The Journal of Nutrition
Nutrition and Disease

Menhaden Oil Decreases High-Fat Diet–Induced Markers of Hepatic Damage, Steatosis, Inflammation, and Fibrosis in Obese *Ldlr*$^{-/-}$ Mice[1–3]

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Abstract

The frequency of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) has increased in parallel with obesity in the United States. NASH is progressive and characterized by hepatic damage, inflammation, fibrosis, and oxidative stress. Because C20–22 (n-3) PUFA are established regulators of lipid metabolism and inflammation, we tested the hypothesis that C20–22 (n-3) PUFA in menhaden oil (MO) prevent high-fat (HF) diet–induced fatty liver disease in mice. Wild-type (WT) and *Ldlr*$^{-/-}$ C57BL/6J mice were fed the following diets for 12 wk: nonpurified (NP), HF with lard (60% of energy from fat), HF–high-cholesterol with olive oil (HFHC-OO; 54.4% of energy from fat, 0.5% cholesterol), or HFHC-OO supplemented with MO (HFHC-MO). When compared with the NP diet, the HF and HFHC-OO diets induced hepatosteatosis and hepatic damage [elevated plasma alanine aminotransferase (ALT) and aspartate aminotransferases] and elevated hepatic expression of markers of inflammation (monocyte chemoattractant protein-1), fibrosis (procollagen 1α1), and oxidative stress (heme oxygenase-1) ($P \leq 0.05$). Hepatic damage (i.e., ALT) correlated ($r = 0.74$, $P < 0.05$) with quantitatively higher (>140%, $P < 0.05$) hepatic cholesterol in *Ldlr*$^{-/-}$ mice fed the HFHC-OO diet than WT mice fed the HF or HFHC-OO diets. Plasma and hepatic markers of liver damage, steatosis, inflammation, and fibrosis, but not oxidative stress, were lower in WT and *Ldlr*$^{-/-}$ mice fed the HFHC-MO diet compared with the HFHC-OO diet ($P < 0.05$). In conclusion, MO [C20–22 (n-3) PUFA at 2% of energy] decreases many, but not all, HF diet–induced markers of fatty liver disease in mice. J. Nutr. 142: 1495–1503, 2012.

Introduction

Nonalcoholic fatty liver disease (NAFLD)[7] has increased in parallel with central obesity, and its prevalence is anticipated to continue to increase (1,2). NAFLD is now the most common cause of liver disease in developed countries (3) and is defined as excessive lipid accumulation in the liver, i.e., hepatosteatosis (4,5). The American Liver Foundation estimates that ~25% of the U.S. population has NAFLD and 75% of obese and 100% of morbidly obese individuals have NAFLD. NAFLD is the hepatic manifestation of metabolic syndrome (MetS) (4); MetS risk factors include obesity, elevated plasma TG and LDL cholesterol, reduced HDL cholesterol, high blood pressure, and fasting hyperglycemia (5).

NAFLD ranges in severity from simple fatty liver (steatosis) to nonalcoholic steatohepatitis (NASH) (6). Simple steatosis is relatively benign until it progresses to NASH, which is characterized by hepatic injury (hepatocyte ballooning and cell death), increased blood levels of hepatic enzymes [alanine aminotransferase (ALT)], hepatic inflammation, oxidative stress, and fibrosis (1,2,7). Approximately 30–40% of individuals with simple steatosis progress to NASH (8), and NASH can progress to cirrhosis (8), which is a major risk factor for hepatocellular carcinoma (2). In the "2 hit hypothesis" for NASH (9), the first hit involves chronic hepatosteatosis as TG and cholesterol (free

[1] Supported by the USDA, National Institute of Food and Agriculture grant 2009-65200-05846, and NIH grant DK-43220.
[2] Author disclosures: C. M. Depner, M. Torres-Gonzalez, S. Tripathy, G. Milne, and D. B. Jump, no conflicts of interest.
[3] Supplemental Figures 1 and 2 and Supplemental Tables 1–3 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.
[7] Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ChREBP, carbohydrate response element binding protein; DNL, de novo lipogenesis; HF, high-fat; HFHC, high-fat, high cholesterol; LF, low-fat; LXR, liver X receptor; MetS, metabolic syndrome; MLX, max-like protein X; MO, menhaden oil; OO, olive oil; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, nonesterified fatty acid; NP, nonpurified; ROS, reactive oxygen species; SREBP1, sterol regulatory element binding protein-1; WT, wild-type.
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Manuscript received January 26, 2012. Initial review completed March 5, 2012. Revision accepted May 3, 2012.
First published online June 27, 2012; doi:10.3945/jn.112.158865.

cholesterol and cholesterol esters) accumulation. Excessive hepatic lipid sensitizes hepatocytes to the second hit, which is manifested by increased inflammation derived from resident (Kupffer cells) and recruited macrophages, induction of oxidative stress, activation of stellate cells, and fibrosis (2,10).

Although the 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 (11). On the basis of the well-established effects of C20–22 (n-3) PUFA to regulate hepatic lipid metabolism, dyslipidemia, and inflammation (12–14), we tested the hypothesis that dietary (n-3) PUFA prevents high-fat (HF) diet–induced fatty liver disease in mice. Recent clinical studies have indicated that dietary (n-3) PUFA have the potential to reduce hepatic lipid content in children and adults (15–19). Our studies, however, go beyond the analysis of hepatic lipids and examine the capacity of (n-3) PUFA to regulate markers of NASH, such as hepatic damage, inflammation, oxidative stress, and fibrosis. We used wild-type (WT) and $Ldlr^{-/-}$ mice and 3 HF diets: HF lard (HF diet; 60% of energy from fat), which induces obesity and insulin resistance (20,21), HF–high cholesterol with olive oil (HFHC-OO diet; 54.4% of energy from fat, 0.5% cholesterol), which induces fatty liver and oxidative stress (22), and the HFHC-OO diet supplemented with menhaden oil (MO) (HFHC-MO) a rich source of EPA [20:5(n-3)] and DHA [22:6(n-3)]. EPA and DHA in the HFHC-MO diet were 2% of total energy, a level comparable to that used to treat dyslipidemia (23). Our studies established that dietary C20–22 (n-3) PUFA have the capacity to regulate some, but not all, HF diet–induced markers of NASH.

Materials and Methods

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 WT and $Ldlr^{-/-}$ mice (on the C57BL/6J background; Jackson Laboratories) at 2 mo of age consumed one of the following diets ad libitum for 12 wk: *1*) Purina chow 5001 [nonpurified (NP); 13.5% of energy from fat and 58.0% of energy from carbohydrates]; *2*) HF [60% of energy from fat; D12492; Research Diets]; *3*) HFHC-OO [54.4% of energy from fat, 0.5% cholesterol (weight%); D08010702; Research Diets]; or *4*) HFHC-MO [54.4% of energy from fat, 0.5% cholesterol (weight%); D08010703; Research Diets] (**Supplemental Table 1**). The HF, HFHC-OO, and HFHC-MO diets were described previously (21,22). Fat energy density in the HFHC-OO and HFHC-MO diets was identical (54.4% energy from fat). C20–22 (n-3) PUFA in the HFHC-MO diet represented ~2% of total energy (Supplemental Table 1). All diets were stored frozen (−20°C) until used to feed the mice; diets were replenished every other day in an effort to reduce the formation of oxidation products.

The study was carried out twice with 8 mice/diet group in each study. Energy intake was monitored every other day, and body weight was monitored weekly. At the end of the 12-wk feeding period, all mice were feed-deprived overnight (1800 to 0800 the next day); then half of the mice were refed their diets for 4 h (0800 to 1200). Feed-deprived and refed mice were killed (isoflurane anesthesia and exsanguination) at 0800 and 1200, respectively, for the collection of blood and liver. Blood was collected in tubes containing EDTA; plasma was collected by centrifugation. Livers were removed, weighed, and rapidly frozen in liquid nitrogen. Plasma, blood cells, and liver were stored frozen (−80°C) until used for specific assays.

Our studies used WT and $Ldlr^{-/-}$ mice fed the NP diet as controls. Because there was no significant difference (Student's t test) in any variable measured between WT and $Ldlr^{-/-}$ mice fed the NP diet, NP-fed $Ldlr^{-/-}$ mice served as the control group for all studies described below. The following 6 group designations are used to describe the study: $Ldlr^{-/-}$ mice fed NP (NP), WT mice fed HF (WT-HF), WT mice fed HFHC-OO (WT-HFHC-OO), WT mice fed HFHC-MO (WT-HFHC-MO), $Ldlr^{-/-}$ mice fed HFHC-OO ($Ldlr^{-/-}$ HFHC-OO), and $Ldlr^{-/-}$ mice fed HFHC-MO ($Ldlr^{-/-}$ HFHC-MO). During the course of our studies, we found that markers of NASH were induced to higher levels in $Ldlr^{-/-}$ mice compared with in WT mice. As such, much of the data presented below describe the capacity of MO to regulate NASH markers in $Ldlr^{-/-}$ mice. A comparison of the effects of the 4 diets on inflammation and fibrosis markers in WT and $Ldlr^{-/-}$ mice is shown in **Supplemental Table 3**.

Measurement of plasma markers. Plasma glucose (Autokit Glucose), TG (L-type TG H triglyceride), nonesterified fatty acids (NEFA; NEFA-C), and cholesterol (Cholesterol E) were measured with the use of kits from Wako. Plasma β-hydroxybutyrate was measured with the use of a kit (β-hydroxybutyrate Liquicolor) from Stanbio. Plasma apo B (ApoB K-Assay) and apo CIII (ApoCIII K-Assay) were measured by immunoturbidimetric assay from Kamiya Biomedical. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by using kits from Thermo Electron.

Measurement of urinary isoprostanes. After 11 wk of being fed the NP or HFHC diets, $Ldlr^{-/-}$ mice were placed in metabolic cages for 24-h urine collection. Collected urine was stored at <80°C until extracted for F2- and F3-isoprostanes and F4-neuroprostanes analysis. Results were normalized to urinary creatinine as described (22,24).

Lipid extraction and analyses. Total lipids were extracted from liver and plasma; FAME were prepared and quantified by gas chromatography (20). To measure hepatic total TG and cholesterol, extracted hepatic lipids were dried, dissolved in 10% Triton X-100 (Fisher Scientific), and assayed for TG and cholesterol content using the L-type TG H triglyceride and total cholesterol assay kits from Wako as described above (20). Hepatic protein was measured by using the Quick Start Bradford Reagent (Bio-Rad) and bovine serum albumin (Sigma) as the standard (20).

RNA extraction and qRT-PCR. Total RNA was extracted from liver, and specific mRNA were quantified by qRT-PCR (20,25). Specific primers for each gene were described previously (20,25) or are listed in **Supplemental Table 2**. Cyclophilin was used as the internal control for all genes.

Immunoblotting. Hepatic nuclear protein extracts were prepared by using both protease (Roche Diagnostics) and phosphatase inhibitors (1 mmol/L β-glycerol phosphate, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L Na_3VO_4) (20). Proteins (25–100 μg) were separated electrophoretically and transferred to nitrocellulose for immunoblotting. TBP (TATA-binding protein) served as a loading control for all immunoblot studies. Antibodies used in these studies were described previously (20); antibodies for NF-κB-p50 and NF-κB-p65 were purchased from Santa Cruz Biotechnology and Cell Signaling, respectively.

Statistical analysis. We used 1- and 2-way ANOVA and Tukey's honestly significant difference post hoc analysis to establish significant differences. One-way ANOVA was used to detect dietary effects when only $Ldlr^{-/-}$ mice were included in the analysis. Two-way ANOVA was used to detect diet-gene interactions, when both WT and $Ldlr^{-/-}$ mice were included in the analysis and for the analysis of refeeding effects (**Fig. 1*A***). Data were analyzed for homogeneous variances by using 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. The Student's t test was used when only 2 groups were compared. $P < 0.05$ was considered different. The correlation analysis used linear regression analysis. Statistical analysis was performed with VassarStats (http://vassarstats.net/) and Statgraphics (StatPoint Technologies, Inc.). All values reported are means ± SD.

Results

Body weight and plasma markers. After 12 wk of being fed the NP and test diets, the body weights of WT mice fed the HF or HFHC diets and $Ldlr^{-/-}$ mice fed the HFHC diets were >39%

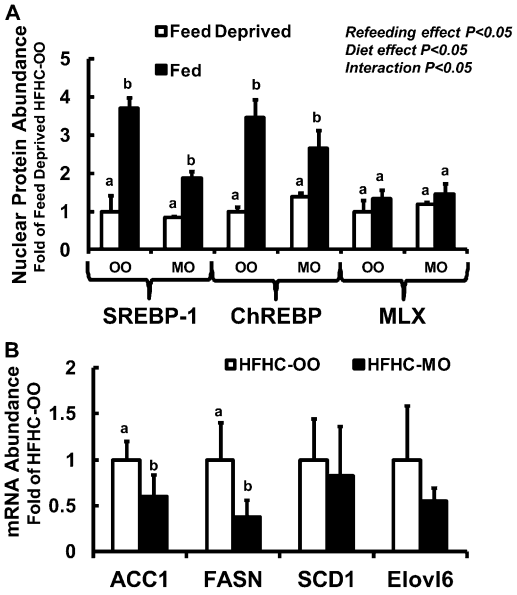

FIGURE 1 The feed-deprived feeding response of hepatic nuclear SREBP1, ChREBP, and MLX (*A*) and hepatic lipogenic gene expression (*B*) in $Ldlr^{-/-}$ mice fed the HFHC-OO or HFHC-MO diets for 12 wk. (*A*) After 12 wk of being fed these diets, the mice were deprived of food overnight. At 0800 the next day, one-half of the mice in each group were fed their diets, i.e., HFHC-OO or HFHC-MO. Feed-deprived and fed mice were killed at 0800 and 1200, respectively, for the quantitation of hepatic nuclear SREBP1, ChREBP, and MLX. Mean (±SD) results are expressed as nuclear protein abundance, fold of feed-deprived HFHC-OO; n = 8. Data were analyzed by 2-way ANOVA. Labeled means without a common letter differ, $P < 0.05$. (*B*) Hepatic RNA from the fed mice described above was used to quantify *Acc1*, *Fasn*, *Scd1*, and *Elovl6* mRNA by qRT-PCR. Mean (±SD) results are expressed as mRNA abundance, fold of HFHC-OO; n = 8. Data were analyzed by Student's t test. Labeled means without a common letter differ, $P < 0.05$. ACC1, acetyl CoA carboxylase-1; ChREBP, carbohydrate response element binding protein; Elov16, fatty acid elongase-6; FASN, fatty acid synthase; HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; MLX, max-like protein X; SCD1, stearoyl CoA desaturase-1; SREBP-1, sterol regulatory element binding protein-1.

higher than those of the NP group ($P < 0.05$) (**Table 1**). Body weights of WT and $Ldlr^{-/-}$ mice were not different between the groups fed the HF, HFHC-OO, or HFHC-MO diets. Plasma markers were measured in feed-deprived mice. Diet and genotype did not significantly affect plasma glucose. Plasma TG were >190% higher in the $Ldlr^{-/-}$ HFHC-OO group compared with the NP group ($P < 0.05$). Plasma TG in the $Ldlr^{-/-}$ HFHC-MO group were ~50% lower than in the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$) and not different from the NP group. Analysis of plasma cholesterol showed a diet effect ($P < 0.05$); plasma cholesterol in all mice fed the HFHC-OO diet was >60% higher than in all mice fed the HFHC-MO diet. There was also a genotype effect ($P < 0.05$): plasma cholesterol in all $Ldlr^{-/-}$ mice was >330% higher than in all WT mice. No significant diet-by-genotype interaction for plasma cholesterol was observed. Plasma apo B and apo CIII paralleled diet-induced changes in plasma TG and cholesterol in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups. When compared with the NP group, plasma NEFA was nearly 90% higher in the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$). Finally, plasma β-hydroxybutyrate was >85% higher in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups when compared with the NP group ($P < 0.05$).

Hepatic damage. Plasma ALT and AST are markers of hepatic damage; these markers were measured in feed-deprived mice. When compared with the NP group, ALT was elevated 100% in the WT and $Ldlr^{-/-}$ mice fed the HF, HFHC-OO, or HFHC-MO diets ($P < 0.05$), whereas AST was elevated 30% in the WT-HFHC-OO, $Ldlr^{-/-}$ HFHC-OO, and $Ldlr^{-/-}$ HFHC-MO groups ($P < 0.05$) (Table 1). Plasma ALT and AST in the $Ldlr^{-/-}$ HFHC-OO group were 90% and 46% higher, respectively, than in the WT-HFHC-OO group ($P < 0.05$). Plasma AST was lower ($P < 0.05$) and ALT tended to be lower ($P = 0.12$) in the $Ldlr^{-/-}$ HFHC-MO group by ~50% compared with the $Ldlr^{-/-}$ HFHC-OO group.

Hepatic lipids. Hepatic TG in the WT-HF, WT-HFHC-OO, and $Ldlr^{-/-}$ HFHC-OO groups were 150% higher than in the NP group ($P < 0.05$) (Table 1). This level of TG accumulation is comparable to that in WT mice fed the HF diet (20,26,27). Hepatic TG in the WT-HFHC-MO and $Ldlr^{-/-}$ HFHC-MO groups were not different from the NP group. Hepatic cholesterol was >140% higher only in the $Ldlr^{-/-}$ HFHC-OO group when compared with the NP group ($P < 0.05$) (Table 1). Supplementing the HFHC-OO diet with C20–22 (n-3) PUFA (i.e., the HFHC-MO diet) prevented the HFHC-OO diet–induced accumulation of hepatic TG and cholesterol in $Ldlr^{-/-}$ mice.

The level of plasma ALT correlated with hepatic cholesterol ($r = 0.74$, $P < 0.05$), but not with hepatic TG, in WT and $Ldlr^{-/-}$ mice. This outcome agrees with recent studies linking hepatic cholesterol to NASH (26–28). Because hepatic damage, as reflected by plasma ALT, was quantitatively greater by 90% in the $Ldlr^{-/-}$ HFHC-OO group compared with the WT-HFHC-OO group ($P < 0.05$) (Table 1), the studies described below examined the effects of the HFHC-OO and HFHC-MO diets on hepatic fatty acids and markers of NASH in $Ldlr^{-/-}$ mice (i.e., oxidative stress, inflammation, and fibrosis).

Hepatic fatty acids. Hepatic fatty acid composition is significantly affected by diet. Because the HFHC-OO and HFHC-MO diets had similar affects on hepatic fatty acid profiles in WT and $Ldlr^{-/-}$ mice, only the $Ldlr^{-/-}$ data are shown (**Table 2**). Oleic acid [18:1(n-9)] is the predominant fatty acid in the HFHC-OO diet (Supplemental Table 1); the mole% of hepatic oleic acid was >260% and >150% higher in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups, respectively, when compared with the NP group ($P < 0.05$). Hepatic oleic acid in the $Ldlr^{-/-}$ HFHC-MO group was >30% lower than in the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$). Hepatic 20:4(n-6) was 48% and 68% lower in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups, respectively, when compared with the NP group ($P < 0.05$). Hepatic C20–22 (n-3) PUFA were ~70% lower in the $Ldlr^{-/-}$ HFHC-OO group compared with the NP group ($P < 0.05$), but was >200% higher in the $Ldlr^{-/-}$ HFHC-MO group ($P < 0.05$). The ratio of 20:4(n-6) to 18:2(n-6) or 20:3(n-6) to 18:2(n-6) was the same in both the NP and HFHC-OO groups, but was 50% lower in the $Ldlr^{-/-}$ HFHC-MO group ($P < 0.05$) (Table 2). Similar diet effects were seen in plasma C20–22 (n-3) PUFA and the ratio of 20:4(n-6) to 18:2(n-6) (**Supplemental Fig. 1**) as well as in liver and plasma of WT mice (not shown).

Whole-body and hepatic oxidative stress. F2- and F3-isoprostanes and F4-neuroprostanes are nonenzymatic oxidation products of 20:4(n-6), 20:5(n-3), and 22:6(n-3), respectively (22,24). Because urinary isoprostanes are a measure of whole-body oxidative stress (29), we analyzed urinary isoprostanes (F2 and F3) and neuroprostanes (F4) in the NP, $Ldlr^{-/-}$ HFHC-OO,

TABLE 1 Phenotypic comparison of WT and *Ldlr*$^{-/-}$ mice fed the NP, HF, HFHC-OO, or HFHC-MO diets for 12 wk[1]

	WT				*Ldlr*$^{-/-}$	
	NP[2]	HF	HFHC-OO	HFHC-MO	HFHC-OO	HFHC-MO
Body weight,[3] *g*	28 ± 2^{a}	45 ± 4^{b}	43 ± 1^{b}	39 ± 2^{b}	40 ± 5^{b}	40 ± 5^{b}
Plasma variables[4]						
Glucose, *mmol/L*	6.0 ± 1.0	9.3 ± 2.7	8.3 ± 2.8	5.6 ± 0.7	11.9 ± 4.6	11.0 ± 4.6
TG,[5,6] *mg/dL*	120 ± 44^{a}	90 ± 16^{a}	66 ± 27^{a}	106 ± 25^{a}	352 ± 141^{b}	185 ± 82^{a}
Cholesterol,[3,5] *mg/dL*	52 ± 21^{a}	108 ± 46^{a}	138 ± 66^{b}	81 ± 6^{a}	957 ± 252^{b}	599 ± 145^{b}
Apo B,[5,6] *mg/dL*	11 ± 2^{a}	14 ± 2^{a}	15 ± 4^{a}	14 ± 2^{a}	160 ± 70^{b}	50 ± 21^{a}
Apo CIII,[5,6] *mg/dL*	13 ± 3^{a}	13 ± 7^{a}	10 ± 4^{a}	8 ± 2^{a}	21 ± 6^{b}	11 ± 6^{a}
NEFA,[5,6] *mEq/ml*	0.8 ± 0.2^{a}	0.7 ± 0.2^{a}	0.6 ± 0.1^{a}	0.7 ± 0.3^{a}	1.5 ± 0.6^{b}	0.9 ± 0.4^{a}
β-Hydroxybutyrate,[5] *mmol/L*	1.4 ± 0.3^{a}	1.3 ± 0.4^{a}	1.5 ± 0.3^{a}	1.7 ± 0.2^{a}	2.9 ± 0.8^{b}	2.6 ± 0.5^{b}
ALT,[3,5,6] *U/L*	4 ± 1^{a}	19 ± 10^{b}	20 ± 11^{b}	8 ± 2^{b}	38 ± 9^{c}	21 ± 16^{b,c}
AST,[3,5,6] *U/L*	10 ± 2^{a}	18 ± 2^{a}	30 ± 12^{b}	13 ± 2^{a}	56 ± 1^{c}	27 ± 12^{b}
Liver variables						
Weight, *g*	1.0 ± 0.1	1.6 ± 0.5	1.9 ± 0.5	1.2 ± 0.1	2.0 ± 0.6	1.6 ± 0.4
% Body weight[3]	3.6 ± 0.1^{a}	3.4 ± 1.0^{a}	4.5 ± 1.2^{b}	3.0 ± 0.2^{a}	4.8 ± 0.9^{b}	3.9 ± 0.7^{a}
TG,[3,7] μ*g/mg*	51 ± 23^{a}	157 ± 88^{b}	141 ± 63^{b}	72 ± 15^{a}	129 ± 13^{b}	64 ± 26^{a}
Cholesterol,[3,5,6,8] μ*g/mg*	7 ± 1^{a}	6 ± 2^{a}	8 ± 1^{a}	8 ± 1^{a}	17 ± 3^{b}	10 ± 5^{a}

[1] Values are means ± SD; n = 8/treatment group. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HF, high-fat; HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; NEFA, nonesterified fatty acid; NP, nonpurified; WT, wild-type.
[2] NP *Ldlr*$^{-/-}$ mice were used as the control group.
[3] Two-way ANOVA diet effect, $P < 0.05$.
[4] To convert from mg/dL to g/L, multiply by 0.0113 for TG and 0.02586 for cholesterol. To convert from mg/dL to g/L, multiply by 0.01 for apo B and apo CIII. To convert from mEq/mL to mEq/L, multiply by 1000 for NEFA.
[5] Two-way ANOVA genotype effect, $P < 0.05$.
[6] Two-way ANOVA diet × gene interaction effect, $P < 0.05$.
[7] Data are expressed as μg TG/mg protein (liver); to convert from μg/mg to mmol/g, multiply by 0.00113.
[8] Data are expressed as μg cholesterol/mg protein (liver); to convert from μg/mg to mmol/g, multiply by 0.002586.

and *Ldlr*$^{-/-}$ HFHC-MO groups (22,24) (**Table 3**). F2- and F3-isoprostanes and F4-neuroprostanes in urine from the NP group were comparable to previous reports (30). F2-isoprostanes were not elevated in urine of the *Ldlr*$^{-/-}$ HFHC-OO group, but increased by >110% in the *Ldlr*$^{-/-}$ HFHC-MO compared with the NP group ($P < 0.05$). Although urinary F3-isoprostanes and F4-neuroprostanes were not significantly affected by the HFHC-OO diet, these oxidized lipids were >490% and >40% higher, respectively, in the urine of the *Ldlr*$^{-/-}$ HFHC-MO group when compared with the NP and *Ldlr*$^{-/-}$ HFHC-OO groups ($P < 0.05$). Supplementing the HFHC-OO diet with C20–22 (n-3) PUFA (i.e., the HFHC-MO diet) increased lipid peroxidation as measured by urinary F2- and F3-isoprostanes and F4-neuroprostanes.

To assess hepatic oxidative stress, we measured hemeoxygenase-1 (*Hmox1*) expression (**Table 4**). *Hmox1* is induced in NAFLD in response to oxidative stress (31–36); it is also cytoprotective (31–33,37). Hepatic *Hmox1* mRNA was induced >300% and >200% ($P < 0.05$) in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups, respectively, when compared with the NP group. Supplementing the HFHC-OO diet with MO did not lower HFHC-OO–induced hepatic oxidative stress (i.e., *Hmox1* expression) in *Ldlr*$^{-/-}$ mice.

Lipid-regulated hepatic transcription factors and gene expression. Transcription factors controlling de novo lipogenesis (DNL), MUFA, and TG synthesis include sterol regulatory element binding protein-1 (SREBP1), carbohydrate response element binding protein (ChREBP), max-like protein X (MLX), liver X receptor (LXR), peroxisome proliferator-activated receptor α (PPARα), and hepatic nuclear factor 4α (12). We examined the effect of feed-deprivation and refeeding on the nuclear abundance of these transcription factors in *Ldlr*$^{-/-}$ mice maintained on the HFHC-OO or HFHC-MO diets (Fig. 1). The nuclear abundance of SREBP1 and ChREBP, but not MLX, was increased by 250% ($P < 0.05$) in *Ldlr*$^{-/-}$ mice 4 h after refeeding the HFHC-OO group. The nuclear abundance of SREBP1 in the *Ldlr*$^{-/-}$ HFHC-MO group increased by 100% 4 h after refeeding ($P < 0.05$). This level of induction, however, is 60% lower when compared with the *Ldlr*$^{-/-}$ HFHC-OO group ($P < 0.05$) (Fig. 1*A*). Because the induction of *Srebp-1* mRNA abundance was not different between the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups (**Supplemental Fig. 2**), MO suppression of hepatic SREBP1 nuclear abundance involved a post-translational mechanism (38). The refeeding response of ChREBP was not affected by MO. The nuclear abundance of other transcription factors controlling hepatic metabolism (i.e., LXRα, PPARα, HNF4α, and forkhead box protein O1,) remained unaffected by diet (not shown).

We next measured the abundance of *Acc1*, *Fasn*, *Scd1*, and *Elovl6* mRNA in livers of the refed mice described above. Of these transcripts, hepatic abundance of *Acc1* and *Fasn* was suppressed by 50% ($P < 0.05$) in the *Ldlr*$^{-/-}$ HFHC-MO group compared with the *Ldlr*$^{-/-}$ HFHC-OO group (Fig. 1*B*). PPARα regulates the expression of multiple genes involved in fatty acid oxidation (12), including acyl CoA oxidase (*Aox*), CYP450-4A10 (*Cyp4A10*), acyl-CoA thioesterase-1 (*Acot1*), carnitine palmitoyl transferase 1 (*Cpt1*), and *Cpt2*. None of these transcripts were affected by the HFHC-OO or HFHC-MO diets when compared with the NP diet (not shown).

Enzymes involved in converting essential fatty acids [18:2(n-6) and 18:3(n-3)] to C20–22 (n-6) and (n-3) PUFA include fatty acid

TABLE 2 Hepatic fatty acid composition in $Ldlr^{-/-}$ mice fed the NP, HFHC-OO, or HFHC-MO diets for 12 wk[1]

	NP	HFHC-OO	HFHC-MO
Fatty acids, *mol%*			
16:0	26.9 ± 1.0[a]	18.0 ± 1.9[b]	14.0 ± 4.7[b]
16:1(n-7)	2.2 ± 0.7	3.4 ± 0.9	2.2 ± 1.9
18:0	11.7 ± 2.7[a]	2.4 ± 0.5[b]	4.0 ± 2.3[b]
18:1(n-9)	15.2 ± 1.8[a]	55.0 ± 4.3[b]	37.6 ± 3.7[c]
18:1(n-7)	3.1 ± 0.3[a]	7.3 ± 1.2[b]	5.1 ± 1.1[c]
18:2(n-6)	24.7 ± 2.8[a]	14.2 ± 1.6[b]	16.4 ± 3.0[b]
18:3(n-3)	0.8 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:0	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1
20:2(n-6)	0.2 ± 0.1[a]	1.8 ± 0.1[b]	0.8 ± 0.2[a]
20:3(n-6)	0.7 ± 0.2	0.6 ± 0.1	0.4 ± 0.1
20:4(n-6)	6.2 ± 0.1[a]	3.2 ± 0.7[b]	2.0 ± 1.1[b]
20:5(n-3)	0.6 ± 0.1[a]	0.2 ± 0.1[a]	7.9 ± 2.3[b]
22:5(n-6)	0.2 ± 0.1	0.8 ± 0.2	0.6 ± 0.2
22:5(n-3)	0.6 ± 0.1[a]	0.2 ± 0.1[a]	2.0 ± 0.3[b]
22:6(n-3)	5.1 ± 0.9[a]	1.4 ± 0.3[b]	9.6 ± 3.8[c]
Σ C20–22[2]			
C20–22(n-3)	6.3 ± 0.6[a]	1.8 ± 0.3[b]	19.5 ± 3.3[c]
C20–22(n-6)	7.3 ± 1.3[a]	6.4 ± 0.8[a]	3.7 ± 1.1[b]
Fatty acid ratios			
20:4(n-6) to 18:2(n-6)	0.26 ± 0.07[a]	0.27 ± 0.08[a]	0.14 ± 0.09[b]
20:3(n-6) to 18:2(n-6)	0.04 ± 0.02[a]	0.05 ± 0.01[a]	0.02 ± 0.01[b]

[1] Values are means ± SD and are representative of 2 separate studies; n = 4/treatment group. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; NP, nonpurified.
[2] Sum C20-22 refers to the sum of all the 20 to 22 carbon fatty acids analyzed.

elongases (*Elovl2* and *Elovl5*) and desaturases (*Fads1* and *Fads2*). Expression of these enzymes is controlled, at least in part, by SREBP1 and PPARα (12,39–41). Hepatic *Elovl2* was induced 120% ($P < 0.05$) in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups when compared with the NP group (Table 4). Hepatic *Elovl5* and *Fads1* mRNA were reduced by 70–80% ($P < 0.05$) in the $Ldlr^{-/-}$ HFHC-MO group compared with the NP or $Ldlr^{-/-}$ HFHC-OO groups. Hepatic *Fads2* was not affected by diet. Changes in *Elovl5* and *Fads1* expression paralleled the decline in the hepatic 20:4/18:2 ratio in $Ldlr^{-/-}$ mice fed the HFHC-MO diet (Table 2).

Because the combination of the HFHC-OO diet and the $Ldlr^{-/-}$ genotype significantly increased hepatic cholesterol (Table 1), we examined pathways controlling hepatic cholesterol content (Table 4). The mRNA abundance of SREBP2 target genes (*Hmg CoA red* and *Hmg CoA syn1*) was not affected. Of the LXR target genes examined, only the ABC transporters (*Abca1*, *Abcg5*) were induced 200% in livers of $Ldlr^{-/-}$ mice fed the HFHC-OO and HFHC-MO diets when compared with the NP group ($P < 0.05$). *Acat1* is expressed in Kupffer cells (42), whereas *Acat2* is expressed in hepatocytes (43). The expression of hepatic *Acat1* and *Acat2* mRNA was induced 380% and 100%, respectively, by the HFHC-OO diet when compared with the NP diet. Hepatic *Acat1* mRNA, however, was 38% lower in the $Ldlr^{-/-}$ HFHC-MO group when compared with the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$) (Table 4).

Hepatic inflammation. We next examined markers of inflammation. Monocyte chemoattractant protein-1 (*Mcp1*) mRNA, an early marker of inflammation (44), was induced 600% in the WT-HF and WT-HFHC-OO groups ($P < 0.05$) (**Supplemental Table 3**) and >2000% in the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$) when compared with the NP group (Table 4). Furthermore, *Mcp1* mRNA was >170% higher in the $Ldlr^{-/-}$ HFHC-OO group compared with the WT-HFHC-OO group ($P < 0.05$). In contrast to the HFHC-OO diet, the HFHC-MO diet did not induce hepatic *Mcp1* mRNA in either genotype (Table 4, Supplemental Table 3).

Cd68 and *Clec4f* are cell surface markers of monocytes and resident macrophages (Kupffer cells), respectively. Hepatic *Cd68* mRNA was induced 170% in the WT-HFHC-OO group, >600% in the $Ldlr^{-/-}$HFHC-OO group, and >300% ($P < 0.05$) in the $Ldlr^{-/-}$ HFHC-MO group when compared with the NP group (all $P < 0.05$) (Table 4, Supplemental Table 3). A comparison of the $Ldlr^{-/-}$ HFHC-OO and the $Ldlr^{-/-}$ HFHC-MO groups had a ~50% reduction in *Cd68* mRNA ($P < 0.05$) (Table 4). *Clec4f* mRNA was elevated by >500% and >370% in the $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$HFHC-MO groups, respectively, compared with the NP group ($P < 0.05$). Hepatic *Clec4f* was not induced by either HF or HFHC-OO diet in WT mice (Supplemental Table 3).

NF-κB-p50 and NF-κB-p65 are transcription factors that control the expression of multiple genes involved in inflammation (45). We examined the hepatic nuclear abundance of NF-κB-p50 and NF-κB-p65 in $Ldlr^{-/-}$ mice fed the NP, HFHC-OO, and HFHC-MO diets. The nuclear content of NF-κB-p50 and NF-κB-p65 was elevated 100% and ~200%, respectively in mice fed the HFHC-OO diet compared with the NP diet (both $P < 0.05$). While mice fed the NP or HFHC-MO diets had comparable levels of nuclear NF-κB-p50, levels of NF-κ-B-p65 in the HFHC-MO group remained elevated compared with the NP group, similar to the HFHC-OO fed group (**Fig. 2**).

Hepatic fibrosis. Markers of fibrosis examined in this study include TGF-β1 (*Tgfβ1*) and procollagen 1α1 (*Procol1α1*) (46–49). These proteins are expressed in Kupffer and stellate cells but not in hepatic parenchymal cells. *Tgfβ1* mRNA was not induced in the WT-HF or WT-HFHC-OO groups compared with the NP group (Supplemental Table 3) but was induced 200% and 110% in $Ldlr^{-/-}$mice fed the HFHC-OO and HFHC-MO diets, respectively ($P < 0.05$) (Table 4). *Procol1α1* mRNA was induced 1400%, 4200%, and 1900% in the WT-HFHC-OO, $Ldlr^{-/-}$ HFHC-OO, and $Ldlr^{-/-}$ HFHC-MO groups, respectively ($P < 0.05$) (Supplemental Table 3; Table 4). *Procol1α1* mRNA abundance in the WT-HFHC-MO and NP groups was not different. *Procol1α1* mRNA abundance in the $Ldlr^{-/-}$ HFHC-MO group, however, was >50% lower when compared with the $Ldlr^{-/-}$ HFHC-OO group ($P < 0.05$).

Discussion

We examined the effect of 3 HF diets on the development of fatty liver disease in WT and $Ldlr^{-/-}$ mice. WT mice fed the HF and HFHC-OO diets and $Ldlr^{-/-}$ mice fed the HFHC-OO diet for 12 wk had increased body weight (39%) and multiple markers of dyslipidemia and NASH, including hepatic damage (plasma ALT and AST), hepatosteatosis, oxidative stress [urinary isoprostanes and hepatic *Hmox1* ($Ldlr^{-/-}$ only)], inflammation (mRNA encoding *Mcp1*, *Cd68*, *Clec4f*), and fibrosis (*Tgfβ1* and *Procol1α1)* (Tables 1, 3, and 4; Supplemental Table 3). Although hepatic TG content increased equally in the WT-HF, WT-HFHC-OO, and $Ldlr^{-/-}$ HFHC-OO groups, the excess accumulation of hepatic cholesterol in $Ldlr^{-/-}$ mice correlated with hepatic damage (plasma ALT vs. hepatic cholesterol; $r = 0.74$, $P < 0.05$) and the induction of markers of inflammation and fibrosis. Thus, the combination of diet lipid composition and genotype contributed to the severity of fatty liver disease in mice.

TABLE 3 Urinary iso- and neuroprostanes in $Ldlr^{-/-}$ mice fed the NP, HFHC-OO, or HFHC-MO diets for 12 wk[1]

	NP	HFHC-OO	HFHC-MO
		ng/mg creatinine	
F2-isoprostane	2.2 ± 0.1[a]	2.6 ± 1.1[a]	4.7 ± 1.2[b]
F3-isoprostane	2.1 ± 0.1 [a]	0.9 ± 0.6[a]	12.5 ± 8.2[b]
F4-neuroprostane	70.7 ± 5.7[a]	45.5 ± 12.8[a]	110.3 ± 57.6[b]

[1] Values are means ± SD of 4 pooled samples/diet group; samples were derived from 2 separate studies. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; NP, nonpurified.

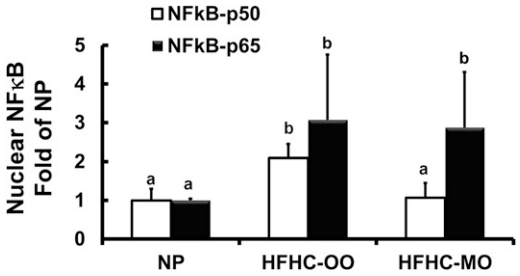

FIGURE 2 Hepatic nuclear abundance of NF-κB-p50 and NF-κB-p65 in $Ldlr^{-/-}$ mice fed the NP, HFHC-OO, or HFHC-MO diets for 12 wk. $Ldlr^{-/-}$ mice were deprived of food overnight before hepatic nuclear extracts were prepared and used to quantify the nuclear abundance of NF-κB-p50 and NF-κB-p65. Mean (±SD) results are expressed as nuclear NF-κB, fold change of NP; $n = 4$. Labeled means without a common letter differ, $P < 0.05$. HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; NP, nonpurified.

Because fatty liver disease was more robust in $Ldlr^{-/-}$ mice fed the HFHC-OO diet than in WT mice fed the same diet, most of our studies focused on determining if MO supplementation of the HFHC-OO diet (i.e., the HFHC-MO diet) could prevent diet-induced fatty liver disease in $Ldlr^{-/-}$ mice. As expected, feeding $Ldlr^{-/-}$ mice the HFHC-MO diet significantly elevated hepatic and plasma C20–22 (n-3) PUFA (Table 2, Supplemental Fig.1) and reduced the expression of many NASH markers when compared with $Ldlr^{-/-}$ mice fed the HFHC-OO diet (Tables 1 and 4). The exception was oxidative stress.

Both $Ldlr^{-/-}$ HFHC-OO and $Ldlr^{-/-}$ HFHC-MO groups expressed the hepatic oxidative stress marker *Hmox1* at levels >300% above those seen in the NP group (Table 4). Moreover, $Ldlr^{-/-}$ mice fed the HFHC-MO diet had increased urinary isoprostanes (Table 3). *Hmox1* expression is controlled by nuclear factor E2-related factor-2 (NRF2), a transcription factor regulated by reactive oxygen species (ROS) (35). NRF2 binds antioxidant response elements in the *Hmox1* promoter as well as other promoters responsive to ROS (e.g., *Gstα1*). Induction of hepatic *Hmox1* by the HFHC-OO and HFHC-MO diets is an indirect marker of elevated hepatic ROS.

TABLE 4 Hepatic gene expression in $Ldlr^{-/-}$ mice fed the NP, HFHC-OO, or HFHC-MO diets for 12 wk[1]

	NP	HFHC-OO	HFHC-MO
Oxidative stress		*Fold of NP*	
Hmox1	1.0 ± 0.3 [a]	4.2 ± 1.0 [b]	3.2 ± 1.1 [b]
PUFA synthesis			
Elovl2	1.0 ± 0.4[a]	2.2 ± 0.9[b]	2.4 ± 1.1[b]
Elovl5	1.0 ± 0.2 [a]	0.8 ± 0.1[a]	0.3 ± 0.1[b]
Fads1	1.0 ± 0.1 [a]	0.8 ± 0.3[a]	0.2 ± 0.1[b]
Fads2	1.0 ± 0.1	1.1 ± 0.2	0.7 ± 0.3
Cholesterol metabolism			
HMG CoA red	1.0 ± 0.3	1.0 ± 0.2	1.2 ± 0.4
HMG CoA syn 1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
Cyp7A1	1.0 ± 0.3	1.2 ± 0.2	2.1 ± 1.3
Abca1	1.0 ± 0.2 [a]	4.7 ± 0.3[b]	4.0 ± 0.4[b]
Abcg5	1.0 ± 0.1[a]	3.2 ± 0.3[b]	4.0 ± 0.5[b]
Acat1	1.0 ± 0.4[a]	4.8 ± 1.5[b]	3.0 ± 1.1[c]
Acat2	1.0 ± 0.1[a]	2.0 ± 0.5[b]	2.3 ± 0.7[b]
Inflammation			
Mcp1	1.0 ± 0.5[a]	22.8 ± 10.4[b]	4.6 ± 2.8[a]
Cd68	1.0 ± 0.5[a]	7.7 ± 2.5[b]	4.1 ± 2.5[c]
Clec4f	1.0 ± 0.5[a]	6.3 ± 1.2[b]	4.7 ± 1.3[b]
Fibrosis			
Tgfβ1	1.0 ± 0.2[a]	3.0 ± 0.9[b]	2.1 ± 0.4[b]
Procol1α1	1.0 ± 0.7[a]	43.0 ± 24.0[b]	20.1 ± 14.0[c]

[1] Values are means ± SD and are fold of NP; $n = 8$. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. HFHC-MO, high-fat–high-cholesterol with olive oil supplemented with menhadin oil; HFHC-OO, high-fat–high-cholesterol with olive oil; NP, nonpurified.

NAFLD patients have elevated oxidative stress (31–36), and dietary supplement approaches that counter oxidative stress have been tested as a potential NAFLD therapy (50,51). Our finding that dietary C20–22 (n-3) PUFA increased urinary isoprostanes may raise concerns for their use in NAFLD therapy (Table 3) (52). Interestingly, urinary F2-isoprostane levels were not different in the NP and HFHC-OO groups, but increased > 100% in the HFHC-MO group. One explanation for this outcome is the impact of dietary fat composition on tissue (n-6) and (n-3) PUFA content. In contrast to the NP diet, the HFHC-OO diet contains high levels of SFA and MUFA, relative to (n-6) PUFA (**Supplemental Table 1**). Hepatic 20:4(n-6), the substrate for F2-isoprostane, was lower in the HFHC-OO group than in the NP group (Table 2). Thus, low tissue 20:4(n-6) content may not favor increased F2-isoprostane formation, even when hepatic ROS is elevated. Moreover, the HFHC-OO diet did not suppress the expression of hepatic enzymes involved in PUFA synthesis (Table 4). The hepatic and plasma ratio of 20:4(n-6) to 18:2(n-6) also indicated that (n-6)PUFA synthesis was not suppressed by the HFHC-OO diet (Table 2, **Supplemental Fig. 1**). One explanation for the decline in hepatic 20:4(n-6) in the $Ldlr^{-/-}$ HFHC-OO group is that synthesized 20:4(n-6) is exported via VLDL and stored in extrahepatic tissues. Additional studies are required to define the impact of HF diets on hepatic and whole-body 20:4(n-6) metabolism.

Although the HFHC-OO and HFHC-MO diets have the same SFA, MUFA, and (n-6) PUFA content, they differ in (n-3) PUFA content; the HFHC-MO diet increased urinary isoprostanes (Table 3). In contrast to the HFHC-OO group, low hepatic 20:4(n-6) in the HFHC-MO group (Table 2) can be explained by low expression of enzymes involved in PUFA synthesis and a 50% reduction in the 20:4(n-6) to 18:2(n-6) ratio (Tables 1, 2; Fig. 1*B*). Moreover, the HFHC-OO and HFHC-MO diets induced hepatic oxidative stress (i.e., *Hmox1*) (Table 4). In livers with elevated C20–22 (n-3) PUFA, F3-isoprostane and F4-neuroprostane content was elevated (22). Increased production of isoprostanes from C20–22 (n-3) PUFA may stimulate F2-isoprostane formation (22,52). F2-isoprostanes activate thromboxane and prostaglandin F2α receptors; they activate platelets and promote smooth muscle cell–mediated vasoconstriction

(52,53), whereas isoprostanes generated from (n-3) PUFA do not (52). By analogy to the proinflammatory (series 2) and antiinflammatory (series 3) cyclooxygenase-derived eicosanoids (54), F3-isoprostanes may be antiinflammatory. If so, some effects of C20–22 (n-3) PUFA on inflammatory markers reported in Table 4 and Figure 2 may be attributed to the action of F3-isoprostanes and F4-neuroprostanes. Clearly, more study is required to establish whether isoprostanes generated from (n-3) PUFA are beneficial.

Because (n-3) PUFA inhibit hepatic fatty acid synthesis and induce fatty acid oxidation (12,55), we hypothesized that increasing hepatic (n-3) PUFA would suppress hepatic accumulation of TG and prevent the induction of all markers linked to fatty liver disease. Major regulators of DNL include SREBP1 and the ChREBP/MLX heterodimer (56,57); these transcription factors play a role in hepatosteatosis (2,12,38,58,59). The nuclear abundance of SREBP1, but not the ChREBP/MLX heterodimer, was vulnerable to C20–22 (n-3) PUFA regulation in obese $Ldlr^{-/-}$ mice (Fig. 1*A*). The suppression of mRNA encoding *Acc1* and *Fasn* parallels the suppression of hepatic nuclear abundance of SREBP1 (Fig. 1*B*). Although C20–22 (n-3) PUFA suppression of nuclear SREBP1 accounts for some reduction of hepatic TG, other mechanisms are likely involved. In humans with NAFLD, fatty acids entering hepatic TG are derived from multiple sources, including DNL, portal circulation, and NEFA mobilized from adipose tissue (10,60,61). In feed-deprived mice, plasma NEFA were reduced by 40% in the $Ldlr^{-/-}$ HFHC-MO group compared with the $Ldlr^{-/-}$ HFHC-OO group (Table 1). Thus, (n-3) PUFA control of lipid mobilization from adipose tissue and delivery of NEFA to the liver may contribute to the low hepatic TG in the $Ldlr^{-/-}$ HFHC-MO group.

The combination of HFHC-OO and the $Ldlr^{-/-}$ genotype was required to increase hepatic cholesterol content by >140% ($P < 0.05$) (Table 1) and induced high levels of expression of inflammation and fibrosis markers (Table 4). Several recent reports have linked elevated hepatic cholesterol to the induction of NASH markers (26–28,62). In those studies, increased hepatic cholesterol activated SREBP2, upregulated the LDL receptor, reduced biotransformation of bile acids, and suppressed canalicular pathways for cholesterol and bile acid export (28,62). In our study, however, the LDL receptor is absent, and SREBP2 target genes and the expression of CYP7A1, the rate-limiting step in bile acid synthesis, were not affected by the HFHC-OO or HFHC-MO diets (Table 4). As such, alternative mechanisms are needed to explain our observation. Because both HFHC-OO and HFHC-MO diets induced *Abca1* and *Abcg5*, cholesterol efflux may not be affected. The very high plasma TG and cholesterol in feed-deprived $Ldlr^{-/-}$ mice (Table 1) likely promotes LDL uptake via hepatic scavenger receptors. Using the same diet and mice as reported herein, Saraswathi et al. (22) found that dietary C20–22 (n-3) PUFA decreased hepatic cholesterol, but increased cholesterol storage in adipose tissue. $Ldlr^{-/-}$ mice fed the HFHC-MO diet had significantly lower apo B, apo CIII (a lipoprotein lipase inhibitor), and plasma TG and cholesterol when compared with the HFHC-OO group (Table 1). Therefore, part of the control of hepatic TG and cholesterol may involve VLDL assembly and secretion as well as VLDL/LDL clearance by nonhepatic tissues. More studies are required to verify this mechanism.

A key finding of our study was the effect of the HFHC-OO and HFHC-MO diets on markers of NASH in nonparenchymal hepatic cells. *Cd68* and *Clec4f* are expressed in macrophage and Kupffer cells (27), whereas *Tgfβ1* is expressed predominantly in Kupffer cells and stellate cells, but not in parenchymal cells. *Procol1α1* is expressed in stellate cells (46). Feeding WT and $Ldlr^{-/-}$ mice the HFHC-OO diet induced expression of mRNA and nuclear proteins linked to inflammation (*Mcp1*, *Cd68*, *Clec4f*, and nuclear NF-κB-p50 and -p65) and fibrosis (*Tgfβ1*, *Procol1α1*) (Fig. 2, Table 4, Supplemental Table 3). More important, supplementing the diet with MO (HFHC-MO) lowered the levels of most of these markers (except for *Clec4f* and *Tgfβ1*) when compared with HFHC-OO–fed mice.

Although we have a good understanding of C20–22 (n-3) PUFA control of hepatic parenchymal cell gene expression (12), much less is known about (n-3) PUFA regulation of Kupffer and stellate cells. *Mcp1* is an early marker of inflammation and is regulated by NF-κB (44,63). In the $Ldlr^{-/-}$ HFHC-OO group, the nuclear abundance of NF-κB-p50 and NF-κB-p65 was significantly induced when compared with the NP group (Fig. 2). In mice fed the HFHC-MO diet, however, the nuclear abundance of NF-κB-p50 was not induced when compared with the NP group (Fig. 2). Changes in *Mcp1* mRNA paralleled changes in hepatic nuclear NF-κB-p50 content. This outcome suggests that genes requiring the classical p50/p65 heterodimer or the p50/50 homodimer for transcriptional activation will be attenuated by C20–22 (n-3) PUFA, whereas inflammatory genes that are regulated by p65 heterodimerization with other partners, such as c/EBPβ, will be less sensitive to C20–22 (n-3) PUFA control. In addition to NF-κB, other regulatory factors control the expression of hepatic inflammatory and fibrotic markers, including TGF-β, cJun, TNF-α, and the Toll-like receptors 2 and 4 (26,64). *Procol1α1* expression in stellate cells is induced by TGF-β1 through a H_2O_2-c/EBPβ-dependent mechanism (48,49). The HFHC-MO diet had a modest effect on *Tgfβ1* expression in $Ldlr^{-/-}$ mice but significantly attenuated *Procol1α1* expression in the WT and $Ldlr^{-/-}$ HFHC-MO groups when compared with the WT and $Ldlr^{-/-}$ HFHC-OO groups (Table 4, Supplemental Table 3). A systematic analysis of C20–22 (n-3) PUFA effects on hepatic target genes controlled by these regulatory factors will clarify the breadth of C20–22 (n-3) PUFA effects on hepatic inflammation and fibrosis markers.

In summary, we used an established model of HF- diet–induced fatty liver disease in mice (22) and assessed the capacity of dietary C20–22 (n-3) PUFA in MO to prevent fatty liver disease in WT and $Ldlr^{-/-}$ mice. $Ldlr^{-/-}$ mice developed more severe fatty liver disease than did WT mice when fed the HFHC-OO diet. Mice fed the HFHC-MO diet, which was supplemented with a physiologically relevant level (2% of total energy) of C20–22 (n-3) PUFA, had lower plasma and hepatic lipids (TG and cholesterol), hepatic damage, inflammation, and fibrosis than did mice fed the HFHC-OO diet. The HFHC-MO diet, however, did not prevent the induction of other HF diet–induced markers of fatty liver disease or MetS, including obesity, hyperglycemia, and hepatic or whole-body oxidative stress. Thus, the C20–22 (n-3) PUFA in MO reduced many, but not all, metabolic abnormalities associated with HF diet–induced fatty liver disease in mice.

Acknowledgments

The authors thank Karin Hardin and Sam Brown for excellent technical assistance in these studies. They thank Dr. E. Ho (Oregon State University) for her insight into the differential regulation of NF-κB subunits. C.M.D., M.T.-G. and D.B.J. designed the research; C.M.D., M.T.-G., S.T., G.M., and D.B.J. conducted the research and analyzed the data; C.M.D. and D.B.J. wrote the manuscript; and D.B.J. had primary responsibility for the final content. All authors read and approved the final manuscript.

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