Polyunsaturated fat in the methionine-choline-deficient diet influences hepatic inflammation but not hepatocellular injury[1,S]

Gene S. Lee,[*,†] Jim S. Yan,[*,†] Raymond K. Ng,[*,†] Sanjay Kakar,[*,§] and Jacquelyn J. Maher[2,*,†]

Liver Center* and Departments of Medicine† and Pathology,§ University of California, San Francisco, San Francisco, CA 94110

Abstract Methionine-choline-deficient (MCD) diets that cause steatohepatitis in rodents are typically enriched in polyunsaturated fat. To determine whether the fat composition of the MCD formula influences the development of liver disease, we manufactured custom MCD formulas with fats ranging in PUFA content from 2% to 59% and tested them for their ability to induce steatohepatitis. All modified-fat MCD formulas caused identical degrees of hepatic steatosis and resulted in a similar distribution of fat within individual hepatic lipid compartments. The fatty acid composition of hepatic lipids, however, reflected the fat composition of the diet. Mice fed a PUFA-rich MCD formula showed extensive hepatic lipid peroxidation, induction of proinflammatory genes, and histologic inflammation. When PUFAs were substituted with more saturated fats, lipid peroxidation, proinflammatory gene induction, and hepatic inflammation all declined significantly. Despite the close relationship between PUFAs and hepatic inflammation in mice fed MCD formulas, dietary fat had no impact on MCD-mediated damage to hepatocytes. Indeed, histologic apoptosis and serum alanine aminotransferase levels were comparable in all MCD-fed mice regardless of dietary fat content. Together, these results indicate that dietary PUFAs promote hepatic inflammation but not hepatotoxicity in the MCD model of liver disease. These findings emphasize that individual dietary nutrients can make specific contributions to steatohepatitis.—Lee, G. S., J. S. Yan, R. K. Ng, S. Kakar, and J. J. Maher. **Polyunsaturated fat in the methionine-choline-deficient diet influences hepatic inflammation but not hepatocellular injury. *J. Lipid Res.* 2007.** 48: **1885–1896.**

Supplementary key words steatohepatitis • fatty liver • lipotoxicity • inflammation • apoptosis

Dietary formulas that are completely devoid of methionine and choline (MCD) induce steatohepatitis in mice (1–4). MCD diets are often used to investigate the pathogenesis of fatty liver disease because they rapidly induce steatosis and necroinflammation and over longer periods cause hepatic fibrosis (2, 5). MCD formulas provoke hepatic steatosis by limiting the availability of substrates for phosphatidylcholine synthesis, which in turn inhibits VLDL assembly and blocks hepatic triglyceride secretion (6, 7). Why this model of fat accumulation progresses to hepatic inflammation and fibrosis is uncertain.

Liver injury in MCD-fed mice is not likely the result of impaired VLDL secretion per se. This is evident from studies of mice with a liver-specific knockout of microsomal triglyceride transfer protein (MTP), a peptide that, like phosphatidycholine, is essential for the proper assembly and trafficking of VLDL particles (8). MTP deficiency impairs hepatic triglyceride secretion and induces hepatic steatosis in much the same manner as methionine and choline deprivation. Unlike MCD-mediated steatosis, however, the fatty liver caused by MTP deficiency does not progress spontaneously to hepatitis or fibrosis (9). Experimental data indicate that hepatic steatosis is critical to the pathogenesis of liver injury in MCD-fed mice. Specifically, studies have shown that when MCD-fed mice are treated simultaneously with drugs that prevent hepatic lipid accumulation, they do not develop liver injury (10, 11). This suggests that the lipid composition of MCD-fed livers plays an important role in MCD-mediated hepatotoxicity.

The lipids that accrue in the livers of MCD-fed mice are directly related to the nutrient composition of the MCD

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Abbreviations: ALT, alanine aminotransferase; COX-2, cyclooxygenase-2; CXCL-2, CXC chemokine ligand-2; CYP2E1, cytochrome P4502E1; I-kB, inhibitor of NF-κB; iNOS, inducible nitric oxide synthase; MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient; MTP, microsomal triglyceride transfer protein; NF-κB, nuclear factor-κB; PL, phospholipid; SFA, saturated fatty acid; TAG, triacylglycerol; TBARS, thiobarbituric acid-reactive substances; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling.

[1] A portion of this work appeared previously in abstract form (*Hepatology.* 2005. **42:** 630A).

[2] To whom correspondence should be addressed.
e-mail: jmaher@medsfgh.ucsf.edu

[S] The online version of this article (available at http://www.jlr.org) contains an additional figure.

formula. The standard MCD formula contains 10% corn oil, which is highly enriched in unsaturated fat. Because unsaturated fatty acids are excellent substrates for lipid peroxidation, and because hepatic lipid peroxidation is a prominent feature of MCD-related liver disease (1, 12), we reasoned that unsaturated fat in the MCD formula is critical to its hepatotoxicity. We posited that substituting corn oil with more saturated fat would ameliorate lipid peroxidation and steatohepatitis in MCD-fed mice. To test this theory, we prepared custom MCD formulas with varying amounts of unsaturated fat and monitored their effects on steatosis, lipid peroxidation, and liver injury. The results indicated that hepatic lipid peroxidation was directly related to the amount of unsaturated fat in the MCD diet, and lipid peroxidation in turn correlated with hepatic induction of proinflammatory cytokine genes and hepatic inflammation. In contrast to its effect on hepatic inflammation, unsaturated fat in the MCD formula had no impact on MCD-mediated hepatocellular injury. Histology scores, measures of hepatocyte apoptosis, and serum alanine aminotransferase (ALT) were all comparable in MCD-fed mice regardless of the fat composition of the MCD formula.

EXPERIMENTAL PROCEDURES

Dietary studies in mice

Male C3H/HeOuJ mice (Jackson Laboratory, Bar Harbor, ME) weighing 23–28 g were used for all studies. They were divided into eight groups, each of which received a different dietary formula as described below for 21 days. All animals had free access to drinking water for the entire study period. On the final day of the experiment, mice were fasted for 4 h before euthanasia. Procedures for animal care and euthanasia followed the guidelines set by the American Veterinary Medical Association. All protocols were approved by the Committee on Animal Research at the University of California, San Francisco.

The experimental diets used in this study were based on lipogenic methionine-choline-sufficient (MCS) and MCD formulas used in the investigation of steatohepatitis (1–4). These formulas comprise 18% protein (as defined amino acids), 65% carbohydrate (70:30 sucrose-starch), and 10% fat (as corn oil) by weight. MCS formulas contain 2 g/kg L-methionine and 2 g/kg choline chloride; MCD formulas contain no methionine or choline. We modified the standard MCS and MCD formulas by substituting the 10% corn oil with either 10% beef tallow or 10% coconut oil. We also created low-fat versions of the MCS and MCD formulas, which contained 5% fat as corn oil and an additional 5% carbohydrate. Hereafter, all modified fat formulas are designated by their methionine and choline contents as well as their fat content: MCS-corn, MCD-corn, MCS-low-fat, MCD-low-fat, MCS-tallow, MCD-tallow, MCS-coconut, and MCD-coconut. The composition of each formula is shown in **Table 1**. All formulas were manufactured by Dyets, Inc. (Bethlehem, PA).

Triglyceride and fatty acid analysis

Lipids were extracted from fresh liver tissue by the method of Folch, Lees, and Sloane Stanley (13). Extracts were evaporated under a stream of nitrogen and resuspended in choloroform-methanol (2:1) containing 0.01% butyrated hydroxytoluene (5 ml/g liver). Aliquots were dried and resuspended in 1-butanol for analysis of total triglyceride (TR0100; Sigma Chemical Co., St. Louis, MO). Results are reported as milligrams of triglyceride per gram of liver.

Separate pieces of liver tissue were flash-frozen in liquid nitrogen for the analysis of individual fatty acids in various hepatic lipid compartments. Lipid extraction and TrueMass™ analysis were performed by Lipomics Technologies, Inc. (West Sacramento, CA). Tissue samples were subjected to a combination of liquid- and solid-phase extraction procedures to separate neutral lipids from phospholipids (PLs), followed by thin-layer chromatography to separate neutral lipid classes and gas chromatography to quantitate individual fatty acids. All samples were

TABLE 1. Composition of the MCS and MCD formulas

	Diet							
Nutrient	MCS-Corn	MCD-Corn	MCS-Low-Fat	MCD-Low-Fat	MCS-Tallow	MCD-Tallow	MCS-Coconut	MCD-Coconut
L-Amino acids (g/kg)	175.7	175.7	175.7	175.7	175.7	175.7	175.7	175.7
L-Methionine (g/kg)	2.0	—	2.0	—	2.0	—	2.0	—
Choline chloride (g/kg)	2.0	—	2.0	—	2.0	—	2.0	—
Sucrose (g/kg)	437.9	441.9	472.9	476.9	437.9	441.9	437.9	441.9
Cornstarch (g/kg)	150.0	150.0	165.0	165.0	150.0	150.0	150.0	150.0
Dextromaltose (g/kg)	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Cellulose (g/kg)	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Corn oil (g/kg)	100.0	100.0	50.0	50.0	—	—	—	—
Beef tallow (g/kg)	—	—	—	—	100.0	100.0	—	—
Coconut oil (g/kg)	—	—	—	—	—	—	100.0	100.0
Sodium bicarbonate (g/kg)	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
Salt mix (g/kg)	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mix (g/kg)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Total (g/kg)	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0
Saturated fatty acid (%)	13	13	13	13	50[a]	50[a]	92	92
MUFA (%)	28	28	28	28	43[a]	43[a]	6	6
PUFA (%)	59	59	59	59	4[a]	4[a]	2	2
PUFA (g/kg)	59	59	30	30	4	4	2	2
Caloric density (kcal/g)	4.3	4.3	3.9	3.9	4.3	4.3	4.3	4.3

MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient.
[a] Totals for beef tallow are <100% because of the presence of cholesterol and other minor constituents.

processed in the presence of internal standards to monitor extraction efficiency and verify measurement accuracy.

Measurement of hepatic lipid peroxidation and hepatic glutathione

Hepatic lipid peroxidation was assessed by measuring the amount of thiobarbituric acid-reactive substances (TBARS) in whole liver extracts. Liver tissue was homogenized in 1.15% potassium chloride containing 4 mM desferrioxamine; TBARS was measured as described by Pyles, Stejskal, and Einzig (14). Results are expressed as nanomoles of TBARS per milligram of liver. For the measurement of glutathione, preweighed pieces of whole liver were homogenized in a solution comprising 20 mM HCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM ascorbic acid, and 5 mM trichloroacetic acid. Reduced glutathione was measured fluorometrically according to the method of Senft, Dalton, and Shertzer (15), and the results are expressed as micrograms of glutathione per gram of liver.

Serum chemistries

Glucose and ALT were measured in mouse serum using a BAX autoanalyzer (Bayer Corp., Tokyo, Japan) in the clinical chemistry laboratory at San Francisco General Hospital. Insulin was measured by ELISA (Linco, St. Charles, MO).

Measurement of hepatic gene expression by real-time quantitative PCR

Total RNA was extracted from mouse liver by homogenization in TRI reagent (Molecular Research Center, Cincinnati, OH), followed by chloroform extraction and ethanol precipitation. RNA was incubated with DNase (Qiagen, Inc., Valencia, CA) to remove contaminating DNA; the enzyme was then inactivated and removed according to the manufacturer's specifications (RNeasy; Qiagen). cDNA was synthesized from 1 μg of RNA in a reaction mixture containing 2.5 U/μl Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and 5 μM random hexamer primers (Invitrogen).

Real-time PCR analysis was performed using an AB Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). Assays-on-Demand® primer and probe sets (Applied Biosystems) were used for all of the genes of interest. The expression of each test gene was normalized to that of mouse β-glucuronidase. Quantitative detection of specific nucleotide sequences was based on the fluorogenic 5′ nuclease assay (16). Relative gene expression was calculated using the method of Livak and Schmittgen (17).

Measurement of hepatic nuclear factor-κB activity

Nuclear factor-κB (NF-κB) DNA binding activity was measured in liver homogenates using an oligonucleotide-based ELISA (Trans-AM NF-κB; Active Motif, Carlsbad, CA). Spectrophotometric results were normalized to protein and expressed as relative values in comparison with the activity measured in the livers of chow-fed mice.

Liver histology and quantitative scoring system

Paraffin sections of liver tissue were stained with hematoxylin and eosin. Slides were viewed blindly and scored for steatosis, ballooning, inflammation, and necrosis using the following criteria. Steatosis (0–4): 0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%. Ballooning (0–3): 0 = absent; 1 = mild (focal involving fewer than three hepatocytes); 2 = moderate (focal involving more than three hepatocytes or multifocal); 3 = prominent (multifocal with more than two foci of three or more hepatocytes). Inflammation (0–4): 0 = absent; 1 = minimal (zero to one focus per 20× field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci). Necrosis (0–4): 0 = absent; 1 = minimal (rare scattered necrotic cells); 2 = mild (occasional scattered necrotic cells); 3 = moderate (many scattered cells or confluent necrosis); 4 = marked (multiple areas of confluent necrosis).

TABLE 2. Clinical and biochemical data from mice fed MCS and MCD formulas

	MCS				MCD			
Variable	Corn (n = 10)	Low-Fat (n = 5)	Tallow (n = 5)	Coconut (n = 5)	Corn (n = 10)	Low-Fat (n = 10)	Tallow (n = 10)	Coconut (n = 10)
Body weight change (%)	33 ± 3	24 ± 2	19 ± 2[a]	33 ± 2	−30 ± 1[b]	−31 ± 1[b]	−30 ± 1[b]	−29 ± 1[b]
Liver weight to body weight (%)	4.3 ± 0.1	4.3 ± 0.1	5.0 ± 0.3[a]	5.0 ± 0.2[a]	5.7 ± 0.1[b]	6.0 ± 0.2[b]	5.8 ± 0.1[b]	6.2 ± 0.2[b,c]
Adipose weight to body weight (%)	4.47 ± 0.17	4.06 ± 0.17	4.20 ± 0.18	4.05 ± 0.05	0.54 ± 0.08[b]	0.51 ± 0.07[b]	0.55 ± 0.12[b]	0.46 ± 0.08[b]
Hepatic triglyceride (mg/g liver)	25.4 ± 4.0	32.2 ± 7.9	32.4 ± 6.2	43.3 ± 7.6[a]	127.6 ± 6.3[b]	152.2 ± 15.7[b]	140.8 ± 7.2[b]	134.1 ± 13.1[b]
Hepatic triglyceride (mg/g protein)	170.9 ± 23.3	228.6 ± 42.6	206.8 ± 42.3	277.5 ± 55.6[a]	1137.0 ± 77.7[b]	1323.5 ± 88.0[b]	1113.7 ± 46.4[b]	1317 ± 6.4[b]
Hepatic phospholipid (μmol/g liver)	39.9 ± 0.8	ND	ND	ND	32.7 ± 1.2[b]	32.9 ± 1.8[b]	32.8 ± 0.7[b]	31.6 ± 1.1[b]
Serum triglyceride (mg/dl)	123.0 ± 9.4	118.8 ± 6.0	139.2 ± 6.2	78.8 ± 5.8[a]	53.7 ± 3.8[b]	74.8 ± 6.7[b,c]	61.6 ± 3.5[b]	64.6 ± 3.0[b]
Serum cholesterol (mg/dl)	190.4 ± 7.6	165.8 ± 5.1[a]	217.4 ± 15.6	204.8 ± 5.3	75.7 ± 3.5[b]	80.0 ± 4.0[b]	93.6 ± 3.2[b,c]	109.0 ± 3.5[b,c]
Serum glucose (mg/dl)	359.1 ± 13.6	158.5 ± 3.8[a]	221.6 ± 19.1[a]	418.2 ± 29.0	155.5 ± 15.3[b]	145.9 ± 20.8	119.4 ± 9.8[b]	170.1 ± 10.0[b]
Serum insulin (ng/ml)	1.86 ± 0.52	0.55 ± 0.07	1.01 ± 0.11	1.10 ± 0.09	0.37 ± 0.02[b]	0.42 ± 0.05[b]	0.36 ± 0.03[b]	0.35 ± 0.01[b]

Values are shown for mice fed MCS or MCD formulas for 21 days. Values represent means ± SEM.
ND, not determined.
[a] $P < 0.05$ versus MCS-corn.
[b] $P < 0.01$ versus nutrient-matched MCS formula.
[c] $P < 0.05$ versus MCD-corn.

Quantitation of apoptotic cells in mouse liver sections

Apoptotic cells were identified in mouse liver by terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). Deparaffinized tissue sections were immersed in citrate buffer (pH 6.0) for 20 min at 95°C and subsequently in 3% H_2O_2 for 15 min at room temperature. After these pretreatments, biotin-16-dUTP was incorporated into nicked DNA by application to the slides in the presence of terminal deoxynucleotide transferase (Roche Applied Science, Indianapolis, IN) for 2 h at 42°C. Slides were then incubated with streptavidin-HRP (1:250) for 20 min followed by 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA) to highlight cells with DNA fragmentation. Tissue sections were counterstained with hematoxylin and viewed by light microscopy at 20× magnification. The number of cells with TUNEL-positive nuclei was determined by manual counting of >100 microscopic fields per liver. Results are reported as the average number of TUNEL-positive cells per microscopic field.

Statistical analysis

All experiments included at least five mice per study group. Mean data from each study group were compared by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Modifying the fat in the MCD formula did not influence any of the major metabolic abnormalities induced by MCD feeding

Mice fed the standard MCD formula containing 10% corn oil (MCD-corn) for 21 days exhibited several metabolic abnormalities. First, the mice developed hypolipidemia and hepatic steatosis, based on a 50% decrease in serum triglycerides and cholesterol and a 5-fold increase in hepatic triglyceride concentration compared with MCS control mice (MCS-corn) (**Table 2**). Second, mice fed the MCD-corn formula lost weight over the 3 week study interval. This is characteristic of MCD feeding and has been attributed to a diet-induced increase in metabolic rate (4). Third, serum glucose and insulin levels were significantly lower in MCD-corn mice than in control mice at 21 days. This is consistent with previous reports that MCD-fed mice retain peripheral insulin sensitivity despite eating a lipogenic diet (18). Over the same interval, control mice eating the MCS-corn formula gained weight and developed hyperglycemia and hyperinsulinemia.

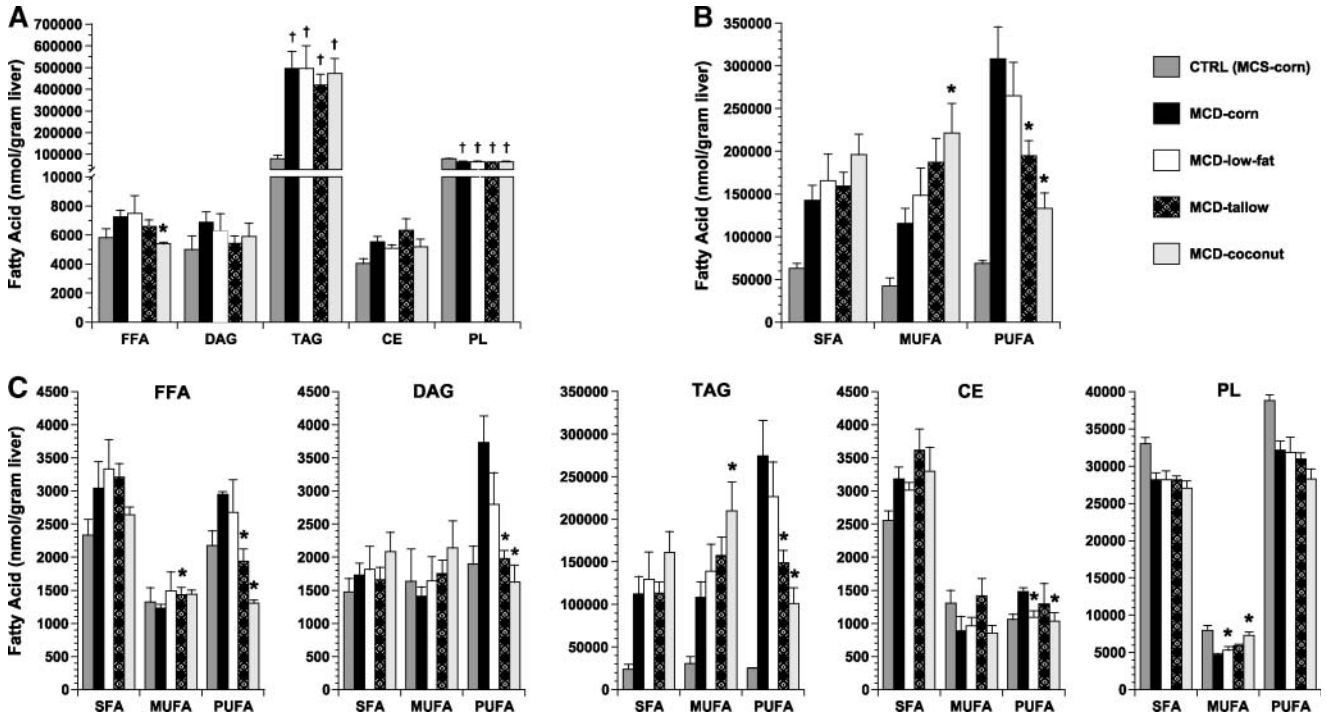

Fig. 1. Hepatic fatty acid content in methionine-choline-deficient (MCD) mice. A: Total amount of fatty acid present in individual hepatic lipid compartments in mice fed four different MCD formulas (MCD-corn, MCD-low-fat, MCD-tallow, and MCD-coconut) for 21 days. The amount of fatty acid present in control livers [fed methionine-choline-sufficient (MCS)-corn] is shown for comparison [control (CTRL)]. Compared with MCS feeding, MCD feeding did not alter the amount of fatty acid present in hepatic FFA, diacylglycerol (DAG), or cholesteryl ester (CE). By contrast, MCD feeding significantly increased the amount of fatty acid in hepatic triacylglycerol (TAG) ($P < 0.005$) and significantly decreased the amount of fatty acid in hepatic phospholipid (PL) ($P < 0.007$) compared with that in control mice. Comparing the four MCD groups, no differences in hepatic fatty acids were observed except that FFAs were less abundant in MCD-coconut livers than in MCD-corn livers. B: Type of fatty acids present in the livers of MCS and MCD mice. All four groups of MCD-fed mice had markedly higher levels of hepatic saturated fatty acids (SFAs), MUFAs, and PUFAs than MCS control mice. The PUFA content of hepatic fatty acids varied significantly in MCD-fed mice, in a pattern that mirrored the PUFA content of the diet (corn > low-fat > tallow > coconut). MUFAs, and to a lesser degree SFAs, increased in a reciprocal manner. C: Breakdown of saturated and unsaturated fatty acids in MCS and MCD livers by individual lipid compartment. The PUFA content of hepatic FFA, diacylglycerol, and TAG reflected the PUFA content of the MCD diet, but the PUFA content of hepatic PL remained constant in all four MCD-fed groups. Values represent means ± SEM for n = 5. † $P < 0.01$ versus control; * $P < 0.05$ versus MCD-corn.

Changing the fat composition of the MCD diet from corn oil to either beef tallow or coconut oil did not affect the degree of hepatic steatosis in MCD-fed mice (Table 2). Reducing the fat content of the MCD formula from 10% to 5% also had no impact on hepatic triglyceride accumulation when the remaining 5% of the diet was substituted with carbohydrate. Altering the fat content or composition of the MCD formula did not influence the degree of weight loss induced by methionine and choline deprivation. Serum glucose and insulin remained within the same range in all four MCD-fed groups, and all MCD-fed mice displayed hypolipidemia. Notably, manipulation of the fat content of the MCS control formula had some significant consequences. One was that the MCS-coconut diet caused more hepatic steatosis and less triglyceridemia than the other MCS formulas. Excess lipid accumulation in MCS-coconut livers likely resulted from the induction of stearoyl-CoA desaturase-1 by dietary saturated fat (19), which in the presence of high concentrations of palmitate synthesized de novo from sucrose in the MCS formula (4) would yield excess hepatic triglyceride. Indeed, we measured stearoyl-CoA desaturase-1 gene expression and found it to be 2.5-fold higher in MCS-coconut than in MCS-corn livers (2.50 ± 0.20 vs. 1.00 ± 0.25; $P = 0.002$). Also remarkable was that the low-fat version of MCS preserved normal serum glucose and insulin levels and mildly reduced serum cholesterol. This is consistent with a recent report indicating that hyperglycemia and hyperinsulinemia occur more readily in response to a high-fat diet than a high-sucrose diet (20).

Modifying the fat in the MCD formula altered hepatic lipid composition

A detailed analysis of the major lipid compartments of the liver revealed that the total amount of fatty acids in the FFA, diacylglycerol, triacylglycerol (TAG), cholesteryl ester, and PL fractions remained nearly identical among all four MCD groups (**Fig. 1A**). In all cases, total hepatic TAGs were increased and total PLs were decreased in MCD mice relative to MCS control mice, as would be expected in the setting of methionine and choline deprivation. By contrast, total FFA, diacylglycerol, and cholesteryl esters were no different in MCD mice than in MCS controls. This indicates tight homeostatic control of these compartments even in the setting of methionine and choline deprivation. Although varying the fat composition of the MCD formula had no influence on the total amount of fatty acids in the liver, it significantly affected the fatty acid composition of hepatic lipids (Fig. 1B). Specifically, as PUFAs were removed from the diet, the PUFA content of hepatic lipids decreased accordingly. Alterations in the PUFA content of the diet were reflected in all major hepatic lipid compartments except PL, which retained a high PUFA content regardless of the composition of the diet (Fig. 1C). The two major PUFAs in hepatic PL were arachidonic acid (C20:4n6) and docosahexaenoic acid (C22:6n3), which were equivalent in abundance among all four dietary groups (see supplementary Fig. I). Also noteworthy in the fatty acid analysis was that saturated fatty acids (SFAs) did not increase in the liver as PUFAs decreased, even though SFAs were used as substitutes for PUFAs in the modified MCD diets. The reason may be that mice fed MCD diets enriched in saturated fat (MCD-tallow, MCD-coconut) converted the SFAs efficiently to MUFAs for incorporation into hepatic lipids. This was most evident in the TAG and PL fractions Fig. 1C), which contained a relatively high concentration of oleic acid (C18:1n9), but was actually true of all lipid compartments in which significant increases in palmitoleic acid (C16:1n7) and vaccenic acid (C18:1n7) were observed (see supplementary Fig. I).

Hepatic lipid peroxidation correlated positively with the amount of PUFAs in the diet and the liver

To determine whether hepatic lipid peroxidation in MCD-fed mice was associated with the concentration of

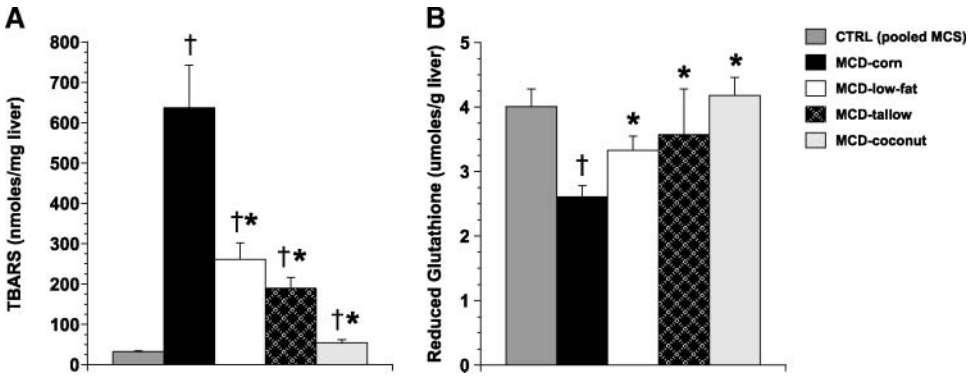

Fig. 2. Hepatic thiobarbituric acid-reactive substances (TBARS) and reduced glutathione in the livers of MCD-fed mice. The histograms illustrate TBARS (A) and reduced glutathione (B) as measures of hepatic lipid peroxidation and oxidant stress in mice fed MCS or MCD formulas for 21 days. The control values (CTRL) represent pooled results from mice fed all four MCS formulas (MCS-corn, MCS-low-fat, MCS-tallow, and MCS-coconut). The MCD-corn formula caused substantial lipid peroxidation (16 times control levels). MCD formulas with a lower PUFA content caused less lipid peroxidation, to as little as 1.5 times control levels (MCD-coconut). Hepatic glutathione varied inversely with TBARS in MCD-fed mice. The lowest values were in MCD-corn mice and increased progressively to control levels in MCD-coconut mice. Values represent means ± SEM for n = 20 (MCS) or n = 10 (MCD). † $P < 0.05$ versus control; * $P < 0.05$ versus MCD-corn.

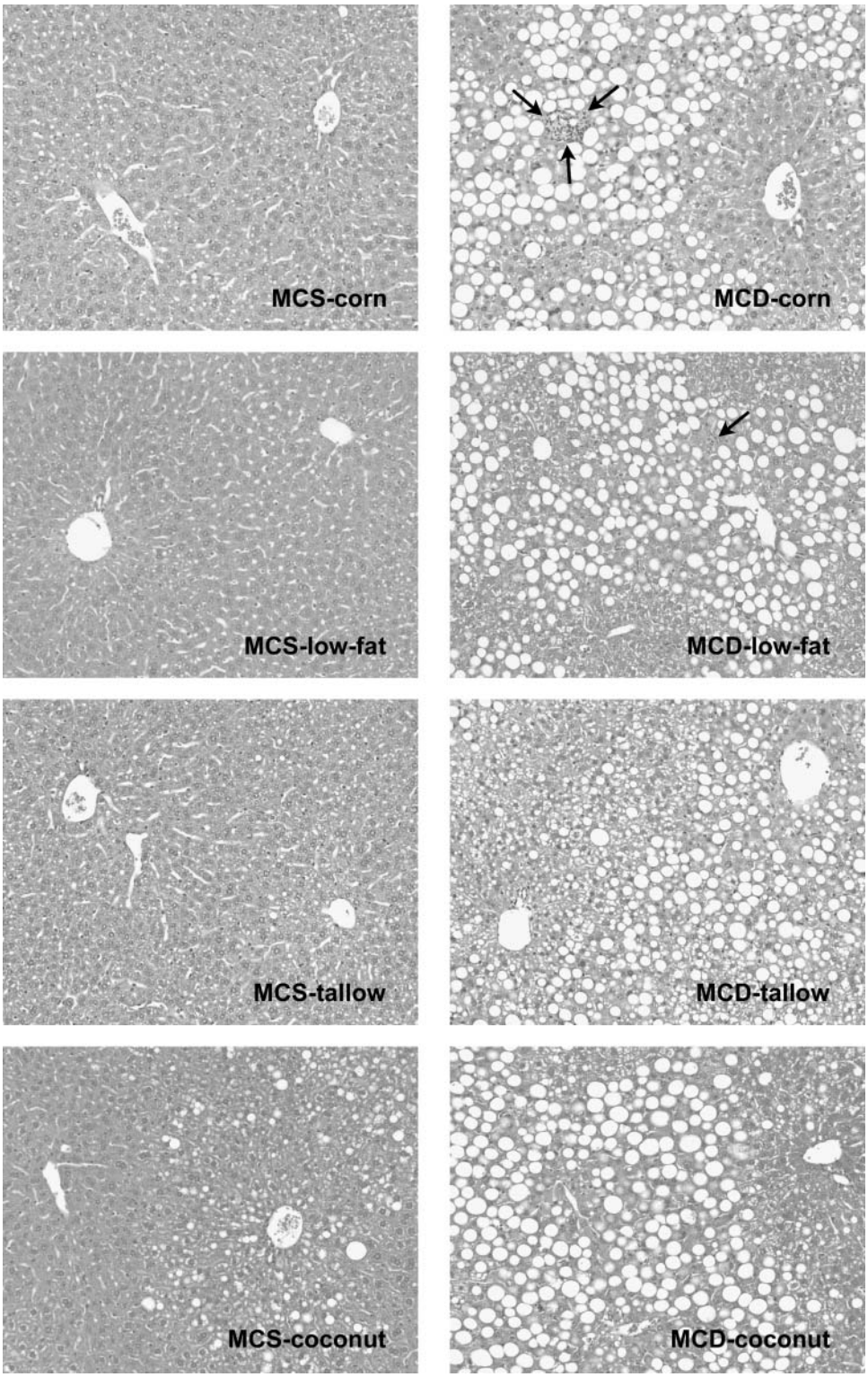

Fig. 3. Liver histology in MCD-fed mice. Photomicrographs depict liver histology in mice fed MCS or MCD formulas for 21 days (hematoxylin and eosin stain; 10× original magnification). In MCS control livers, hepatocytes displayed slight vacuolization, indicative of mild steatosis. More prominent steatosis was evident in MCS-coconut livers; this coincides with their higher triglyceride content (Table 2). All four groups of MCD mice displayed hepatic steatosis in a pericentral distribution expanding toward periportal zones. Inflammatory foci (arrows) were readily visible in MCD-corn livers but were less apparent in MCD-low-fat, MCD-tallow, and MCD-coconut livers.

PUFAs in the diet and the liver, we measured TBARS and reduced glutathione in the livers of mice fed MCS and MCD formulas. TBARS were low in MCS control mice but varied significantly among the four MCD groups (**Fig. 2A**). TBARS were highest in MCD-corn mice, which had the highest hepatic PUFA content. They declined significantly as the PUFA content of the diet and the liver diminished (cf. Figs. 1B and 2A). In MCD-coconut mice, which had the lowest concentration of hepatic PUFAs, TBARS were only 1.5 times control levels. Hepatic levels of reduced glutathione varied inversely with hepatic TBARS in MCD-fed mice (Fig. 2B). Glutathione levels were lowest in MCD-corn mice and increased as PUFAs were removed from the diet. Glutathione in MCD-coconut mice was no different from that in MCS control mice.

PUFAs in the MCD formula coincided with histologic inflammation and the induction of proinflammatory genes

All MCD-fed mice displayed similar degrees of hepatic steatosis by histology (**Figs. 3, 4**), in keeping with their comparable levels of hepatic lipids (Table 1, Fig. 1). Hepatic inflammation, however, was more severe in mice fed the PUFA-rich MCD-corn formula than in mice fed the other three MCD formulas (Figs. 3, 4). MCD-corn mice had an inflammatory score of 2.3 ± 0.2 on a scale from 0 to 4, whereas MCD-low-fat, MCD-tallow, and MCD-coconut mice all had comparable scores ranging from 1.1 to 1.5 (Fig. 4). Unlike hepatic TBARS, the histologic inflammatory score did not vary continuously with the composition of dietary or hepatic lipids.

MCD feeding is known to induce the hepatic expression of genes encoding several proinflammatory mediators (21–24). To determine whether this induction was influenced by the fat content or composition of the MCD diet, we measured mRNA levels for a panel of proinflammatory compounds in the livers of mice fed the four different MCD formulas. Mice fed the PUFA-rich MCD-corn formula showed significant hepatic induction of the genes encoding tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and CXC chemokine ligand-2 (CXCL-2) compared with MCS-corn controls (**Fig. 5A**). In mice fed reduced-PUFA versions of MCD, these same genes were less strongly induced. mRNAs encoding these genes declined in a step-wise manner in parallel with the amount of PUFAs in the diet (MCD-corn > MCD-low-fat > MCD-tallow > MCD coconut). Despite differences in proinflammatory gene expression, mice in all four MCD groups displayed comparable induction of hepatic cytochrome P4502E1 mRNA. None of the MCD-fed mice showed any induction of the gene encoding osteopontin, despite previously reported observations to the contrary (23). When hepatic gene expression was plotted as a function of hepatic lipid peroxidation (TBARS), TNF, COX-2, iNOS, and CXCL-2 all displayed close relationships (Fig. 5B).

Because proinflammatory gene expression is inducible by the transcription factor NF-κB, we sought evidence of NF-κB activation in mice fed MCD formulas. At 21 days, MCD-fed mice displayed no increase in NF-κB DNA binding relative to control mice (**Fig. 6A**). Nor did MCD mice show any indication of inhibitor of NF-κB degradation in the liver (Fig. 6B). Thus, at the 3 week interval, proinflammatory gene expression in MCD-fed mice was not directly attributable to heightened NF-κB activity.

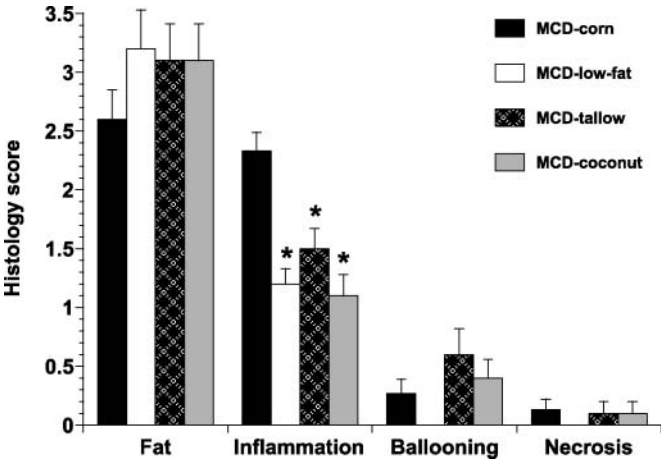

Fig. 4. Liver histology scores in MCD-fed mice. The histogram depicts the itemized histology scores in mice fed MCD formulas for 21 days. Livers were analyzed using the scoring system described in Experimental Procedures. MCS control mice scored values of 0 for all categories (data not shown). MCD-low-fat, MCD-tallow, and MCD-coconut mice all had less hepatic inflammation than MCD-corn mice. All other parameters of steatosis and liver injury were similar among the four MCD groups. Values represent means ± SEM for n = 10. * $P < 0.05$ versus MCD-corn.

PUFAs in MCD-fed livers had no impact on hepatocellular injury

Although reducing the PUFA content of the MCD formula significantly suppressed the inflammatory features of MCD-mediated liver disease, it had no effect on MCD-related injury to hepatocytes. Hepatocyte ballooning and necrosis were no different among the four MCD groups (Fig. 4), and apoptosis was readily demonstrable in all four groups by TUNEL staining (**Fig. 7**). When TUNEL-positive cells were quantitated in the livers of MCD-fed mice, the numbers were significantly higher in MCD mice than in MCS controls. In fact, TUNEL-positive cells were more prominent in mice fed reduced-PUFA MCD formulas than in those fed the PUFA-rich MCD-corn formula (**Fig. 8A**). Serum ALT was increased in all four groups of MCD-fed mice, regardless of the fat content or composition of the diet (Fig. 8B). ALT was proven to derive from liver rather than muscle, based on identical levels of creatine kinase in MCD and control mice (data not shown).

DISCUSSION

The current study demonstrates that polyunsaturated fatty acids, which are the main constituents of corn oil, are directly responsible for the hepatic lipid peroxidation and much of the hepatic inflammation that occur in mice fed commercial MCD diets. When PUFAs are reduced or eliminated from the MCD formula, hepatic lipid perox-

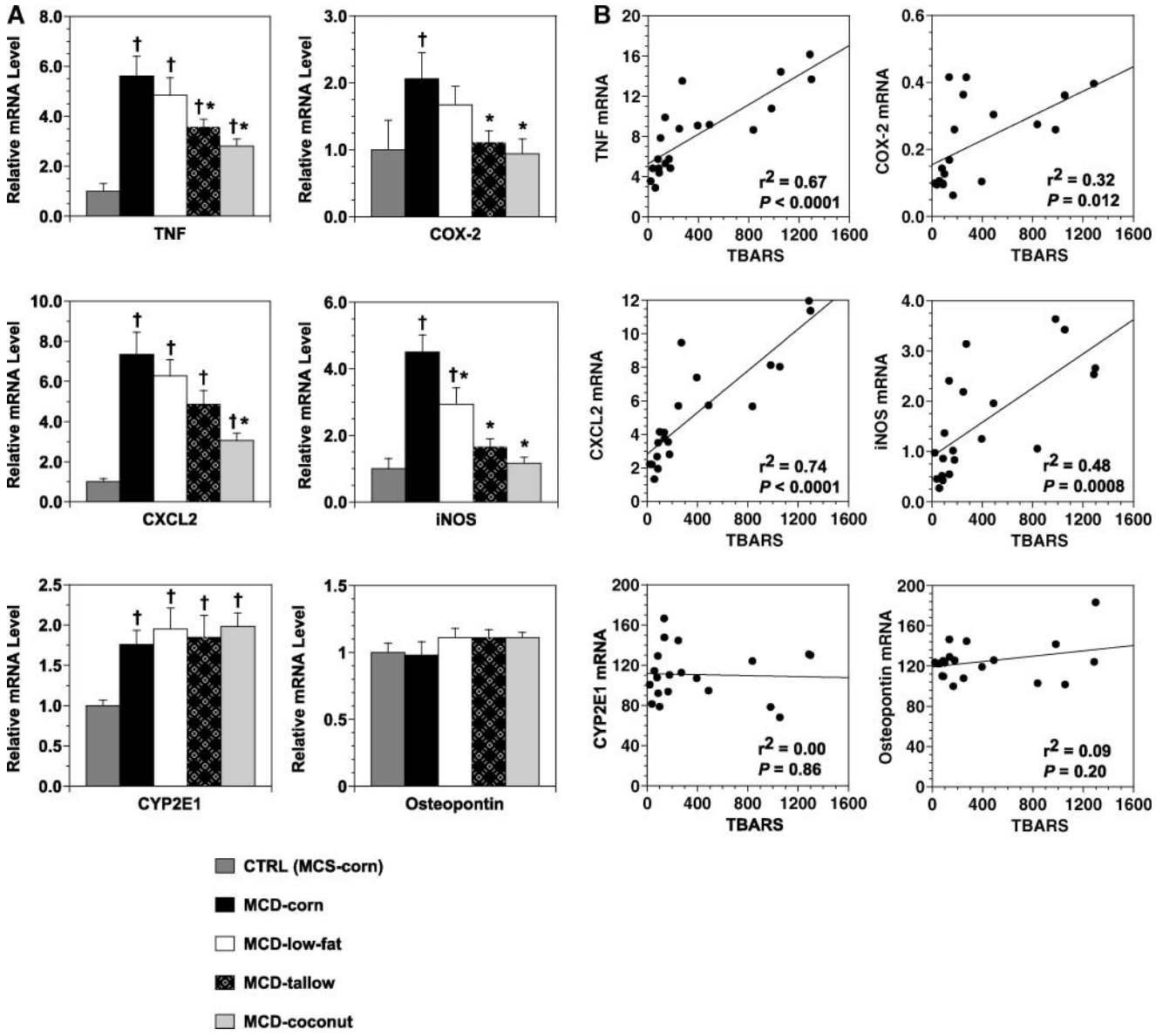

Fig. 5. Proinflammatory gene expression in the livers of MCD-fed mice. A: Effects of MCD feeding on proinflammatory gene expression in the liver. mRNA levels were measured by quantitative PCR as described in Experimental Procedures. Gene expression in MCS-corn control livers (CTRL) is shown for comparison. Relative to control, mRNAs encoding tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), CXC chemokine ligand-2 (CXCL-2), and inducible nitric oxide synthase (iNOS) were all significantly induced in the livers of mice fed the MCD-corn formula. The MCD-low-fat, MCD-tallow, and MCD-coconut formulas caused less pronounced if any induction of these genes. In contrast to the results for proinflammatory markers, cytochrome P4502E1 (CYP2E1) mRNA was upregulated similarly by all four MCD formulas. Osteopontin mRNA was not induced by any of the MCD formulas. Values represent means ± SEM for n = 10. † $P < 0.05$ versus control; * $P < 0.05$ versus MCD-corn. B: Relationship between hepatic gene expression and hepatic TBARS. TNF, COX-2, CXCL-2, and iNOS mRNAs are each strongly linked to hepatic lipid peroxidation, whereas CYP2E1 and osteopontin mRNAs are not.

idation and the induction of proinflammatory cytokines are also reduced, in a progressive manner that directly mirrors the PUFA content of the diet (corn oil > low-fat corn oil > tallow > coconut oil). Removal of unsaturated fat from the MCD formula results in significant suppression of MCD-mediated hepatic inflammation; it does not, however, have any impact on MCD-mediated injury to hepatocytes. These findings indicate that hepatocellular injury and hepatic inflammation are distinct pathophysiologic events in MCD-fed mice, which may provide an important clue to the pathogenesis of liver disease in this animal model of steatohepatitis.

MCD feeding offers a unique opportunity to examine the impact of specific fatty acids on the development of liver disease in the setting of hepatic steatosis. Methionine and choline deprivation blocks hepatic lipid secretion; thus, in animals fed an MCD diet, any fatty acids that are either taken up by or produced within the liver, if not required for cellular metabolism, are trapped within hepatocytes. By modifying the nutrient composition of the

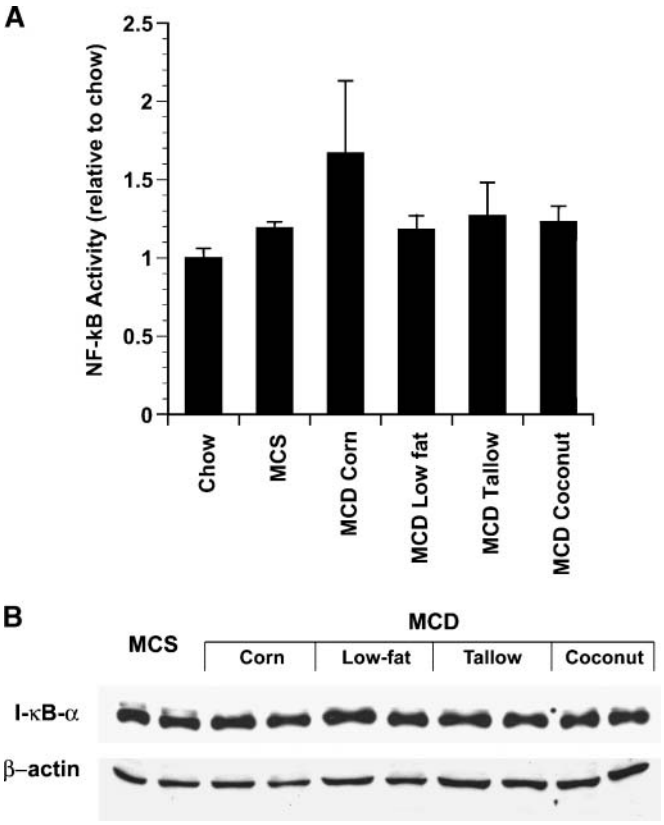

Fig. 6. Nuclear factor-κB (NF-κB) expression in the livers of MCD-fed mice. A: NF-κB DNA binding activity in the livers of mice fed MCS or MCD formulas for 21 days as a relative value compared with chow-fed control mice. Although a slight increase in NF-κB activity was observed in MCD-corn mice, the change was not statistically significant. Values represent means ± SEM for n = 5. B: inhibitor of NF-κB (I-κB) expression in the livers of mice after 21 days of MCS or MCD feeding. No I-κB degradation was observed in any of the MCD mice compared with the MCS controls.

MCD diet, one can exploit the abnormal physiology of MCD-fed animals to manipulate the fatty acid composition of the liver. Moreover, by maintaining the MCD formula at a constant caloric density, one can control the overall degree of hepatic steatosis and eliminate it as an experimental variable. Our study confirmed these principles by demonstrating that four different MCD formulas with similar caloric densities produced identical degrees of hepatic steatosis in mice. In addition, we demonstrated that the PUFA content of hepatic lipids could be decreased progressively by simply changing the fat source of the MCD formula from corn oil to beef tallow to coconut oil. Interestingly, the reverse was not completely true for SFAs. Although the SFA content of the MCD formulas increased progressively (corn oil < tallow < coconut oil) as unsaturated fatty acids were decreased, this led to only modest increases in SFAs within hepatic lipids (Fig. 1B). High-saturated fat MCD diets instead caused an increase in MUFAs within the liver. This reflects the requirement for SFAs to undergo desaturation before incorporation into triglyceride storage pools (Fig. 1C) (25). Notably, SFAs remained remarkably constant among all hepatic lipid compartments, despite wide variations in the SFA content of the MCD formula. This suggests the presence of a strong stimulus to control the SFA content of the liver, which may represent an effort to limit SFA-induced lipotoxicity (26).

One of the major findings in our study was that mice fed the MCD formula with the largest amount of polyunsaturated fat accumulated the most PUFAs in their hepatic lipids and exhibited the greatest degree of hepatic lipid peroxidation. This is reminiscent of earlier results with alcohol-fed animals, in which ethanol diets containing large amounts of polyunsaturated fat provoked a PUFA-rich hepatic steatosis together with significant lipid peroxidation (27–29). Like MCD diets, ethanol diets that are formulated with saturated fat cause much less hepatic lipid peroxidation than those formulated with polyunsaturated fat. The use of saturated fat in ethanol diets, however, markedly suppresses hepatic steatosis as well (30–32). The tendency for dietary saturated fat to cause less hepatic steatosis than dietary unsaturated fat was confirmed recently in a nonalcoholic model by Sampath and colleagues (19). They attributed the difference to sequestration of dietary unsaturated fat into an intracellular compartment unavailable for secretion as VLDL. Because methionine and choline deprivation causes a fixed defect in hepatic triglyceride secretion, all dietary lipids are retained regardless of their composition. This may explain why MCD-mediated steatosis does not vary with dietary fat, as does alcoholic steatosis. One advantage of the MCD-related defect in triglyceride export is that it permits the direct demonstration of a link between hepatic PUFAs and hepatic lipid peroxidation, even when the total amount of fat in the liver remains constant. This underscores the importance of hepatic PUFAs as substrates for lipid peroxidation and enables close scrutiny of the relationship between PUFAs, lipid peroxidation, and clinical outcomes in MCD-fed mice.

Deliberately reducing the PUFA content of dietary, and thus hepatic, lipids in MCD-fed mice blunted the induction of a number of proinflammatory genes in the liver and suppressed MCD-related hepatic inflammation. Proinflammatory gene expression varied linearly with hepatic TBARS, emphasizing the role of lipid peroxidation as a key event in the pathogenesis of MCD-related steatohepatitis. Among the genes influenced by dietary fat were COX-2 and TNF; these two compounds are believed to be upregulated in MCD-fed animals by oxidant stress in a process that involves the activation of NF-κB (22, 24). Interestingly, dietary fat also modulated the hepatic expression of iNOS and CXCL-2, two genes whose expression in MCD-fed livers does not require NF-κB activation (22). Together, these observations suggest that MCD-mediated oxidant stress and hepatic lipid peroxidation can lead to hepatic inflammation independently of NF-κB. In support of this theory, we found no evidence of NF-κB activation in mice fed MCD formulas at 21 days when proinflammatory gene expression was strongly induced. This is consistent with prior observations that MCD feeding causes early and transient activation of NF-κB in the liver, not necessarily coincident with the time course of proinflammatory gene expression (24). Our results implicate lipid peroxidation

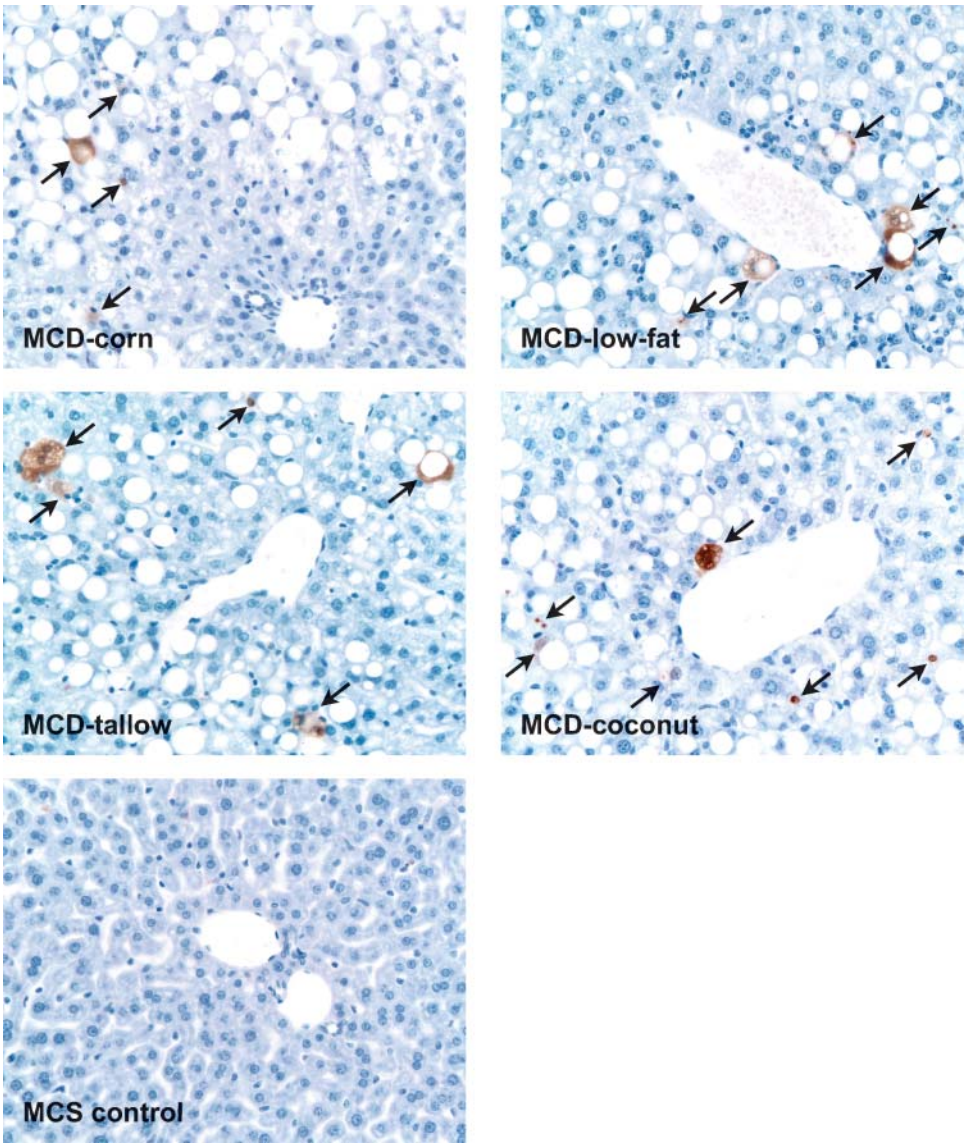

Fig. 7. Terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining in the livers of MCD-fed mice. Photomicrographs depict TUNEL staining in the livers of mice fed MCS and MCD formulas for 21 days. Liver sections were processed as described in Experimental Procedures, and TUNEL-positive cells were photographed at 10× original magnification. The chosen fields illustrate the most prominent TUNEL staining (arrows) within each dietary group. TUNEL-positive cell counts, performed on >100 unselected microscopic fields per liver, are shown in Fig. 8.

as an important stimulus to hepatic inflammation in MCD-fed mice. Even so, lipid peroxidation is not solely responsible for leukocyte infiltration, because we could not eliminate inflammation completely from MCD-fed mice even when we reduced hepatic lipid peroxidation to nearly normal levels. This suggests that other inflammatory mediators also promote leukocyte infiltration in the MCD model of steatohepatitis. Arachidonic acid and its derivatives are potential candidates for consideration, because MCD feeding provokes a significant increase in arachidonic acid levels in the liver. Our experiments do not support this theory, because we found no difference in arachidonic acid levels in mice fed the four versions of the MCD diet (see supplementary Fig. I), even though they had marked differences in hepatic inflammatory score. Another possibility is that factors released by dying hepatocytes could provoke leukocyte recruitment (33). Yet another option is that bacterial endotoxins in the portal circulation contribute to the pathogenesis of MCD-mediated hepatic inflammation. This is controversial, because mice with defects in Toll-like receptor 2 signaling have been reported to be even more susceptible to MCD-mediated liver disease than wild-type mice (34).

In striking contrast to the close interrelationship among dietary fat, hepatic lipid peroxidation, and hepatic inflammation in MCD-fed mice, dietary fat had virtually no impact on hepatocellular injury. Indeed, reducing or eliminating polyunsaturated fat from the MCD formula failed to ameliorate histologic apoptosis or ALT release, even though these same manipulations caused significant

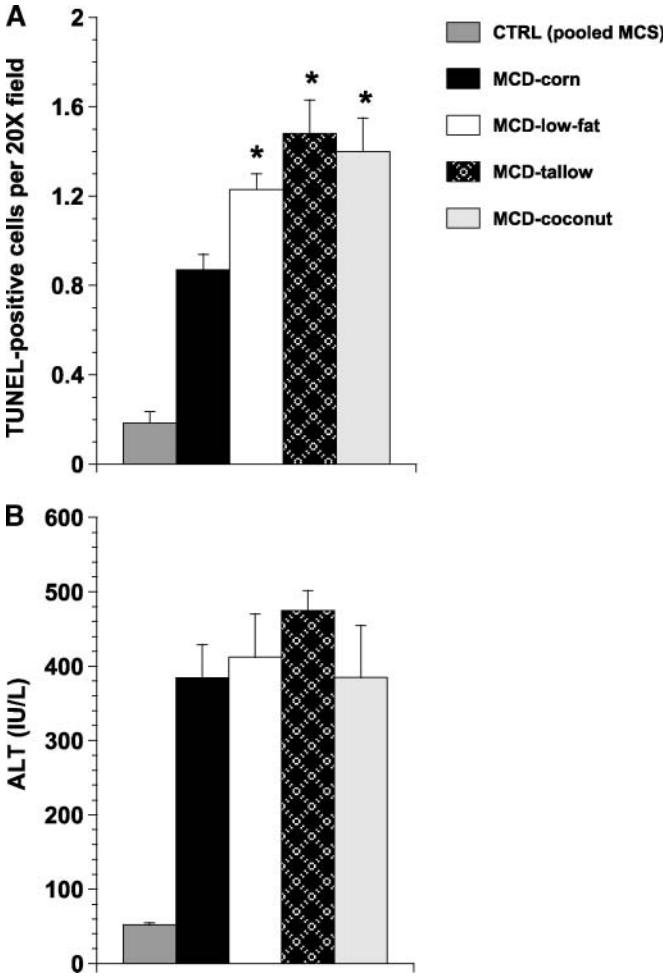

Fig. 8. TUNEL-positive cell counts and serum alanine aminotransferase (ALT) as measures of liver cell death in MCD-fed mice. A: Number of TUNEL-positive cells in the livers of mice fed MCS or MCD formulas for 21 days. TUNEL-positive cells were counted under direct microscopic visualization in >100 unselected 20× microscopic fields per liver. Many more TUNEL-positive cells were present in the livers of MCD mice than in MCS control (CTRL) mice ($P < 1 \times 10^{-7}$ vs. control for all MCD groups). Mice fed MCD formulas with reduced amounts of unsaturated fat (MCD-low-fat, MCD-tallow, and MCD-coconut) had more apoptosis than mice fed the MCD-corn formula. Values represent means ± SEM for n = 5 livers. * $P < 0.05$ versus MCD-corn. B: Serum ALT in mice fed MCS or MCD formulas for 21 days. Control data are from pooled MCS control groups (MCS-corn, MCS-low-fat, MCS-tallow, and MCS-coconut). All four MCD formulas caused a significant increase in serum ALT relative to control formulas. There was no difference in ALT among any of the individual MCD groups. Values represent means ± SEM for n = 10.

improvements in hepatic lipid peroxidation and proinflammatory gene expression. This indicates the presence of a clear-cut distinction between hepatic lipid peroxidation and hepatocellular injury in the MCD model of steatohepatitis and suggests that hepatocyte death in MCD-fed mice is caused by some factor that was consistently abnormal among all animals fed the four modified-fat MCD formulas. The factor in question is probably a lipid, because experiments have shown that elimination of all hepatic lipid from MCD-fed mice corrects MCD-mediated liver injury (10, 11). We speculate that long-chain SFAs are the cytotoxic species, because they remained nearly invariant in mice fed the four modified-fat MCD formulas and because long-chain SFAs are directly toxic to hepatocytes in culture (35, 36). Because the bulk of long-chain SFAs in MCD-fed livers are probably produced from dietary sucrose (37), our results suggest that dietary sugar is responsible for hepatocellular injury in the MCD model. Studies are under way to address this issue directly.

Whether dietary PUFAs influence the development of MCD-mediated hepatic fibrosis could not be determined from our study. Fibrosis is a delayed event in the MCD model of steatohepatitis, occurring only after 12 or more weeks of MCD feeding (12). Our studies ended at 3 weeks, because this represents the interval at which MCD-fed mice lost 30% of their body weight. Longer term experiments will be required to assess whether dietary PUFAs affect the liver fibrosis that results from prolonged steatohepatitis in MCD-fed mice.

In the context of human fatty liver disease, it is worth noting that corn oil, the most noxious of the three fats we incorporated into the MCD formula, is rich in n-6 fatty acids. High intake of n-6 fatty acids was recently implicated in the pathogenesis of nonalcoholic steatohepatitis in humans, even after correction for total fat intake and body mass index (38). In contrast, n-3 fatty acids have been reported to improve nonalcoholic fatty liver disease (39). Our study did not address the role of n-3 fatty acids in the MCD model of steatohepatitis. One can speculate, however, that these PUFAs may be therapeutic by virtue of their ability to enhance peroxisome proliferator-activated receptor α activity and reduce arachidonic acid concentrations (40, 41).

In summary, this study demonstrates that MCD feeding causes hepatocellular injury and hepatic inflammation by independent mechanisms. Whereas MCD-mediated inflammation is provoked by oxidant stress and lipid peroxidation, MCD-mediated death of hepatocytes follows a different pathway, possibly involving lipotoxicity. Because the inflammatory component of MCD-mediated liver injury is accentuated by high intrahepatic concentrations of PUFAs, which are excellent substrates for lipid peroxidation, MCD-related hepatic inflammation can be selectively ameliorated by reducing the polyunsaturated fat content of the MCD formula. Similarly, the cytotoxic component of MCD-mediated liver injury may be attributable to high intrahepatic concentrations of SFAs, which may respond favorably to a reduction in the sucrose content of the MCD diet. The MCD model, whose unique features permit fine regulation of hepatic lipid composition, makes it possible to distinguish how individual nutrients provoke liver disease in vivo. This information should prove useful to researchers investigating the role of dietary carbohydrate and fat in the pathogenesis of nonalcoholic steatohepatitis.

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