Dietary Saturated Fatty Acids Down-Regulate Cyclooxygenase-2 and Tumor Necrosis Factor Alfa and Reverse Fibrosis in Alcohol-Induced Liver Disease in the Rat

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We investigated the potential of dietary saturated fatty acids to decrease endotoxemia and suppress expression of cyclooxygenase 2 (Cox-2) and tumor necrosis factor α (TNF-α) in established alcohol-induced liver injury. Six groups (five rats/group) of male Wistar rats were studied. Rats in group 1 were fed a fish oil-ethanol diet for 6 weeks. Rats in groups 2, 3, and 4 were fed fish oil and ethanol for 6 weeks. Ethanol administration was stopped at this time, and the rats were switched to isocaloric diets containing dextrose with fish oil (group 2), palm oil (group 3), or medium-chain triglycerides (group 4) as the source of fat for an additional 2 weeks. Rats in groups 5 and 6 were fed fish oil-ethanol and fish oil-dextrose, respectively, for 8 weeks. Liver samples were analyzed for histopathology, lipid peroxidation, and levels of messenger RNA (mRNA) for Cox-2 and TNF-α. Concentrations of endotoxin were determined in plasma. The most severe inflammation and fibrosis were detected in groups 1 and 5, as were the highest levels of endotoxin, lipid peroxidation, and mRNA for Cox-2 and TNF-α. After ethanol was discontinued, there was minimal histological improvement in group 2 but near normalization of the histology, including regression of fibrosis, in groups 3 and 4. Histological improvement was associated with decreased levels of endotoxin, lipid peroxidation, and reduced expression of Cox-2 and TNF-α. The data indicate that a diet enriched in saturated fatty acids (groups 3 and 4) effectively reverses alcohol-induced liver injury, including fibrosis. The therapeutic effects of saturated fatty acids may be explained, at least in part, by reduced endotoxemia and lipid peroxidation, which in turn result in decreased levels of TNF-α and Cox-2. (HEPATOLOGY 1997;26:1538-1545.)

Abbreviations: CD, conjugated dienes; Cox, cyclooxygenase; dNTP, deoxynucleotide triphosphate; MCT, medium-chain triglycerides; PCR, polymerase chain reaction; RT, reverse transcription; TBARS, thiobarbituric acid–reactive substances; TNF-α, tumor necrosis factor α.

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Treatment of alcohol-induced liver disease remains limited to supportive measures. Development of effective therapy depends on understanding the mechanisms that contribute to liver injury. Thus, although a number of hepatotoxic effects have been described for alcohol or a combination of alcohol and dietary imbalance,[1] the relationship between demonstrably toxic effects of alcohol and clinically significant liver injury remains uncertain.

Several lines of investigation indicate that dietary fat affects the severity of alcohol-induced liver injury. In experimental animals, for example, diets containing saturated fatty acids protect against alcohol-induced liver injury, whereas polyunsaturated fatty acids enhance the toxic potential of ethanol, as measured by fatty liver, inflammation, necrosis, and fibrosis.[2-5] Diets enriched in saturated fatty acids, e.g., palm oil[6] and medium-chain triglycerides,[7] also reduce the severity of established experimental alcohol-induced liver disease. Epidemiologic observations support the idea that saturated fat protects against alcohol-induced liver injury in humans.[8]

An important experimental issue is to extend the observations relating saturation of dietary fat and alcohol-induced liver injury to an underlying mechanism that accounts for the benefits of saturated fat, in order to target therapies to specific pathophysiological events in the evolution of alcohol-induced liver injury. At the level of mechanism of liver injury, it is known that saturated dietary fat, fed in association with ethanol, diminishes lipid peroxidation,[9] the activity of CYP 2E1,[10,11] which can contribute to lipid peroxidation, and the synthesis of vasoactive and proinflammatory eicosanoids by cyclooxygenase (Cox).[12,13] Although the causal relationships between these events is uncertain, lipid peroxides[14,15] and endotoxin,[16-18] which are believed to be important contributors to alcohol-induced liver injury, induce Cox-2.[19-22] Moreover, Cox-2–deficient (Cox-2 −/−) mice are less sensitive than control mice to endotoxin-mediated liver injury.[23] These observations, taken together with the known proinflammatory effects of the products of Cox-2–catalyzed reactions, have led us to propose that induction of Cox-2 is an important final pathway for mediation of alcohol-induced liver injury. We hence have proposed[24] that elevated levels of endotoxin and lipid peroxides in alcohol-fed animals induce Cox-2, the impact of which is to increase levels of proinflammatory and vasoactive substances that are the proximate causes of alcohol-induced liver injury (Fig. 1). We have tested this idea in the present work by determining whether treatment with saturated but not polyunsaturated

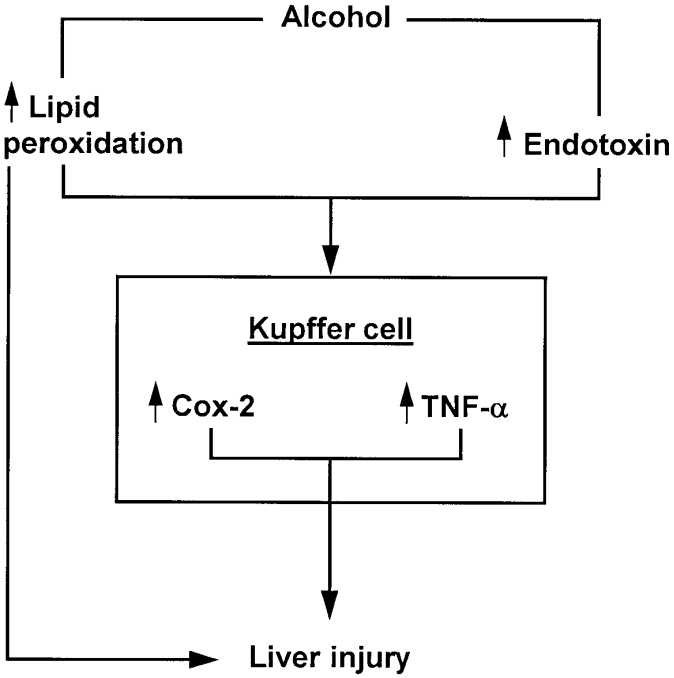

FIG. 1. Schematic diagram of proposed mechanism of interaction between endotoxemia and lipid peroxidation in alcohol-induced liver injury. Both endotoxin and lipid peroxidation up-regulate TNF-α and Cox-2 in Kupffer cells. Synergism between the actions of TNF-α, Cox-2, and lipid peroxidation promotes liver injury.

fatty acids down-regulates Cox-2 after cessation of an alcohol-containing diet that induces liver injury. The data indicate that diets enriched with saturated fatty acids, which improve all measured parameters of alcohol-induced liver disease, down-regulate Cox-2. By contrast, a diet containing polyunsaturated fatty acids neither causes significant improvement in indices of alcohol-induced liver disease nor down-regulates Cox-2.

MATERIALS AND METHODS

Animal Model. Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing between 225 and 250 g were fed a liquid diet by continuous infusion through permanently implanted gastric tubes, as previously described.[25,26] The rats received their total nutrient intake by intragastric infusion. Vitamins and minerals were given as described previously.[27] Ethanol and diet were administered continuously by a single gastric cannula. This was achieved by joining two tubes, one carrying ethanol from one syringe pump and the other carrying diet from a second pump, so that ethanol and diet could be varied at will. The dose of ethanol was increased slowly, as tolerance developed, to maintain blood alcohol levels in the range of 150 to 300 mg/dL. The starting dose was 8 g/kg/d; the final dose was 16 g/kg/d. Each ethanol-fed rat underwent at least two measurements of blood alcohol level.

Six groups of rats (5 rats/group) were studied to evaluate the effects of dietary fatty acids on pathological changes. The experimental design is shown schematically in Fig. 2. Rats in group 1 were fed a fish oil-ethanol diet for 6 weeks, then they were killed. Rats in groups 2 through 4 were fed the same fish oil-ethanol diet for 6 weeks, after which they were switched to a diet containing either fish oil with dextrose (FE-FD, group 2), palm oil with dextrose (FE-PD, group 3), or medium-chain triglycerides (MCT) with dextrose (FE-MCTD, group 4) for 2 more weeks and then killed. A liver biopsy was performed for histopathology before the animals began receiving the dextrose-containing diets. Rats in groups 5 and 6 received fish oil-ethanol and fish oil-dextrose, respectively, for 8 weeks before they were killed. The percentage of calories derived from fish oil, palm oil, or MCT was 35% of total calories. The caloric intake in all groups was identical. The fatty acid compositions of the palm oil, fish oil, and MCT have been described previously.[6,7,9] When the animals were killed, a sample of liver was taken for histopathology; the remainder of the liver was excised rapidly, washed with ice-cold 1.15% (wt/vol) KCl, and cut into small pieces, which were transferred to plastic vials and placed in liquid nitrogen. The vials were stored at −80°C. The studies were conducted according to the guidelines on care and use of laboratory animals established by the National Institutes of Health.

Histopathological Analysis Including Sirius Red Staining for Collagen. A small sample of liver was obtained by biopsy or at death and fixed in formalin. Hematoxylin-eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat), 1+, ≤25% of cells containing fat; 2+, 26%-50%; 3+, 51%-75%; 4+, >75%. Necrosis was evaluated as the number of necrotic foci per square millimeter; inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample of liver. The pathologist evaluating these sections was unaware of the treatment the rats had received.

For evaluation of fibrosis around the central veins, sections were stained with sirius red and analyzed using computerized image analysis. The area of collagen deposition around each central vein was measured using a Macintosh-based morphometric analysis system (Apple Computer Inc., Brea, CA) with NIH Image version 1.52 software. The cross-sectional area of the central vein lumen was measured using the same technique. The area of collagen deposition was divided by the area of the central vein lumen to correct for the size of the lumen and provide a standardized measurement of peri–central vein collagen deposition. The coefficient of variation of parameters measured was determined by assessment of a single central vein on six occasions (<5%). Pericellular fibrosis was estimated as the number of positively staining sites on adjacent hepatocyte surfaces per 100 hepatocytes around the central vein.

Measurements of Blood Alcohol Levels. Blood was collected from the tail vein, and ethanol concentration was measured using the alcohol dehydrogenase kit from Sigma Chemical Co. (St. Louis, MO).

Measurement of Plasma Endotoxin Levels. Blood samples were collected in endotoxin-free vials (Sigma Chemical Co.) and centrifuged at 400g for 15 minutes at 4°C. Samples were then diluted 1:10 in pyrogen-free water and heated to 75°C for 30 minutes to remove inhibitors of endotoxin from plasma. The Limulus Amoebocyte Lysate test (Kinetic-QLC; Whittaker Bioproducts, Walkersville, MD) was used for endotoxin measurements. Samples were incubated at 37°C for 10 minutes with limulus amebocyte lysate. The substrate

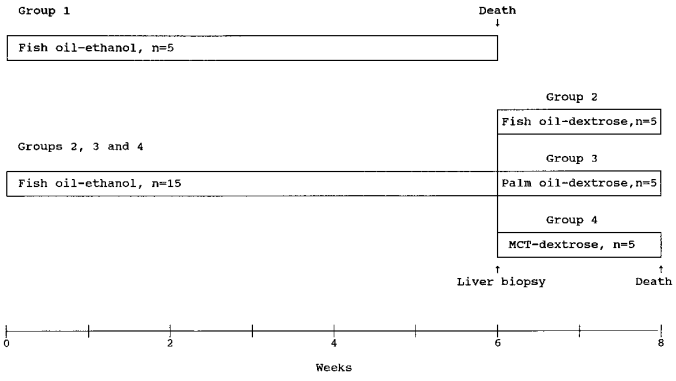

FIG. 2. Schematic diagram of experimental design.

TABLE 1. Severity of Pathological Changes in Treatment Groups

Treatment Group	Duration of Feeding (d)	Fatty Liver (0-4)	Necrosis (foci/mm^2)	Inflammation (cells/mm^2)
Group 1 (FE)				
Fish oil-ethanol	6	4.0 ± 0.0	1.4 ± 0.4	32.4 ± 7.4
Group 2 (FE-FD)				
Fish oil-ethanol	6	3.8 ± 0.4	1.2 ± 0.4	29.3 ± 9.1
Fish oil-dextrose	2	2.2 ± 0.4	0.7 ± 0.3	22.5 ± 5.9
Group 3 (FE-PD)				
Fish oil-ethanol	6	3.6 ± 0.5	1.0 ± 0.6	30.6 ± 12.6
Palm oil-dextrose	2	0.8 ± 0.7*,†	0.2 ± 0.1*,†	2.0 ± 1.0*,†
Group 4 (FE-MCTD)				
Fish oil-ethanol	6	3.8 ± 0.4	1.3 ± 0.3	32.0 ± 10.6
MCT-dextrose	2	1.0 ± 0.6*	0.2 ± 0.2*,†	1.9 ± 1.6*,†
Group 5 (FE)				
Fish oil-ethanol	8	4.0 ± 0.0	1.3 ± 0.5	33.9 ± 8.2
Group 6 (FD)				
Fish oil-dextrose	8	0.0	0.08 ± 0.04	0.7 ± 0.3

* $P < .01$ *v* fish oil-ethanol in same group.
† $P < .01$ *v* fish oil-dextrose (group 2).

solution was added, and the incubation continued for 20 minutes. The reaction was stopped with 25% acetic acid. Samples were read spectrophotometrically at 410 nm.

Determination of Thiobarbituric Acid–Reacting Substances and Conjugated Dienes. Liver thiobarbituric acid–reacting substances (TBARS) and conjugated dienes were determined as in previous studies.[6,7]

Analysis of Messenger RNAs for Cox-1, Cox-2, Tumor Necrosis Factor α, and β-Actin by Reverse Transcription–Polymerase Chain Reaction. To examine the expression of Cox-1, Cox-2, tumor necrosis factor α (TNF-α), and β-actin in liver tissue, total RNA was isolated according to the guanidinium isothiocyanate method.[28] The integrity of RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. To transcribe into complementary DNA (cDNA), 0.5-1 μg of total RNA was added to 30 μL of a master mix with reverse transcriptase buffer (0.6 mmol/L $MgCl_2$, 15 mmol/L KCl, 10 mmol/L TRIS HCl [pH 8.3]), 40 pmol of downstream primer, 0.5 mmol/L deoxynucleotide triphosphate (dNTP) mixture, 1 U/μL RNase inhibitor, and 13.3 U/μL Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY; final concentrations indicated). Samples were incubated, first for 60 minutes at 42°C and then for 10 minutes at 75°C, and then chilled on ice. Then 2 μL of each sample were added to 20 μL of 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L TRIS HCl (pH 8.3), 0.2 mmol/L each of dNTP and 0.01% gelatin, 5 U/100 μL Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) and 50 pmol of sense primer and 10 pmol of antisense primer. The sequences of primer pairs, 5′ and 3′, and predicted sizes of the amplified polymerase chain reaction (PCR) fragments of Cox-1, TNF-α, and β-actin have been reported previously.[24,29] The sequences for the Cox-2 sense and antisense primers were 5′-CCG TGG TGA ATG TAT GAG CAT AGG-3′ and 5′-GGA TGA ACT CTC TCC TCA GAA GGA CC-3′, respectively. The size of the amplified fragment was 440 base pairs (bp). Amplification was performed in an automated thermal cycler at 94°C for 60 seconds, 50°C for 90 seconds, and 72°C for 2 minutes for 35 cycles, followed by an additional 10-minute extension period at 72°C. To account for variations in the amount of reverse-transcribed RNA between samples, all data were normalized to β-actin, which was measured by the same technique. PCR products and molecular weight markers were subjected to electrophoresis on 1% agarose gels and visualized by means of ethidium bromide staining. The gels were analyzed by laser scanning densitometry using a Molecular Dynamics Densitometer and Image Quant Software (Molecular Dynamics, Sunnyvale, CA). Each experiment included a negative control (sample RNA that had not been subjected to reverse transcription [RT]). This sample did not yield a PCR product, confirming the absence of extraneous genomic DNA or PCR product contaminating the samples.

Sequence of RT-PCR Product. The identity of Cox-2 mesenger RNA (mRNA) and TNF-α mRNA bands was confirmed by sequence analysis of the RT-PCR products using an automated sequence analyzer (Applied Biosystems, Model 373A; Dana Farber Cancer Institute, Boston, MA).

Statistical Analysis. All data are expressed as means ± SD unless otherwise indicated. Differences between groups were analyzed using analysis of variance with *post hoc* analysis using the Bonferroni test.

RESULTS

There were no significant differences in weight gain among the different experimental groups (data not shown). Blood alcohol levels ranged from 150 to 300 mg/dL and were similar among the groups.

Effect of Experimental Diets on Liver Pathology. Feeding rats the fish oil-ethanol diet for 6 (group 1) or 8 (group 5) weeks caused fatty infiltration, necrosis, and inflammation (Table 1). As previously reported,[6,7] there was minimal improvement in these measures of hepatic pathology when ethanol was discontinued and rats were switched to a fish oil-dextrose diet (group 2). However, larger and statistically significant improvement in hepatic pathology occurred when the fish oil-ethanol diet was discontinued and replaced with either palm oil-dextrose (group 3) or MCT-dextrose (group 4). This was true for all measures of hepatic injury, e.g., degree of fatty liver, foci of necrosis, and numbers of inflammatory cells. Especially striking was a greater than 10-fold decline in numbers of inflammatory cells in groups 3 and 4.

In addition to the above changes, the type of fatty acid in the diet affected the amount of collagen and the extent of pericellular fibrosis (Figs. 3 and 4). For example, there was no improvement in the abundance of fibrous tissue when ethanol was stopped and rats were fed the fish oil-dextrose diet for 2 weeks (group 2), but the degree of fibrosis improved markedly ($P < .01$) when ethanol was stopped and the rats were switched to either the palm oil-dextrose diet

(group 3) or an MCT-dextrose diet (group 4) for 2 weeks (Figs. 3 and 4).

Dietary Modulation of Endotoxemia and Lipid Peroxidation. The effects of different dietary fatty acids on levels of known mediators of hepatic inflammation and injury, e.g., endotoxin and lipid peroxidation, were measured after cessation of the ethanol phase of the experiment (Table 2). Concentrations of endotoxin in serum and TBARS and conjugated dienes (CD) in liver decreased significantly in response to discontinuation of alcohol in all experimental groups; however, there were significant quantitative differences in the responses of groups fed different fatty acids. The smallest decreases in levels of endotoxin, TBARS, and CD occurred in rats fed the fish oil-dextrose diet in place of ethanol. The changes in these indices of liver injury were far greater in rats fed saturated fatty acids (groups 3 and 4). The levels of endotoxin, TBARS, and CD were about 70% lower in groups 3 and 4 than in group 2.

Effect of Experimental Diets on Cox-2 and TNF-α. As mentioned already, we have proposed that endotoxin and lipid peroxidation induce TNF-α and Cox-2 in alcohol-induced liver disease.[24] Thus, we also measured the effects of the different diets on levels of mRNA for TNF-α, Cox-2, and Cox-1 (Figs. 5 and 6, Table 2). We attempted to measure mRNA for TNF-α and Cox-2 by Northern blot and by ribonuclease protection assays, but levels of either mRNA were too low to detect by these analyses. Therefore, RT-PCR was used for these measurements. Data for TNF-α are shown in Fig. 5 for individual animals in groups 1 through 4. The band labeled TNF-α had the expected size of 276 bp, based on the primers used. Additionally, sequencing of the band labeled TNF-α confirmed that the material appearing here had the sequence of TNF-α. By comparison with the fish oil-ethanol group, the data for the other groups show that discontinuing ethanol led to a decrease in mRNA for TNF-α (group 1 vs. group 2). Treatment with saturated fatty acids was associated with a further decrease in TNF-α compared with the fish oil-dextrose diet. There was nonspecific staining of bands smaller than the expected 276-bp fragment of TNF-α that was amplified. The presence of these bands does not alter interpretation of the data for the band designated TNF-α because, as mentioned already, sequencing of this band identified it as TNF-α.

Changes in Cox-2 in the different groups paralleled those for TNF-α (Fig. 6 and Table 2). The highest levels of Cox-2 mRNA were seen in rats that were fed ethanol. A small decrease occurred when ethanol was discontinued and replaced with the fish oil-dextrose diet; no mRNA was detected in rats fed the palm oil-dextrose or MCT-dextrose diets. Levels of mRNA for Cox-1, which is a constitutive isoform of the enzyme, were similar in all groups.

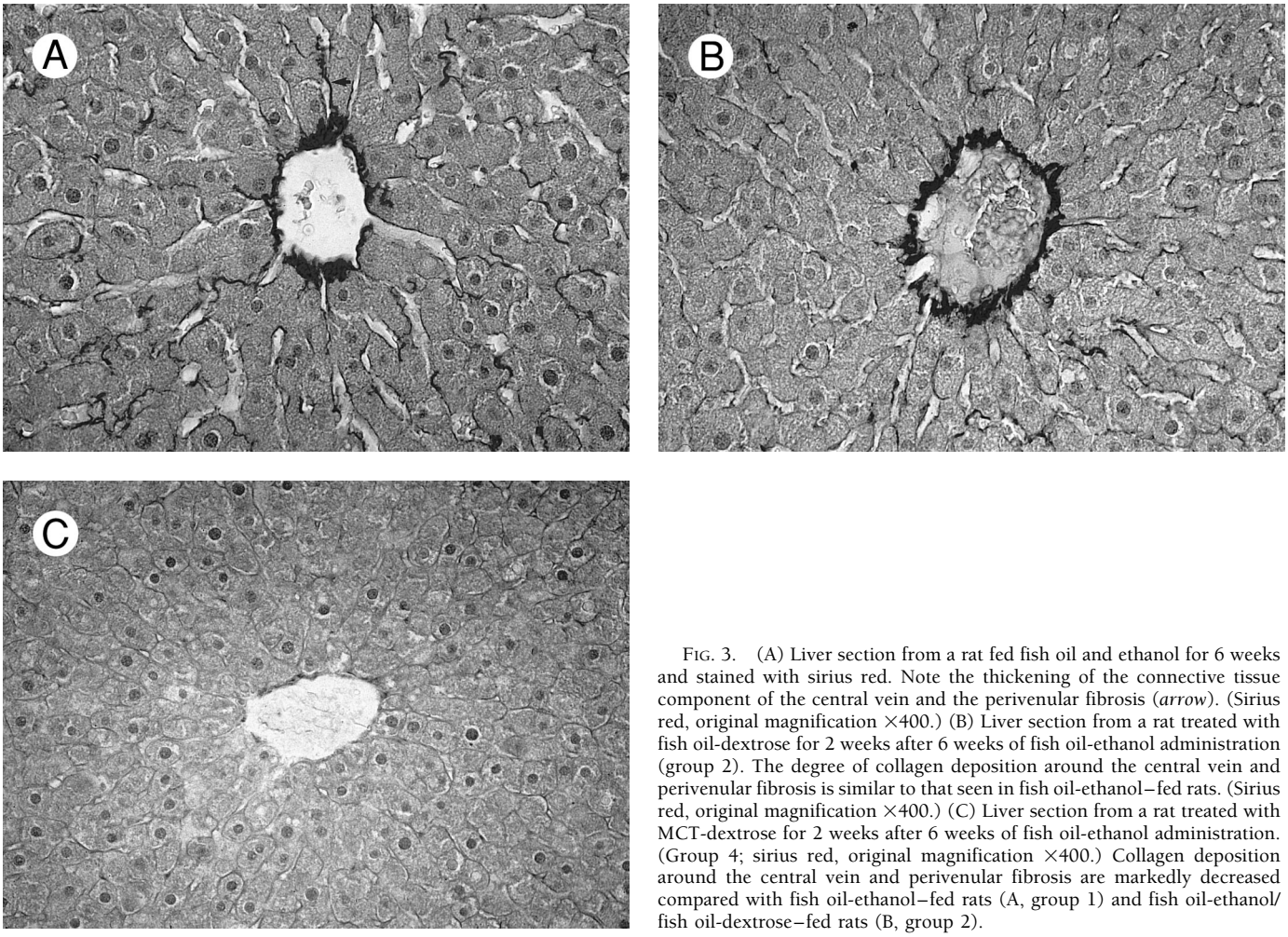

FIG. 3. (A) Liver section from a rat fed fish oil and ethanol for 6 weeks and stained with sirius red. Note the thickening of the connective tissue component of the central vein and the perivenular fibrosis (*arrow*). (Sirius red, original magnification ×400.) (B) Liver section from a rat treated with fish oil-dextrose for 2 weeks after 6 weeks of fish oil-ethanol administration (group 2). The degree of collagen deposition around the central vein and perivenular fibrosis is similar to that seen in fish oil-ethanol–fed rats. (Sirius red, original magnification ×400.) (C) Liver section from a rat treated with MCT-dextrose for 2 weeks after 6 weeks of fish oil-ethanol administration. (Group 4; sirius red, original magnification ×400.) Collagen deposition around the central vein and perivenular fibrosis are markedly decreased compared with fish oil-ethanol–fed rats (A, group 1) and fish oil-ethanol/fish oil-dextrose–fed rats (B, group 2).

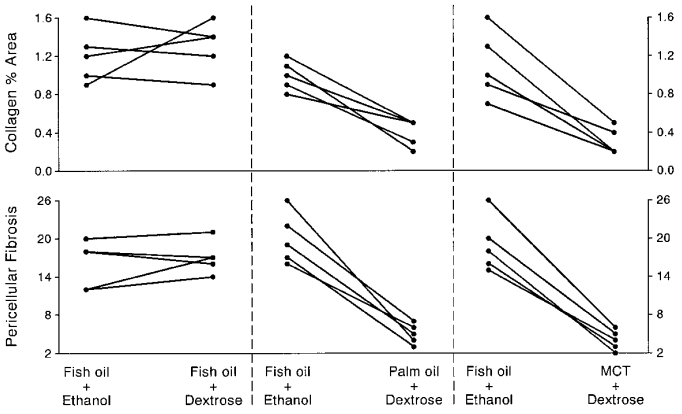

FIG. 4. Changes in amounts of collagen and pericellular fibrosis in rats fed fish oil and ethanol for 6 weeks followed by fish oil and dextrose (group 2), palm oil and dextrose (group 3), or MCT and dextrose (group 4) for 2 weeks. All animals underwent liver biopsy after 6 weeks of fish oil-ethanol treatment. The results of these initial biopsies served as a baseline for comparison with the results after treatment with the three experimental diets. In the fish oil-ethanol/fish oil dextrose group (group 2), there were no significant decreases in the amounts of central vein collagen (% area) (1.2% ± 0.5% to 1.3% ± 0.4%) or pericellular fibrosis (16% ± 9% to 17% ± 6%) after 2 weeks of treatment with fish oil-dextrose. The amounts of collagen and pericellular fibrosis in the fish oil-ethanol/palm oil-dextrose group (group 3) and the fish oil-ethanol/MCT-dextrose group (group 4) were significantly lower ($P < .01$) than those in the fish oil ethanol/fish oil-dextrose group (group 2) at the end of 2 weeks of treatment. For group 3, central vein collagen decreased from 0.97% ± 0.13% to 0.4% ± 0.1% ($P < .01$) and pericellular fibrosis from 18% ± 7% to 5% ± 3% ($P < .01$) after 2 weeks of palm oil-dextrose administration. In group 4, central vein collagen decreased from 1.1% ± 0.4% to 0.3% ± 0.2% ($P < .01$) and pericellular fibrosis from 19% ± 8% to 4% ± 2% ($P < .01$) after 2 weeks of MCT-dextrose administration.

DISCUSSION

Relationship Between the Rat Model of Alcohol-Induced Liver Injury and Alcohol-Induced Liver Disease in Humans. There are two possible strategies for preventing alcohol-induced liver disease. One, obviously, is to prevent the disease by preventing or stopping alcohol abuse. The other is to alter the natural course of the disease in the alcoholic, especially during and after the first clinical episode of alcohol-induced hepatitis. Unfortunately, there is no reason to be optimistic about primary prevention of alcohol abuse; realistically, we are more likely to arrive at better management of the hepatic complications of alcohol abuse than we are to interdict alcoholism. The current data are important in this regard because the experimental model used has striking similarities to the clinical setting in which alcohol-induced liver disease often occurs.

The rats in the current experiments had alcohol-induced liver injury before the application of a therapeutic regime. Typically, the alcoholic patient also stops drinking at the time of presentation. Many patients become sicker rather than better after being hospitalized, despite discontination of alcohol use.[30] The reason(s) for deterioration of these patients after discontinuation of alcohol use and the reason(s) for the rapid progression of acute alcohol-induced hepatitis to cirrhosis in a large percentage of patients,[31] even in the absence of further exposure to alcohol, remain unclear. However, the present data show that the injurious processes activated by alcohol can persist after alcohol use is stopped, e.g., high rates of lipid peroxidation, necrosis, inflammation, and fibrosis. It is clear that whatever pathological events are activated by ethanol cannot be down-regulated to premorbid levels by withdrawal of ethanol alone. The persistence of these indices of alcohol-induced liver injury in animals fed fish oil cannot be attributed exclusively to a diet enriched in polyunsaturated fatty acids because this type of diet has no injurious effect on the liver when fed in the absence of alcohol.[4] It seems instead that ethanol and diet interact in a concerted manner to set in motion injurious events that become self-sustaining. The key to improving the current therapy of alcohol-induced liver disease is to down-regulate the events that, once activated by ethanol, are not down-regulated by withdrawal of ethanol alone.

The current data and work published elsewhere show that it is possible to down-regulate the phenomena activated by exposure to ethanol in a surprisingly simple way, i.e., by manipulating the saturation of dietary fat. Thus, discontinuing ethanol administration and placing rats on a diet enriched in saturated fatty acids reduced indices of inflammation and necrosis and reduced the amounts of fibrous tissue accumulated during the ingestion of ethanol. But whereas diets enriched in saturated fatty acids led to healing of alcohol-induced injury, evidence of injury persisted in the absence of ethanol in rats fed a diet enriched in polyunsaturated fatty acids. Some of the reduction in levels of endotoxin, lipid peroxidation, and TNF-α mRNA can be attributed to cessation of alcohol. Substitution of the fish oil-dextrose diet led

TABLE 2. Evaluation of Endotoxin, Lipid Peroxidation, and mRNA Levels (Normalized Using β-Actin) for TNF-α, Cox-2, and Cox-1 in Experimental Groups

Experimental Group*	Endotoxin (pg/mL)	CD A_{232}	TBARS (nmol/mg protein)	TNF-α mRNA	Cox-2 mRNA	Cox-1 mRNA
FE (6 weeks)	79 ± 13	0.46 ± 0.16	1.37 ± 0.26	2.5 ± 0.9	1.3 ± 0.6	1.4 ± 0.2
FE-FD	42 ± 11§	0.29 ± 0.08	0.74 ± 0.19†	1.3 ± 0.3	1.1 ± 0.3	1.2 ± 0.3
FE-PD	17 ± 6‡	0.14 ± 0.01‡	0.28 ± 0.08‡	0.5 ± 0.2‡	ND	1.3 ± 0.1
FE-MCTD	13 ± 6‡	0.09 ± 0.03‡	0.22 ± 0.07‡	0.5 ± 0.1‡	ND	1.4 ± 0.1
FE (8 weeks)	83 ± 11	0.51 ± 0.14	1.40 ± 0.24	2.3 ± 0.6	1.2 ± 0.4	1.5 ± 0.4
FD (8 weeks)	9 ± 3	0.17 ± 0.07	0.37 ± 0.12	0.4 ± 0.2	ND	1.2 ± 0.4

NOTE. Data presented as means ± SD (n = 5). ND, not detected.

* Definitions as in Table 1.

† $P < .02$ *v* FE group (6 weeks).

‡ $P < .01$ *v* FE and FE-FD groups.

§ $P < .01$ *v* FE group (6 weeks).

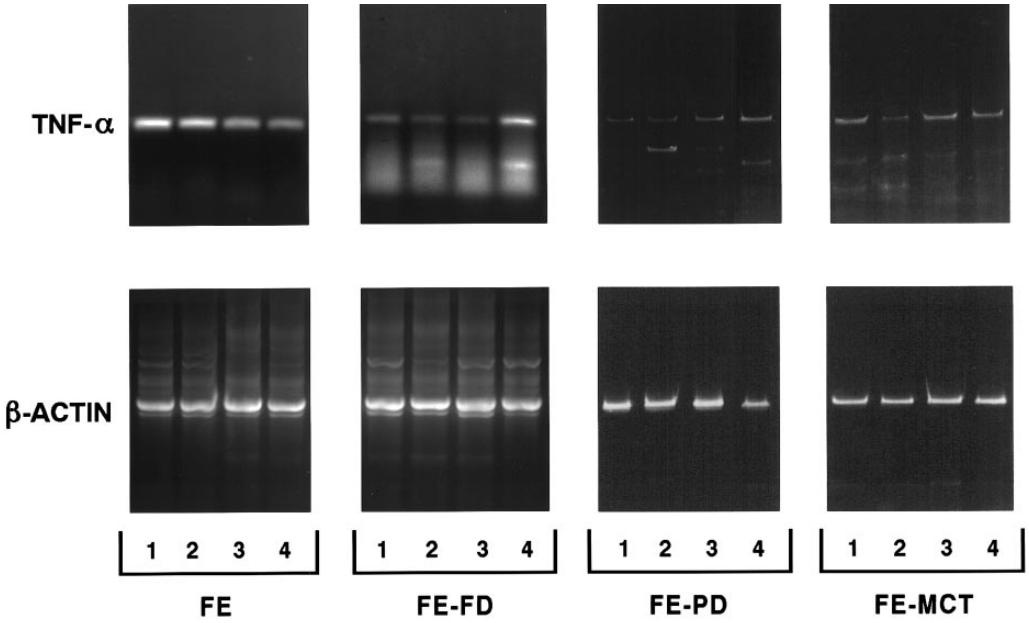

FIG. 5. RT-PCR analysis of mRNAs for TNF-α and β-actin in liver samples obtained from the various experimental groups: FE, fish oil-ethanol for 6 weeks; FE-FD, fish oil-ethanol for 6 weeks followed by fish oil-dextrose for 2 weeks; FE-PD, fish oil-ethanol for 6 weeks followed by palm oil-dextrose for 2 weeks; FE-MCT, fish oil-ethanol for 6 weeks followed by MCT-dextrose for 2 weeks. RNA (1 μg) was subjected to RT as detailed in Materials and Methods. The amplified products were separated on an ethidium bromide–stained agarose gel. The numbers at the bottom of each panel represent four different rats in each experimental group.

to a nearly 50% decline in chemical mediators of inflammation, e.g., endotoxin and TNF-α mRNA, whereas the saturated fatty acid diets led to 80% to 85% reductions in these indices. Taken together with the histological data in Table 1, these results suggest that reductions in chemical indices of inflammation on withdrawal of ethanol alone were not large enough to suppress completely the evidence of hepatic injury. Lieber et al.,[32] using a different lipid-based strategy, protected baboons against alcohol-induced fibrosis and cirrhosis by feeding polyunsaturated lecithin. Feeding this lipid increased degradation of collagen and decreased lipid peroxidation. These results are not incompatible with the present data because our approach and the experiments of Lieber et al.[32] are different. For example, metabolism of lecithin and triglyceride is unlikely to be the same. Additionally, the choline moiety of lecithin could have effects on the course of alcohol-induced liver injury that are independent of those attributable to the fatty acid moieties.[1] On the other hand, the improvement in severity of pathology, in association with a decrease in lipid peroxidation with the use of polyunsaturated lecithin, is consistent with our observations.

Mechanism by Which Saturated Fats Down-Regulate Pathological Events Triggered by Ethanol. It is well known that endotoxin is hepatotoxic and that concentrations of endotoxin increase in alcohol-induced liver injury.[16-18] Lipid peroxides also are hepatotoxic, and levels increase during alcohol-induced liver injury.[9] A concerted effect of endotoxin and lipid peroxidation in promoting liver injury also probably occurs. Thus Liu et al.[33] have shown that inhibition of lipid peroxidation protected against endotoxin-induced liver injury. Moreover, TNF-α, which is increased in alcohol-induced liver disease,[34,35] also can injure liver cells.[36-38] In other words, it is difficult, if not impossible, to delineate the contributions of each of these toxic factors to the overall problem of alcohol-induced liver injury. Nevertheless, a growing body of evidence places the activity of Cox-2 at the center of multiple

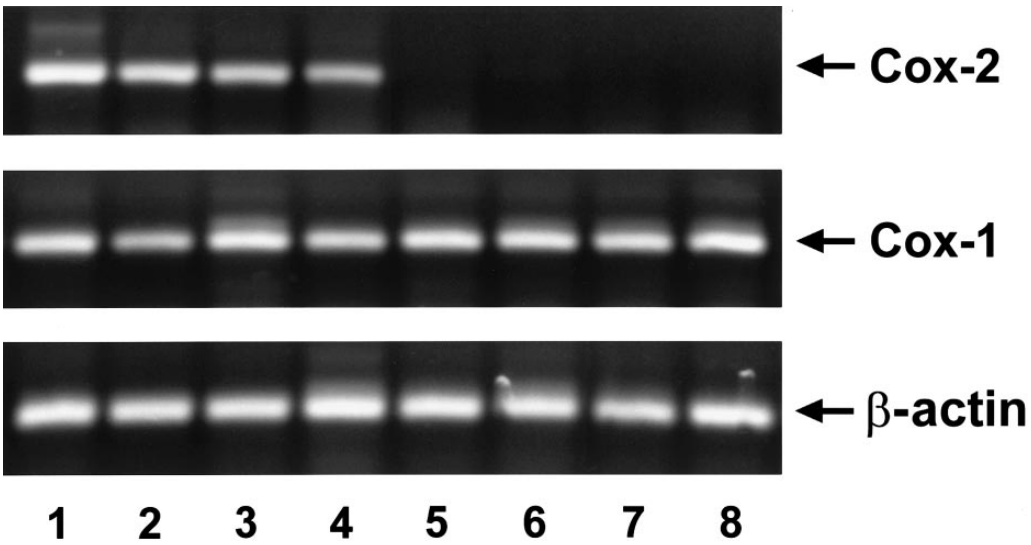

FIG. 6. RT-PCR analysis of mRNAs for Cox-2, Cox-1, and β-actin in liver samples obtained from the different experimental groups analyzed as in Fig. 5. *Lanes 1 and 2*: fish oil and ethanol (group 1); *lanes 3 and 4*: fish oil-ethanol followed by fish oil-dextrose (group 2); *lanes 5 and 6*: fish oil-ethanol followed by palm oil-dextrose (group 3); and *lanes 7 and 8*: fish oil-ethanol followed by MCT-dextrose (group 4).

mechanisms of tissue injury via production of vasoactive and proinflammatory compounds.[22] This is an important idea in the context of alcohol-induced liver injury because the current experiments show that alcohol-induced liver injury induces Cox-2, that a high level of induction can be sustained even after ethanol is discontinued, and that levels of mRNA for Cox-2 returned to pre-experimental levels only when ethanol was discontinued and rats were fed a diet enriched in saturated fat, which repaired all other indices of alcohol-induced liver injury. In addition, it is known already that two proposed mediators of alcohol-induced liver injury, e.g., endotoxin and lipid peroxides, induce Cox-2[14,15,19,20] and that Cox-2–deficient mice are protected against the toxic effects of endotoxin.[23]

An exact causal relationship between induction of Cox-2 and alcohol-induced liver injury remains speculative, but an obvious mechanism for such a relationship is Cox-2–dependent synthesis of proinflammatory and vasoconstrictive eicosanoids. Because drugs are emerging that are specific inhibitors of Cox-2[39-41] and there are agents that block the expression of Cox-2,[42] the present data suggest that these types of drugs, independent of diet, could ameliorate the toxic potential of ethanol by down-regulating some events that are activated by ethanol and that persist after withdrawal of ethanol.

In conclusion, our results show that improvement in pathological changes such as fatty liver, necrosis, and inflammation by saturated fatty acids is accompanied by a reduction in the expression of TNF-α and Cox-2. The improvement in fibrosis in the same groups, as well as the decrease in TNF-α and Cox-2, was associated with a decrease in lipid peroxidation. We believe the reduction in lipid peroxidation and endotoxemia in the treatment groups fed saturated fatty acids decreases proinflammatory stimuli mediated via induction of Cox-2 in alcohol-induced liver disease.

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