous gift from Martek Bioscience. This product is a mix of TGs; 39% of the fatty acyl chains are DHA. Other fatty acids found in this product include: 12:0 (5.7%), 14:0 (15%), 16:0 (8.9%), 16:1 (n-7) (3.5%), 18:1 (n-9) (27%), and 18:2(n-6) (1.2%). DHA is the only C20–22 (n-3) or (n-6) PUFA in this product. The fatty acid composition of both products was verified in our laboratory by GC analysis of FAMEs. Once the diets were prepared, the fatty acid composition was again examined by GC of FAME (Supplemental Table 1). The total fat content in all the WD diets was 44.7% total energy as fat. The C20-22 (n-3) PUFA content in the WD + EPA, WD + DHA, and WD + EPA + DHA diets was 2% of total energy. The diets were stored frozen (−20°C) until used and replenished every other day to reduce the formation of oxidation products. Food consumption was monitored every other day and body weight was monitored weekly. At the end of the 16-wk feeding period, all mice were feed deprived overnight (1800 to 0800 h the next day) and then killed (isoflurane anesthesia and exsanguination) at 0800 h for blood and liver harvesting as described (15).

Liver histology. Liver (~100 mg) was fixed in formalin overnight then transferred to methanol. Livers were embedded, sliced, and stained with either hematoxylin-eosin or trichrome at the Veterinary Diagnostic Laboratory at Oregon State University.

Analysis of body composition. One week prior to the termination of the study, body mass and lean and fat mass were quantified using a Pixamus-dual energy X-ray absorptiometer (PIXImus2, Lunar). Animals were anesthetized by isoflurane and scanned for body composition. Isoflurane was administered using a precision vaporizer (Summit Anesthesia Solutions). A mixture of 2% isoflurane, 98% O_2 was delivered. The head was excluded from analysis. Percent body fat mass is reported (**Table 1**).

Measurement of plasma and hepatic variables. Plasma markers, hepatic lipids, RNA, and proteins were quantified as previously described (15). The quantitation of specific mRNA and proteins used qRT-PCR and immunoblotting, respectively, as previously described. Primers used for qRT-PCR are listed in **Supplemental Table 2**. Extracted hepatic lipids were also analyzed by TLC (silica gel 60A, Whatman); the chromatogram was developed in hexane:diethyl ether:acetic acid (60:40:0.8), dried, stained with iodine, and photographed. Lipid standards, e.g., cholesterol esters, TGs, NEFAs, diacylglycerols, and polar lipids, were used to verify type.

Statistical analysis. One-way ANOVA was used to detect dietary effects when >2 groups were included in the analysis (15). Data were analyzed for homogeneous variances by the Levene test. If unequal variances were detected, the data were log-transformed. ANOVA was performed on both transformed and untransformed data. Untransformed data are presented for interpretation purposes. When only 2 groups were compared, Student's *t* test was used for analysis. $P < 0.05$ was considered significantly different. All values are reported as mean ± SD.

Results

Body weight, body composition, plasma, and liver variables. The WD + OO was used to induce obesity and fatty liver. **Supplemental Table 3** compares body weight, plasma, and liver variables in *Ldlr*$^{-/-}$ mice fed the HFHC + OO diet, as previously reported (15), and the WD + OO diet used here. Of all the parameters measured, hepatic TG (154%; $P < 0.05$) and cholesterol (100%; $P < 0.05$) were significantly elevated in the *Ldlr*$^{-/-}$ mice fed the WD + OO diet compared with the mice fed the HFHC + OO diet. Of the gene expression markers examined, C-type lectin domain family 4f (*Clec4f*) a Kupffer cell marker (24), procollagen-1α1 mRNA (*Procol1α1*), a stellate cell marker (25) and *Tgfβ1* were elevated 56% ($P < 0.05$), 106%, ($P < 0.05$), and 55% ($P < 0.05$), respectively, after feeding *Ldlr*$^{-/-}$ mice the WD + OO compared with the HFHC + OO diet (**Supplemental**

TABLE 1 Phenotypic comparison of *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, and WD + DHA diets for 16 wk[1]

	NP[2]	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
Body weight, *g*	31 ± 3^{b}	42 ± 2^{a}	48 ± 3^{a}	45 ± 3^{a}	47 ± 4^{a}
Fat mass, *%*	20 ± 4^{b}	42 ± 3^{a}	46 ± 3^{a}	45 ± 2^{a}	46 ± 3^{a}
Plasma variables					
Glucose, *mmol/L*	11 ± 3	11 ± 1	10 ± 1	9 ± 2	11 ± 1
TG,[3] *mg/dL*	86 ± 19^{d}	229 ± 51^{a}	167 ± 59^{b,c}	128 ± 20^{c,d}	180 ± 44^{b}
Cholesterol,[3] *mg/dL*	232 ± 30^{c}	1018 ± 54^{a}	880 ± 127^{a,b}	737 ± 139^{b}	809 ± 151^{b}
ApoB,[4] *mg/dL*	20 ± 6^{c}	101 ± 26^{a}	52 ± 13^{b}	38 ± 8^{b}	41 ± 14^{b}
ApoC3,[4] *mg/dL*	16 ± 6^{c}	31 ± 10^{a}	30 ± 8^{a}	19 ± 4^{b,c}	24 ± 6^{a,b}
NEFA,[5] *mEq/mL*	0.7 ± 0.1^{c}	1.1 ± 0.1^{a}	0.8 ± 0.1^{b}	0.9 ± 0.1^{a,b}	0.8 ± 0.2^{b}
β-Hydroxybutyrate, *mmol/L*	0.9 ± 0.4^{b}	2.2 ± 0.4^{a}	2.0 ± 0.3^{a}	2.0 ± 0.3^{a}	1.7 ± 0.1^{a}
ALT, *U/L*	5 ± 1^{c}	44 ± 9^{a}	32 ± 8^{b}	29 ± 10^{b}	29 ± 10^{b}
AST, *U/L*	9 ± 3^{c}	36 ± 7^{a}	20 ± 4^{b}	20 ± 6^{b}	19 ± 5^{b}
Liver variables					
Weight, *g*	1.1 ± 0.1^{b}	2.1 ± 0.3^{a}	2.4 ± 0.4^{a}	1.7 ± 0.3^{a}	2.2 ± 0.4^{a}
Body weight, *%*	4.0 ± 0.5^{b}	5.2 ± 0.5^{a}	5.2 ± 0.7^{a}	4.2 ± 0.6^{b}	5.1 ± 0.8^{a}
TG,[6] *μg/mg*	77 ± 23^{c}	328 ± 55^{b}	421 ± 103^{a,b}	387 ± 76^{b}	487 ± 39^{a}
Cholesterol,[7] *μg/mg*	12 ± 4^{c}	34 ± 5^{a,b}	45 ± 14^{a}	31 ± 6^{b}	46 ± 9^{a}

[1] Values are mean ± SD, n = 8/treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; NEFA, nonesterified fatty acid; NP, nonpurified diet; WD + DHA, Western diet supplemented with DHA; WD + EPA, Western diet supplemented with EPA; WD + EPA + DHA, Western diet supplemented with EPA and DHA; WD + OO, Western diet supplemented with olive oil.
[2] NP *Ldlr*$^{-/-}$ mice were used as the control group.
[3] To convert from mg/dL to mmol/L, multiply by 0.0113 for TG and 0.02586 for cholesterol.
[4] To convert from mg/dL to g/L, multiply by 0.01 for ApoB and ApoCIII.
[5] To convert from mEq/mL to mEq/L, multiply by 1000 for NEFA.
[6] Data are expressed as μg TG/mg protein (liver); to convert from μg/mg to mmol/g, multiply by 0.00113.
[7] Data are expressed as μg cholesterol/mg protein (liver); to convert from μg/mg to mmol/g, multiply by 0.002586

Table 4). These results indicate that 16 wk of feeding *Ldlr*$^{-/-}$ mice the WD induced a more severe form of NASH than the 12 wk of feeding the HFHC + OO diet (15).

Feeding *Ldlr*$^{-/-}$ mice the WD + OO diet for 16 wk induced weight gain (≥30%) compared with age-matched NP-fed *Ldlr*$^{-/-}$ mice ($P < 0.05$) (Table 1). Body composition analysis revealed an increase (≥100%; $P < 0.05$) in fat mass in the WD + OO group. None of the C20–22 (n-3) PUFA-containing diets reduced body weight or fat mass below the levels in the WD + OO fed mice. None of the diets significantly affected blood glucose in feed-deprived mice. The plasma TG in feed-deprived mice was elevated 167% in the WD + OO group compared with the NP group ($P < 0.05$). Whereas plasma TG in feed-deprived mice fed the WD + DHA diet did not differ from that in the NP group, feed-deprived mice in the WD + EPA and WD + EPA + DHA groups had higher plasma TG (≥95%) than the NP group ($P < 0.05$) (Table 1). The plasma total cholesterol in feed-deprived mice was elevated ~340% in the WD + OO group relative to the NP group ($P < 0.05$). Although WD + DHA and WD + EPA + DHA lowered total cholesterol ($P < 0.05$), neither fully prevented WD-induced increases in blood cholesterol (Table 1). Plasma ApoB and ApoC3 were also induced 400 and 96%, respectively, in the WD + OO group compared with the NP group ($P < 0.05$). ApoB was attenuated (≥48%; $P < 0.05$) by the addition of C20–22 (n-3) PUFA to the WD. In contrast, WD + DHA was the only (n-3) PUFA that attenuated (~40%; $P < 0.05$) WD + OO induction of ApoC3 (Table 1), a result consistent with a previous human study (26). Plasma NEFAs were elevated (~57%; $P < 0.05$) by WD + OO feeding relative to NP feeding. This response was attenuated by WD + EPA and WD + EPA + DHA but not by WD + DHA ($P < 0.05$) (Table 1). Plasma β-hydroxybutyrate was induced (≥80%; $P < 0.05$) in all mice fed the WD compared with those fed the NP. Liver weight as percent body weight increased in the WD-fed mice; this was prevented ($P < 0.05$) only in the WD + DHA group (Table 1).

Hepatic histology and lipid content. Histology of livers from the 5 groups is shown in **Supplemental Figure 1**. Evidence of hepatosteatosis is shown by the appearance of highly vacuolated liver; both micro- and macro-hepatosteatosis was observed. Chemical analysis of each group showed that mice fed the WD + OO diet had a ≥320% ($P < 0.05$) increase in hepatic TG concentration and a ≥160% ($P < 0.05$) increase in hepatic total cholesterol relative to the NP-fed mice (Table 1). Further analysis of liver lipids is shown in **Supplemental Figure 2**, where hepatic lipid extracts were fractionated by TLC. Concentrations of hepatic TG and cholesterol esters were higher in mice fed the WD, supporting the chemical analysis in Table 1. In contrast to our previous studies (15), no EPA- or DHA-containing diet significantly lowered the WD-induced accumulation of hepatic TG or total cholesterol (Table 1; Supplemental Figs. 1 and 2).

Hepatic fatty acid composition. Oleic acid [18:1 (n-9)] is the most abundant fatty acid in the WD (Supplemental Table 1); hepatic 18:1(n-9) was elevated >2000% ($P < 0.05$) in the WD + OO group compared with the NP group. The addition of EPA or DHA to the WD diet was equally effective in reducing hepatic 18:1(n-9) by >50% ($P < 0.05$) compared with the WD + OO group. Hepatic ARA is formed by desaturation and elongation of the essential fatty acid linoleic acid [18:2 (n-6)]. Feeding mice the WD + OO increased 20:4 (n-6) by 87% ($P < 0.05$) relative to NP. Of the EPA- or DHA-containing diets tested, the DHA-containing diets were most effective at reducing (>75%; $P < 0.05$) WD-induced hepatic ARA content (**Table 2**).

The addition of EPA or DHA to the WD elevated hepatic EPA and DHA compared with either the NP or WD + OO groups. Feeding mice the WD + EPA increased hepatic EPA >1300 and >5000% compared with the NP and WD + OO groups, respectively ($P < 0.05$). However, feeding mice the WD + EPA increased the hepatic DHA content only ~100% compared with the NP or WD + OO groups. Feeding mice the WD + DHA, however, increased hepatic DHA >570 and >670% compared with the NP and WD + OO groups, respectively ($P < 0.05$). Dietary DHA was clearly more effective than dietary EPA at increasing the hepatic DHA content. Dietary DHA, however, increased hepatic EPA (≥720%; $P < 0.05$) and 22:5 (n-3) (≥580%; $P < 0.05$) compared with the NP and WD + OO groups (Table 2). These diets had similar effects on plasma fatty acid profiles (not shown).

The ratio of hepatic 20:4(n-6):18:2(n-6) and 20:3(n-6):18:2 (n-6) is an indicator of hepatic PUFA synthesis (Table 2). The 20:4(n-6):18:2(n-6) and 20:3(n-6):18:2(n-6) ratios increased 54% ($P < 0.05$) and 100% ($P < 0.05$), respectively, in the WD + OO group compared with the NP group. The WD + DHA diet was the most effective (n-3) PUFA diet at lowering these ratios.

Hepatic fatty acid synthesis. Fatty acid synthase (FASN), stearoyl CoA desaturase-1 (SCD1), and fatty acid elongases (ELOVL)-1, 3, 6, and 7 play a major role in de novo fatty acid synthesis, MUFAs, and sphingolipid synthesis (27,28). The WD + OO group suppressed *Elovl3* expression by ~80% ($P < 0.05$) compared with the NP group. In contrast, this diet induced *Elovl1* (100%; $P < 0.05$), *Elovl6* (50%; $P < 0.05$), *Elovl7* (450%; $P < 0.05$), and *Scd1* (500%; $P < 0.05$), respectively (**Table 3**). The *Elovl3* mRNA abundance in mice fed the EPA- or DHA-containing diets did not differ from mice in the WD + OO group. The hepatic abundance of *Fasn* (66%; $P < 0.05$), *Scd1* (60%; $P < 0.05$), *Elovl1* (45%; $P < 0.05$), *Elovl6* (34%; $P < 0.05$), and *Elovl7* (62%; $P < 0.05$) was significantly lower in the WD + DHA group than in the WD + OO group. These results indicate that the WD + DHA diet is more effective than the WD + EPA diet at reversing the effects of the WD + OO diet on pathways controlling hepatic SFA, MUFA, and sphingolipid production.

The enzymes involved in PUFA synthesis include Elovl2 and Elovl5 and fatty acid desaturase 1 (Fads1) and 2 (Fads2) (Table 3). These enzymes are responsible for the conversion of 18:2(n-6) and 18:3(n-3) to C20–22 (n-6) and (n-3) PUFAs, respectively. The hepatic abundance of *Elovl2*, *Elovl5*, and *Fads2* mRNA was elevated (≥50%; $P < 0.05$) by feeding mice the WD + OO diet compared with the NP group. These changes in expression paralleled the induction of both hepatic 20:4(n-6) and the ratio of 20:4(n-6):18:2(n-6). Unlike the WD + EPA or WD + EPA + DHA diets, the WD + DHA diet completely blocked the WD + OO-mediated induction of all 3 enzymes ($P < 0.05$). The mRNA encoding *Fads1*, in contrast, was not induced in livers of mice fed the WD + OO diet. This mRNA, however, was suppressed (>60%) in livers of mice in the WD + DHA and WD + EPA + DHA groups compared with mice in the NP and WD + OO groups ($P < 0.05$). The changes in mRNA abundance for the fatty acid elongases and desaturases paralleled WD + DHA- and WD + EPA + DHA-induced changes in hepatic 20:4(n-6) (Table 2).

Hepatic TG hydrolysis and fatty acid oxidation. Impairment of TG hydrolysis and fatty acid oxidation contributes to

TABLE 2 Hepatic fatty acid composition in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk[1]

Fatty acid	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			μmol/g protein		
14:0	5 ± 2^{c}	92 ± 11^{a}	57 ± 11^{b}	52 ± 12^{b}	56 ± 9^{b}
16:0	339 ± 77^{c}	1970 ± 284^{a}	1720 ± 320^{a,b}	1360 ± 327^{b}	1490 ± 206^{b}
16:1 (n-7)	30 ± 13^{c}	461 ± 57^{a}	445 ± 81^{a}	293 ± 82^{b}	363 ± 86^{a,b}
18:0	141 ± 11^{b}	178 ± 25^{a}	178 ± 18^{a}	153 ± 23^{a,b}	158 ± 14^{a,b}
18:1 (n-9)	196 ± 65^{d}	4140 ± 591^{a}	2930 ± 599^{b}	2030 ± 610^{c}	2460 ± 457^{b,c}
18:1 (n-7)	39 ± 9^{d}	847 ± 127^{a}	411 ± 104^{b}	204 ± 87^{c}	279 ± 103^{b,c}
18:2 (n-6)	320 ± 107^{a,b}	355 ± 46^{a,b}	413 ± 76^{a}	278 ± 44^{b}	333 ± 52^{a,b}
18:3 (n-3)	10 ± 5^{b,c}	9 ± 2^{c}	16 ± 4^{a}	11 ± 3^{a,b,c}	15 ± 4^{a,b}
20:0	4 ± 1^{c}	134 ± 20^{a}	74 ± 16^{b}	53 ± 22^{b}	62 ± 13^{b}
20:2 (n-6)	2.6 ± 0.3^{c}	8.1 ± 1.0^{a}	7.4 ± 1.7^{a}	2.3 ± 0.7^{c}	5.2 ± 0.9^{b}
20:3 (n-6)	8 ± 1^{c}	22 ± 3^{a}	24 ± 3^{a}	9 ± 1^{c}	16 ± 2^{b}
20:4 (n-6)	76 ± 8^{b}	142 ± 20^{a}	67 ± 8^{b}	31 ± 5^{c}	39 ± 5^{c}
20:5 (n-3)	8 ± 2^{c}	2 ± 1^{c}	115 ± 32^{a}	65 ± 8^{b}	92 ± 18^{a}
22:5 (n-6)	2 ± 1^{b}	26 ± 8^{a}	8 ± 1^{b}	6 ± 3^{b}	6 ± 3^{b}
22:5 (n-3)	7 ± 1^{d}	7 ± 2^{d}	126 ± 27^{a}	47 ± 13^{c}	77 ± 13^{b}
22:6 (n-3)	63 ± 11^{c}	55 ± 8^{c}	121 ± 21^{c}	424 ± 97^{a}	313 ± 84^{b}
Sum C20-22[2]					
C20-22 (n-3)	78 ± 14^{c}	64 ± 9^{c}	362 ± 78^{b}	536 ± 113^{a}	482 ± 109^{a}
C20-22 (n-6)	91 ± 8^{b,c}	211 ± 30^{a}	108 ± 13^{b}	51 ± 7^{d}	68 ± 7^{c,d}
Fatty acid ratios[3]					
20:4 (n-6):18:2 (n-6)	0.26 ± 0.02^{b}	0.40 ± 0.04^{a}	0.16 ± 0.02^{c}	0.11 ± 0.02^{c}	0.12 ± 0.02^{c}
20:3 (n-6):18:2 (n-6)	0.03 ± 0.003^{c}	0.06 ± 0.007^{a}	0.06 ± 0.007^{a,b}	0.03 ± 0.005^{c}	0.05 ± 0.004^{b}

[1] Values are mean ± SD, n = 8/treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, nonpurified diet; WD + DHA, Western diet supplemented with DHA; WD + EPA, Western diet supplemented with EPA; WD + EPA + DHA, Western diet supplemented with EPA and DHA; WD + OO, Western diet supplemented with olive oil.
[2] Sum C20-22 refers to the sum of all the 20 to 22 carbon fatty acids analyzed.
[3] Fatty acid ratios refers to the mole ratio of 20:4 (n-6):18:2 (n-6) or 20:3 (n-6):18:3 (n-6).

TABLE 3 Hepatic gene expression in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk and effects on mRNA abundance of enzymes involved in fatty acid synthesis and TG hydrolysis[1]

Fatty acid synthesis	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			fold of NP		
Fasn	1.0 ± 0.4^{a}	0.6 ± 0.2^{a,b}	0.4 ± 0.1^{b,c}	0.2 ± 0.1^{c}	0.4 ± 0.1^{b,c}
Elovl1	1.0 ± 0.2^{c}	2.0 ± 0.4^{a}	1.8 ± 0.2^{a,b}	1.1 ± 0.2^{c}	1.5 ± 0.3^{b}
Elovl2	1.0 ± 0.4^{c,d}	1.5 ± 0.4^{a}	1.2 ± 0.3^{a,b}	0.6 ± 0.2^{d}	0.9 ± 0.2^{b,c}
Elovl3	1.0 ± 0.3^{a}	0.2 ± 0.1^{b}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}
Elovl5	1.0 ± 0.3^{c}	2.1 ± 0.6^{a,b}	1.8 ± 0.4^{a,b}	1.0 ± 0.4^{c}	1.6 ± 0.5^{b,c}
Elovl6	1.0 ± 0.2^{b,c}	1.5 ± 0.4^{a}	1.4 ± 0.2^{a,b}	1.0 ± 0.4^{c}	1.3 ± 0.3^{a,b,c}
Elovl7	1.0 ± 0.2^{b}	5.5 ± 2.2^{a}	2.3 ± 1.6^{b}	2.1 ± 0.9^{b}	2.4 ± 0.9^{b}
Fads1	1.0 ± 0.3^{a,b}	1.1 ± 0.4^{a}	0.7 ± 0.2^{b,c}	0.2 ± 0.2^{d}	0.4 ± 0.2^{c,d}
Fads2	1.0 ± 0.2^{b,c}	2.0 ± 0.6^{a}	1.5 ± 0.2^{b}	0.8 ± 0.4^{c}	1.2 ± 0.2^{b,c}
Scd1	1.0 ± 0.7^{c}	6.0 ± 2.6^{a}	6.1 ± 2.6^{a}	2.4 ± 1.5^{b,c}	4.1 ± 1.5^{a,b}
TG hydrolysis					
Atgl	1.0 ± 0.3	1.2 ± 0.3	1.2 ± 0.1	1.0 ± 0.3	1.3 ± 0.2
Tgh	1.0 ± 0.3^{b}	0.9 ± 0.4^{b}	1.2 ± 0.2^{a,b}	1.2 ± 0.5^{a,b}	1.5 ± 0.3^{a}
Aadac	1.0 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	0.8 ± 0.3	0.9 ± 0.2
Adrp	1.0 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	1.2 ± 0.4	1.3 ± 0.5
Adpn	1.0 ± 0.3^{b}	2.1 ± 0.9^{a}	1.3 ± 0.2^{b}	0.9 ± 0.2^{b}	1.4 ± 0.5^{b}

[1] Values are mean ± SD, n = 8/treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, nonpurified diet; WD + DHA, Western diet supplemented with DHA; WD + EPA, Western diet supplemented with EPA; WD + EPA + DHA, Western diet supplemented with EPA and DHA; WD + OO, Western diet supplemented with olive oil.

hepatosteatosis (29). We analyzed the mRNA abundance of several hepatic TG lipases, key enzymes involved in VLDL assembly and fatty acid oxidation (Table 3). Of these, only adiponutrin (*Adpn*) was induced (110%) by the WD + OO diet compared with mice fed the NP ($P < 0.05$). This induction was blocked in the C20–22 (n-3)-supplemented groups. We also quantified mRNAs for several PPARα target genes, including ones involved in fatty acid oxidation, i.e., carnitine palmitoyl transferase 1 and 2 and acyl CoA oxidase. The hepatic abundance of these transcripts was not affected by diet (not shown).

Hepatic damage. Increased concentrations of plasma alanine aminotransferase (ALT) and aspartate aminotransferase reflect hepatic damage. Plasma concentrations of both enzymes were induced ≥270% ($P < 0.05$) by WD + OO feeding compared with the NP (Table 1). All EPA- and DHA-containing WD diets attenuated the WD + OO induction for ALT and aspartate aminotransferase ($P < 0.05$).

Hepatic inflammation. Hepatic damage is a result of increased hepatic inflammation and cell death. Histological evidence of inflammation is shown by leukocyte (monocytes and macrophage) infiltration in liver; this phenomenon was detected only in livers of mice fed the WD + OO diet (Supplemental Fig. 1). In agreement with this observation, monocyte chemo-attractant protein-1 (*Mcp1*) mRNA, an early marker of inflammation (30), was induced 3100% ($P < 0.01$) in the WD + OO group compared with the NP group. Moreover, the cell surface markers for Kupffer cells and macrophages [*Clec4f*, *Clec10a*, cell determination-68 (*Cd68*) and *F4/80*] were also induced ≥180% ($P < 0.05$). In every case, the expression levels of these transcripts was lower in livers of mice in the WD + DHA group than in mice in the WD + EPA or WD + EPA + DHA groups (**Table 4**).

Cytokines produced by Kupffer cells and macrophages play a major role in promoting cell damage. The hepatic abundance of mRNA encoding *Il-1β* and *Tnf-α* was induced 290% ($P < 0.01$) and 760% ($P < 0.01$), respectively, in livers of mice fed the WD + OO diet compared with the NP group. The WD + EPA and WD + DHA diets attenuated ($P < 0.01$) the induction of *Il-1β* and *Tnf-α* relative to the WD + OO group. The WD-mediated induction of *Clec4* and *F4/80* was lower in mice fed the WD + DHA diet compared with mice fed the WD + EPA or WD + EPA + DHA diets ($P < 0.05$) (Table 4).

Toll-like receptors (TLR) -2, -4, and -9 play a role in the progression of NASH (31). Stimulation of these pattern recognition receptors augments NFκB nuclear content and the induction of cytokines. *Tlr9* mRNA was the most responsive to WD + OO; *Trl9* was induced nearly 700% relative to the NP group ($P < 0.01$). The induction of *Tlr4* and *Tlr9* was attenuated in the WD + DHA group but not in the WD + EPA group ($P < 0.01$). All C20–22 (n-3)-containing diets attenuated the WD + OO induction of *Tlr2*. Cluster of differentiation-14 (CD14), myeloid differentiation factor-2 (MD-2), and myeloid differentiation primary response gene-88 (MyD88) are also involved in TLR signaling. Of these, *Cd14* and *Myd88* mRNA were induced in the WD + OO group; only the WD + DHA group blocked induction of both these genes ($P < 0.05$) (Table 4).

NFκB, a downstream target of TLR-signaling, is a transcription factor that controls a wide range of genes involved in inflammation, including cytokines and chemo-attractant proteins (32). We quantified the hepatic nuclear abundance of NFκB-p50 and NFκB-p65 subunits (**Supplemental Fig. 3**). Feeding mice the WD + OO or WD + EPA diet increased the hepatic nuclear content of NFκB-p50 (~100%; $P < 0.01$) relative to the NP. Diets containing DHA blocked the WD-induced accumulation of NFκB-p50 in hepatic nuclei. We also analyzed *NfκB-p105* mRNA, the precursor to NFκB-p50. *NfκB-p105* mRNA abundance changed in parallel with NFκB-p50 nuclear protein abundance. The hepatic nuclear content of NFκB-p65, a NFκB-p50 heterodimer partner, was significantly elevated in all WD groups.

Hepatic oxidative stress. Oxidative stress is implicated in the progression of fatty liver to NASH (33). Nuclear factor-E2-related factor-2 (NRF2) is a transcription factor that responds

TABLE 4 Hepatic gene expression in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk and effects on mRNA abundance of proteins involved in inflammation, oxidative stress, and fibrosis[1]

Inflammation	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			fold of NP		
Mcp1	1.0 ± 0.4^{c}	32 ± 11^{a}	13 ± 4^{b}	9.0 ± 3^{b,c}	10 ± 4^{b}
Cd68	1.0 ± 0.2^{d}	7.6 ± 1.3^{a}	5.0 ± 1.4^{b,c}	3.5 ± 1.0^{c}	5.5 ± 1.5^{b}
Clec4f	1.0 ± 0.3^{c}	3.8 ± 0.9^{a}	2.6 ± 0.8^{b}	1.5 ± 0.4^{c}	2.4 ± 0.5^{b}
Clec10a	1.0 ± 0.4^{b}	2.7 ± 1.6^{a}	1.9 ± 0.6^{a,b}	1.5 ± 1.0^{a,b}	2.7 ± 1.0^{a}
F4/80	1.0 ± 0.4^{c}	4.1 ± 0.7^{a}	3.6 ± 0.9^{a}	2.2 ± 0.9^{b}	3.4 ± 1.0^{a}
Il-1β	1.0 ± 0.6^{c}	3.9 ± 0.7^{a}	2.5 ± 0.6^{b}	1.7 ± 0.2^{b,c}	2.0 ± 0.7^{b}
Tnf-α	1.0 ± 0.4^{c}	8.6 ± 2.0^{a}	4.2 ± 1.3^{b}	3.8 ± 1.2^{b}	4.6 ± 1.7^{b}
Trl2	1.0 ± 0.3^{c}	5.6 ± 1.3^{a}	3.5 ± 1.1^{b}	2.6 ± 0.5^{b}	3.3 ± 0.9^{b}
Tlr4	1.0 ± 0.3^{c}	4.0 ± 1.6^{a}	2.9 ± 0.9^{a,b}	1.7 ± 0.4^{b,c}	2.7 ± 0.7^{b}
Trl9	1.0 ± 0.3^{c}	7.7 ± 1.8^{a}	6.2 ± 2.3^{a,b}	4.2 ± 1.1^{b}	5.9 ± 1.7^{a,b}
Cd14	1.0 ± 0.3^{c}	6.1 ± 1.5^{a}	3.4 ± 1.5^{b}	2.4 ± 0.5^{b,c}	3.7 ± 1.4^{b}
Md-2	1.0 ± 0.3	1.4 ± 0.4	1.4 ± 0.5	1.6 ± 0.9	1.0 ± 0.3
Myd88	1.0 ± 0.2^{b}	1.6 ± 0.4^{a}	1.4 ± 0.3^{a,b}	1.0 ± 0.3^{b}	1.3 ± 0.2^{a,b}
Oxidative stress					
Nrf2	1.0 + 0.2^{c}	1.9 + 0.5^{a}	1.9 + 0.4^{a,b}	1.4 + 0.4^{b,c}	1.7 + 0.3^{a,b}
Gsta1	1.0 ± 0.4^{b}	1.9 ± 0.6^{b}	3.6 ± 0.9^{a}	1.9 ± 0.8^{b}	3.6 ± 1.9^{a}
Hmox1	1.0 ± 0.3^{c}	5.0 ± 1.6^{a}	3.5 ± 1.9^{b}	2.7 ± 0.5^{b}	3.7 ± 1.1^{a,b}
Nox1	1.0 ± 1.5	1.8 ± 0.6	1.8 ± 1.2	0.8 ± 1.2	0.8 ± 0.3
Noxa1	1.0 ± 0.5^{b}	6.2 ± 2.5^{a}	3.9 ± 1.7^{a}	3.9 ± 1.2^{a}	3.5 ± 1.7^{a}
Noxo1	1.0 ± 0.3^{b}	1.6 ± 0.4^{a}	2.0 ± 0.3^{a}	1.7 ± 0.5^{a}	1.9 ± 0.3^{a}
Nox2 (Gp91phox)	1.0 ± 0.3^{b}	6.7 ± 0.3^{a}	5.9 ± 2.2^{a}	2.5 + 0.9^{b}	5.1 ± 1.9^{a}
Nox4	1.0 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	0.7 ± 0.3	0.8 ± 0.2
P22phox	1.0 ± 0.3^{c}	5.7 ± 1.2^{a}	3.7 ± 1.1^{b}	2.9 ± 0.8^{b}	3.4 ± 1.1^{b}
P40phox	1.0 ± 0.3^{d}	5.6 ± 1.3^{a}	4.5 ± 1.6^{a,b}	2.8 ± 0.6^{c}	3.9 ± 1.1^{b,c}
P47phox	1.0 ± 0.3^{c}	3.9 ± 1.2^{a}	2.9 ± 0.8^{a,b}	2.0 ± 0.3^{b,c}	3.0 ± 0.9^{a,b}
P67phox	1.0 ± 0.6^{b}	7.3 ± 4.4^{a}	2.3 ± 1.2^{b}	1.0 ± 0.7^{b}	3.1 ± 2.5^{b}
Rac1	1.0 ± 0.2^{b}	1.0 ± 0.2^{b}	1.0 ± 0.2^{b}	0.6 ± 0.1^{a}	0.9 ± 0.2^{b}
Fibrosis					
Procol1α1	1.0 ± 0.4^{b}	18 ± 6^{a}	16 ± 11^{a}	6.1 ± 3^{b}	10 ± 5^{a,b}
Tgfβ1	1.0 ± 0.1^{c}	2.5 ± 0.2^{a}	2.0 ± 0.5^{a,b}	1.8 ± 0.4^{b}	1.8 ± 0.5^{b}
Bambi	1.0 ± 0.2	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.3	1.0 ± 0.2
Timp1	1.0 ± 0.5^{c}	39 ± 11^{a}	20 ± 10^{b}	16 ± 10^{b}	19 ± 10^{b}
Pai1	1.0 ± 0.5^{c}	7.4 ± 1.6^{a}	4.6 ± 1.4^{b}	3.9 ± 1.0^{b}	4.3 ± 1.6^{b}

[1] Values are mean ± SD, n = 8/treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, nonpurified diet; WD + DHA, Western diet supplemented with DHA; WD + EPA, Western diet supplemented with EPA; WD + EPA + DHA, Western diet supplemented with EPA and DHA; WD + OO, Western diet supplemented with olive oil.

to oxidative stress; it accumulates in nuclei under conditions of oxidative stress and induces expression of multiple genes involved in the antioxidative response, including glutathione *S*-transferase (GST1α) and hemeoxygenase-1 (HMOX1) (34,35). Hepatic mRNA expression of *Nrf2* was induced in the WD + OO group; this induction was attenuated only in the WD + DHA group ($P < 0.05$) (Table 4). The nuclear abundance of hepatic NRF2 protein was not elevated in all WD groups regardless of C20–22 (n-3) PUFA ($P = 0.09$) (not shown). HMOX1 was induced under conditions of oxidative stress (36,37). *Hmox1* mRNA was induced (400%; $P < 0.05$) in the WD + OO group; this induction was moderately attenuated by the WD + EPA and WD + DHA diets ($P < 0.05$) (Table 4). Hepatic HMOX1 protein abundance, however, was elevated in all WD groups (**Supplemental Fig. 4**).

NADPH oxidase is an important pathway for the generation of reactive oxygen species and its role in NASH progression, especially fibrosis, has been well established (38–40). We measured hepatic mRNA abundance of several NADPH oxidase subunits (NOX), including *Nox1*, *Nox2* (*gp91*), *Nox4*, *Noxa1*, *Noxo1*, RAS-related C3 botulinum substrate 1 (*Rac1*), cytochrome b-245 light chain (*P22phox*), neutrophil cytosolic factor 4 (*P40phox*), neutrophil cytosolic factor 1 (*P47phox*), and neutrophil cytosolic factor 2 (*P67phox*) (Table 4). Of these, *Nox2*, *P22phox*, *P40phox*, *P47phox*, and *P67phox* were induced 400–600% ($P < 0.05$) in mice fed the WD + OO compared with NP. Of the C20-22 (n-3) PUFA diets examined, the WD + DHA was most effective at attenuating the WD + OO induction of these transcripts.

Hepatic fibrosis. Histological evidence of hepatic fibrosis was detected in mice fed the WD + OO and WD + EPA diets only. Fibrosis is represented by the blue staining of collagen (Supplemental Fig. 1). The mRNA encoding *Procol1α1* was elevated 1700% ($P < 0.01$) by WD + OO and 1500% in mice fed the WD + EPA compared with mice fed the NP. In mice fed the DHA- and EPA+DHA-containing diets, *Procol1α1* was induced 500 and 870%, respectively. This result indicates that DHA was more effective than EPA at reducing *Procol1α1* expression in WD-fed mice (Table 4). TGFβ-1 is a cytokine involved in the

activation of hepatic stellate cells and the production of *Procol1α1* (41,42). Hepatic *Tgfβ-1* mRNA was induced 150% ($P < 0.01$) by WD + OO feeding compared with the NP and this induction was attenuated ($P < 0.01$) by the WD + DHA and WD + EPA + DHA groups. As such, inclusion of DHA in the WD attenuated *Tgfβ-1* similar to *Procol1α1* (Table 4). BMP and activin membrane-bound inhibitor homolog (BAMBI) is a TGFβ1 pseudo-receptor and has been linked to development of fibrosis (31). However, there were no changes in *Bambi* expression in any of the WD groups. We quantified the mRNA encoding tissue inhibitor of metalloproteinase-1 (TIMP-1) and plasminogen activator inhibitor-1 (PAI-1). Both are involved in inhibiting extracellular matrix degradation and their overexpression is associated with progression of fibrosis (43,44). *Timp-1* and *Pai-1* hepatic mRNA were elevated 3780% ($P < 0.01$) and 640% ($P < 0.01$), respectively, by WD + OO feeding relative to NP. This WD-induced response was attenuated ($P < 0.01$) by all C20-22 (n-3) PUFA-containing groups (Table 4).

Discussion

The goal of this study was to examine the capacity of the major C20-22 (n-3) PUFA in menhaden oil, i.e., EPA and DHA, to attenuate the WD-mediated induction of hepatic markers associated with NASH markers in the $Ldlr^{-/-}$ mouse model. Our previous report established the requirement of using the HFHC + OO diet and the $Ldlr^{-/-}$ mouse to induce hepatosteatosis, inflammation, fibrosis, oxidative stress, and hepatic damage (15). In this report, we used the WD + OO diet and a longer feeding time in an effort to increase the severity of fatty liver disease in $Ldlr^{-/-}$ mice. Clearly, feeding $Ldlr^{-/-}$ mice the WD + OO diet for 16 wk significantly increased hepatosteatosis (TG and cholesterol), inflammation (*Clec4f*), fibrosis (*proCol1α1* and *Tgfβ1*), and hepatic damage (ALT) compared with $Ldlr^{-/-}$ mice fed the HFHC + OO diet for 12 wk (Supplemental Tables 3 and 4). Others using a WD of similar composition and $Ldlr^{-/-}$ mice have reported similar effects on the development of NASH markers (45). In addition to the longer feeding time, the WD + OO diet has a higher sucrose content (26.6% total energy) than the HFHC + OO diet (6.8% total energy). High fructose consumption promotes NASH and significantly alters liver metabolism (13,46).

Using the WD + OO diet and $Ldlr^{-/-}$ mouse model, we tested the hypothesis that DHA was more effective than EPA at preventing the induction of markers associated with NASH. We were surprised to find that no EPA- or DHA-containing diet attenuated WD + OO-induced hepatosteatosis (Table 1; Supplemental Figs. 1 and 2). Although details on the mechanism for this effect remain to be established, we speculate that the high fructose content of the WD overrides the effects of EPA and DHA on lipogenesis and TG synthesis and storage. Despite this outcome, both EPA and DHA attenuated multiple WD + OO-induced markers of inflammation, oxidative stress, and fibrosis. This outcome makes clear that a reduction of hepatosteatosis is not required to lower hepatic inflammation, oxidative stress, fibrosis, and hepatic damage.

Inflammation. Hepatic histology (Supplemental Fig. 1) and gene expression analysis indicated that EPA and DHA attenuated WD + OO-induced expression of *Mcp1*, cell surface markers for macrophages (*Cd68*, *Clec4f*, *Clec10a*, *F4/80*), cytokines (*Il1β*, *Tnfα*), and TLR signaling components (*Tlr2*, *Tlr4*, *Tlr9*, *Cd14*, and *Myd88* but not *Md-2*) (Table 4). No monocyte infiltration was observed in mice fed any of the WD + EPA or WD + DHA diets (Supplemental Fig. 1). In no case was the WD + EPA diet more effective than the WD + DHA diet at suppressing expression of markers of inflammation (Table 4). Notable among the transcripts examined are the TLR signaling components. *Tlr4*, *Tlr9*, and *Cd14* are major targets for DHA. TLR2 and TLR4 are plasma membrane receptors, whereas TLR9 is associated with endosomes in stellate cells (31). CD14 plays a role in antigen presentation to plasma membrane-associated TLR. Antigens for TLR activation are derived from hepatic necrosis, fatty acids, or bacteria, i.e., endotoxin. We are currently assessing these components as regulators of TLR signaling. A key downstream target of TLR is NFκB, a major transcription factor controlling expression of multiple genes involved in inflammation. As previously shown (15) and in Supplemental Figure 3, the WD + OO induced nuclear abundance of both NFκB-p50 and NFκB-p65. TLR regulates NFκB nuclear abundance at least in part by controlling cytosolic sequestration of NFκB subunits by regulating phosphorylation of NFκB inhibitor α and β (IκB α and β). Although regulation at this level was not observed, the results in Supplemental Table 3 suggest that DHA, but not EPA, controls NFκB-p50 by regulating its mRNA abundance.

Oxidative stress. Our previous report established that NASH was associated with the induction of hepatic expression of *Hmox1*, a marker of oxidative stress (15). This report expands the analysis of oxidative stress markers. The WD + OO group had increased hepatic expression of multiple markers of oxidative stress, including transcripts encoding *Nrf2*, *Gsta1*, *Hmox1*, and several NADPH oxidase components (*Nox2*, *Noxa1*, *Noxo1*, *P22phox*, *P40phox*, *P47phox*, *P67phox*). Because all WD-fed groups had similar elevations in NRF2 and HMOX1 protein abundance, it is unlikely these proteins are involved in the protective effects observed with dietary EPA or DHA. In contrast to the other markers, the expression of the WD + OO-mediated induction of NADPH oxidase subunits was attenuated most effectively by the WD + DHA group (Table 4). NADPH oxidase plays a major role in NASH, particularly in fibrosis (38). NADPH oxidase generates superoxide from molecular oxygen by using NADPH as an electron donor. The superoxide is converted to hydrogen peroxide by superoxide dismutase (47). Stellate cells from mice with ablated NOX1 or NOX2 have decreased generation of reactive oxidant species and do not show increased collagen expression (39). As previously observed, and in Table 4 and Supplemental Figure 1, collagen is a major target for induction by the HFHC + OO (15) or WD + OO diets; this response is suppressed by DHA. In our previous report (15), we suggested that C20-22 (n-3) PUFA in menhaden oil did not decrease the expression of genes linked to oxidative stress, i.e., *Hmox1*. Those studies provided a limited view of (n-3) PUFA effects on hepatic oxidative stress. The results presented in this report clearly show dietary C20-22 (n-3) PUFA selectively suppressed the expression of several NADPH oxidase subunits. The effects of EPA and DHA on NADPH oxidase subunits may be one mechanism for the attenuation of *Procol1α1* expression.

Fibrosis. Hepatic histological and gene expression evidence establish that the WD + OO feeding induced fibrosis. Trichrome staining of collagen in liver slices paralleled *Procol1α1* expression. TGFβ1 is a major regulator of fibrosis. Similar to *Procol1α1*, *Tgfβ1* was regulated most potently by inclusion of DHA in the WD. BAMBI is a TGFβ1 pseudo-receptor and a target of NFκB signaling (31). Despite the effect of the WD and dietary DHA on NFκB nuclear abundance, significant changes

in *Bambi* expression are not linked to WD or (n-3) PUFA regulation of fibrosis. *Timp1* induction is essential for the generation of fibrosis (48); TIMP1 essentially blocks the degradation of collagen. Although the WD + OO significantly induced *Timp1* nearly 40-fold, EPA and DHA are equally effective at repressing this response (Table 4). One explanation is that *Timp1* is not regulated by NFκB but by the RUNX1 and RUNX2 transcription factors (48). The effects of EPA and DHA on RUNX1 and 2 are unknown. Like TIMP1, PAI1 also inhibits collagen breakdown (44). Both *Pai1* and *Timp1* are induced by the WD + OO diet and repressed when the WD contains EPA or DHA.

The outcome of our analysis indicates that EPA and DHA are not equivalent in controlling multiple markers associated with NASH. In no case was DHA less effective than EPA in regulating markers of inflammation, fibrosis, or oxidative stress. In contrast, we identified several cases where EPA was less effective than DHA in regulating NASH markers. These effects can be attributed to different mechanisms of EPA and DHA regulation of specific genes. From a prevention perspective, however, DHA is quantitatively more effective at attenuating WD + OO-induced markers of NASH. Whereas dietary EPA significantly elevated hepatic EPA, it had only modest effects on hepatic DHA accumulation. In contrast, dietary DHA significantly induced both hepatic EPA and DHA (Table 2). Moreover, dietary DHA, more than EPA, lowered hepatic ARA content, at least in part by downregulating expression of enzymes involved in its synthesis, i.e., *Fads1*, *Fads2*, and *Elovl5* (Table 3). Our finding of differential impacts on hepatic fatty acid profiles between dietary EPA and DHA is consistent with human studies, i.e., the JELIS trial (23).

In conclusion, the WD + OO diet was used to induce markers associated with NASH in *Ldlr*$^{-/-}$ mice. We tested the hypothesis that dietary DHA was more effective than dietary EPA at preventing the induction of these markers. This is the first study to our knowledge that examines the capacity of EPA compared with DHA to regulate a wide range of markers linked to NASH. More importantly, EPA and DHA supplementation was used at a physiologically relevant dose (2% total energy) to examine their effects on fatty liver disease. Unlike our previous study (15), none of the C20-22 (n-3) PUFA-containing diets prevented diet-induced hepatosteatosis. This is likely due to high dietary fructose and the severity of hepatosteatosis induced following 16 wk of feeding the WD + OO diet. Thus, a reduction in hepatosteatosis is not required for EPA and DHA to attenuate WD + OO-induced hepatic damage, inflammation, oxidative stress, and fibrosis. DHA was more effective than EPA at attenuating inflammation, oxidative stress (NOX subunits), fibrosis, and hepatic damage. Based on these results, DHA may be a more attractive dietary supplement than EPA for the prevention and potential treatment of NASH in obese humans.

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