

Central Role of PPAR α -Dependent Hepatic Lipid Turnover in Dietary Steatohepatitis in Mice

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We have proposed that steatohepatitis results from reactive oxygen species (ROS) acting on accumulated fatty acids to form proinflammatory lipoperoxides. Cytochrome P450 4a (Cyp4a) and Cyp2e1 are potential hepatic sources of ROS. We tested the hypothesis that increasing Cyp4a through activation of peroxisome proliferator-activated receptor α (PPAR α) should aggravate steatohepatitis produced by feeding a methionine and choline deficient (MCD) diet. Conversely, we assessed dietary steatohepatitis in PPAR $\alpha^{-/-}$ mice that cannot up-regulate Cyp4a. Male wild type (wt) or PPAR $\alpha^{-/-}$ mice (C57BL6 background) were fed the MCD diet with or without Wy-14,643 (0.1% wt/wt), a potent PPAR α agonist. Controls were fed the same diet supplemented with methionine and choline. After 5 weeks, wt mice fed the MCD diet developed moderate steatohepatitis and alanine aminotransferase (ALT) levels were increased. Wy-14,643 prevented rather than increased liver injury; ALT levels were only mildly elevated whereas steatohepatitis was absent. Wy-14,643 up-regulated mRNA for liver fatty acid binding protein and peroxisomal β -oxidation enzymes (acyl-CoA oxidase, bifunctional enzyme, and ketothiolase), thereby reducing hepatic triglycerides and preventing steatosis. In wt mice, dietary feeding up-regulated Cyp4a14 mRNA 2.7-fold and increased hepatic lipoperoxides compared with controls. Wy-14,643 prevented hepatic lipoperoxides from accumulating despite an 18-fold increase in both Cyp4a10 and Cyp4a14 mRNA. PPAR $\alpha^{-/-}$ mice fed the MCD diet developed more severe steatohepatitis than wt mice, and were unaffected by Wy-14,643. In conclusion, PPAR α activation both increases Cyp4a expression and enhances hepatic lipid turnover; the latter effect removes fatty acids as substrate for lipid peroxidation and is sufficiently powerful to prevent the development of dietary steatohepatitis. (HEPATOLOGY 2003;38:123-132.)

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Nonalcoholic fatty liver diseases are a common complement to the metabolic disorders: obesity, insulin resistance and type II diabetes mellitus.^{1,2} Among these disorders, nonalcoholic steatohepati-

tis (NASH) is an important form of liver disease that may progress to cirrhosis and liver failure.^{3,4} However, the mechanisms that mediate the transition from steatosis to steatohepatitis remain unknown. One possibility is that an increase in reactive oxygen species (ROS) could act on accumulated fatty acids in the steatotic liver leading to their peroxidation. The end products of lipid peroxidation such as malondialdehyde and 4-hydroxynonenal have known proinflammatory properties, and could therefore initiate hepatic inflammation indirectly or via activation of redox sensitive transcription factors.^{5,6}

The observation that alcoholic steatohepatitis and NASH exhibit similar liver pathology provoked the idea that some pathogenic mechanisms may be shared between the two conditions. Cytochrome P450 2E1 (CYP2E1) is one of several possible sources of ROS increased in the livers of humans with alcohol-induced liver disease⁷ and NASH.⁸ Furthermore, in rodents with steatohepatitis induced by feeding a lipid-rich diet deficient in both methionine and choline (MCD), CYP2E1 is induced, catalytically active, and associated with accumulation of hepatic lipoperoxides.⁹ However, the finding that steatohepatitis also develops in Cyp2e1^{-/-} mice fed the MCD

Abbreviations: NASH, nonalcoholic steatohepatitis; ROS, reactive oxygen species; CYP, cytochrome P-450; MCD, methionine and choline deficient; ACO, acyl-CoA oxidase; PPAR α , peroxisome proliferator activated receptor α ; wt, wild type; ALT, alanine aminotransferase; TBARS, thiobarbituric acid reactive substances; BIEN, peroxisomal L-bifunctional enzyme; KT, peroxisomal ketothiolase; LFABP, liver fatty acid binding protein; mRNA, messenger RNA; NF- κ B, nuclear factor κ B.

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diet showed that Cyp2e1 itself is not an absolute requirement for the development of this lesion. In these animals, Cyp4a10 and Cyp4a14 were up-regulated and shown to be highly capable of peroxidizing lipids.¹⁰ Other evidence that Cyp4a could play a pathogenic role comes from observations that mice deficient in acyl-CoA oxidase (ACO), the rate-limiting enzyme in the peroxisomal β -oxidation of fatty acids, develop severe steatohepatitis, and this is associated with massive up-regulation of Cyp4a.¹¹

Cyp4a and ACO are among several genes involved in fatty acid turnover that are regulated by the ligand-activated transcription factor, peroxisome proliferator-activated receptor α (PPAR α).¹²⁻¹⁴ Unmetabolized fatty acids that accumulate in ACO^{-/-} mice are thought to act as ligands of PPAR α thereby up-regulating Cyp4a and other PPAR α target genes. The potential importance of such induction of Cyp4a and products of lipoperoxides for the pathogenesis of steatohepatitis is lent credence by the observation that ACO/PPAR α double knockout mice develop only mild steatohepatitis.¹⁵ We have therefore proposed an integrated concept whereby microsomal sources of ROS, including either or both Cyp2e1 and the Cyp4a family, contribute centrally to the pathogenesis of steatohepatitis.¹⁶

In the present study, we tested the hypothesis that an increase in Cyp4a activity through activation of PPAR α could lead to an increase in ROS and lipid peroxidation that would accentuate experimental NASH. To do this, we administered the potent and specific PPAR α agonist Wy-14,643 to mice fed the high-fat MCD diet for 5 weeks. We found that the vastly increased expression of PPAR α -dependent Cyp4a10, Cyp4a14, and lipid handling genes by Wy-14,643 is actually associated with reduced hepatic triglyceride and lipoperoxide content and prevention of steatohepatitis. We then further confirmed the primary role of hepatic lipid turnover in pathogenesis of steatohepatitis by analyzing aspects of experimental steatohepatitis in mice deficient in hepatic adaptation to a high-fat intake due to PPAR α deficiency.

Materials and Methods

Animals and Experimental Protocols. Breeding pairs of wild type PPAR α ^{+/+} (wt) and PPAR α ^{-/-}¹⁷ mice (C57BL6 background) were kindly donated by Professor Frank J. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD). Animal breeding and care were conducted within the Animal Care Facility, Westmead Hospital. All animal protocols and studies performed complied with the highest International Criteria of Animal Experimentation, as approved by the Western

Sydney Area Health Service Animal Ethics Committee. Except where otherwise indicated, all experiments were performed on male mice weighing 23 to 30 g at 8 to 10 weeks of age. PPAR α ^{-/-} and wt mice were divided into 3 experimental groups and fed as follows: (1) MCD diet (cat. no. 960439; ICN, Aurora, OH), (2) MCD diet supplemented with Wy-14,643 at 0.1% (wt/wt) (ChemSyn Laboratories, Lenexa, KS), or (3) control diet, which was identical to the MCD diet but supplemented with DL-methionine (3 g/kg) and choline chloride (2 g/kg; cat. no. 960441; ICN). In a limited number of experiments, mice fed the control diet were also treated with Wy-14,643 (0.1% wt/wt). Mice were allowed food and water *ad libitum* for 5 weeks.

Preparation of Tissue and Serum Samples. Mice were anesthetized (ketamine 100 mg/kg and xylazine 20 mg/kg, administered intraperitoneally). Blood was collected by cardiac puncture and livers were rapidly excised and perfused with ice-cold phosphate buffered saline, pH 7.4. A portion of tissue was fixed in 10% neutral buffered formalin and processed and embedded in paraffin for histologic analysis. The remaining liver was snap frozen in liquid nitrogen and stored at -80°C until required.

Morphologic Studies. Liver sections (4 μ m thick) were stained with hematoxylin and eosin, and scored for steatosis and necroinflammation by two expert and independent liver pathologists blinded to the study (P.H. and R.K.). The system devised for this study scored steatosis based on the percentage of hepatocytes showing lipid accumulation as follows: 0, 0%; 1, 0% to 33%; 2, 33% to 66%; 3, 66% to 100%. Necroinflammation was scored as: 0, absent; 1, mild; 2, moderate; 3, severe.

Biochemical Assays. Serum triglyceride, cholesterol, and alanine aminotransferase (ALT) levels were measured using automated techniques within the Department of Clinical Chemistry, Westmead Hospital. Liver triglycerides were assayed using triglyceride GPO-Trinder reagents (Sigma Diagnostics Inc., St. Louis, MO). Liver lipoperoxide levels were estimated as thiobarbituric acid-reactive substances (TBARS) using 1,1,3,3-tetramethoxypropane as a standard (Sigma) as previously described.¹⁰ Microsomal fractions were prepared by differential ultracentrifugation¹⁸ and protein was estimated using bovine serum albumin as standard (DC protein assay; BioRad, Hercules, CA). We determined the extent of NADPH-dependent lipid peroxidation in microsomal fractions essentially as described by Leclercq et al.¹⁰ For inhibition studies, we used rabbit polyclonal anti-rat CYP2E1 antibody (kindly provided by Professor Magnus Ingelman-Sundberg, Department of Physiological Chemistry, Karolinska Institutet, Stockholm, Sweden) to inhibit mouse Cyp2e1¹⁰ and goat polyclonal anti-rat

Table 1. Primer Pairs Used for the Preparation of Riboprobes for Ribonuclease Protection Assays and Northern Blotting Experiments

Target	PCR Product	Primer Pairs	Genbank Accession No.
ACO	2598-3132	F 5'-TGTCATTCTACCAACTGTC-3' R 5'-CCATCTTCTCAACTAACACTC-3'	AF006688
BIEN	527-928	F 5'-ATGCAGTCGTGAAGTCAGACCC-3' R 5'-CAAGAACGCCCACTGAGGAGAC-3'	K03249
KT	426-748	F 5'-CAACAGACAGTGTTTCATCG-3' R 5'-TCACAGGCACAATCTCAG-3'	AI528283
LFABP	33-405	F 5'-TGCCACCATGAACCTCTCC-3' R 5'-TCTCTGTAGACAATGTCGCC-3'	Y14660

CYP4A1 antibody (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) to inhibit with mouse Cyp4a10¹⁹ or nonspecific goat IgG as control immunoglobulin (Sigma). Cyp2e1 protein levels in microsomal fractions were assayed by Western blotting using the rabbit polyclonal anti-rat CYP2E1 antibody.⁹

Preparation of Riboprobes. Regions from the genes encoding ACO, peroxisomal L-bifunctional enzyme (BIEN), peroxisomal ketothiolase (KT), and liver fatty acid binding protein (LFABP) were amplified from mouse liver complementary DNA by polymerase chain reaction using the primers indicated in Table 1 and inserted into pGEM-T or pGEM-T Easy vectors (Promega, Madison, WI). The plasmids were then used as a template for the synthesis of riboprobes using SP6 or T7 polymerase (Promega) and α [³²P]CTP (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). The probes for Cyp4a10 and Cyp4a14 have been described.^{19,20} pTRI- β -actin and pTRI-cyclophilin mouse antisense control templates were purchased from Ambion (Austin, TX).

Messenger RNA Analyses. Total RNA was prepared from frozen liver using TRIzol reagent (Gibco BRL, Melbourne, Australia). Hepatic messenger RNA (mRNA) for

ACO and KT were detected by northern blotting. RNA (10 μ g) was separated by electrophoresis, transferred onto a Hybond N⁺ nylon membrane (Amersham Pharmacia Biotech) and hybridized to α [³²P]CTP-labeled riboprobe overnight at 68°C in UltraHyb solution (Ambion). Bands were visualized with a Phosphorimager screen and quantified using the ImageQuant analysis program (Molecular Dynamics, Sunnyvale, CA). To standardize for equivalence of loading and to show integrity of RNA, membranes were stripped and reprobated with 18S oligonucleotide.¹⁰

Hepatic levels of mRNAs for Cyp4a10, Cyp4a14, BIEN, and LFABP were measured using RNase protection assays. Riboprobes against target mRNA and an internal control were hybridized to 5 μ g RNA overnight at 55°C. Cyclophilin was used as an internal control for LFABP, whereas β -actin was cohybridized with BIEN, Cyp4a10, and Cyp4a14. Unbound single-stranded RNA was digested using RNase T1 (Roche Diagnostics, Indianapolis, IN), and protected double-stranded RNA fragments were separated on 5% polyacrylamide gels containing 8 mol/L urea in 1 $\times$ Tris-borate-ethylenediaminetetraacetic acid. Gels were dried and visualized by autoradiography, and bands were quantified using the Phosphorimager and ImageAnalysis systems.

Statistical Analyses. Results are presented as mean $\pm$ standard deviation. The 2-way ANOVA and Student's *t* test were used to compare data from different treatment groups. When *P* was less than .05, differences were considered significant.

Results

Effects of Wy-14,643 on Steatohepatitis Induced by the MCD Diet in Wt Mice. As previously reported,^{9,10} administration of the MCD diet caused weight loss (Table 2), but the physical appearance and behavior of mice were otherwise similar to control diet-fed animals. In wt

Table 2. Characteristics of wt and PPAR α ^{-/-} Mice Fed the Control or MCD Diet, With or Without Wy-14,643

	wt			PPAR α ^{-/-}		
	Control	MCD		Control	MCD	
	-	-	Wy-14,643	-	-	Wy-14,643
Number of animals	4	5	4	5	6	3
Weight (% change)	+21	-34	-39	+23	-35	-34
Relative liver weight (% of body weight)	4.0 $\pm$ 0.8	4.1 $\pm$ 0.1	8.0 $\pm$ 1.4*	3.7 $\pm$ 0.5	5.5 $\pm$ 1.1	5.8 $\pm$ 1.4
Serum triglyceride (mmol/L)	0.88 $\pm$ 0.11	0.69 $\pm$ 0.15†	0.21 $\pm$ 0.05*	1.20 $\pm$ 0.68	0.49 $\pm$ 0.19†	0.47 $\pm$ 0.07
Serum cholesterol (mmol/L)	2.05 $\pm$ 0.55	0.70 $\pm$ 0.16†	2.53 $\pm$ 0.90*	2.92 $\pm$ 1.07	0.85 $\pm$ 0.34†	0.87 $\pm$ 0.21

NOTE. Data are mean $\pm$ SD.

**P* < .05 relative to same genotype fed the MCD diet.

†*P* < .05 relative to same genotype fed the control diet.

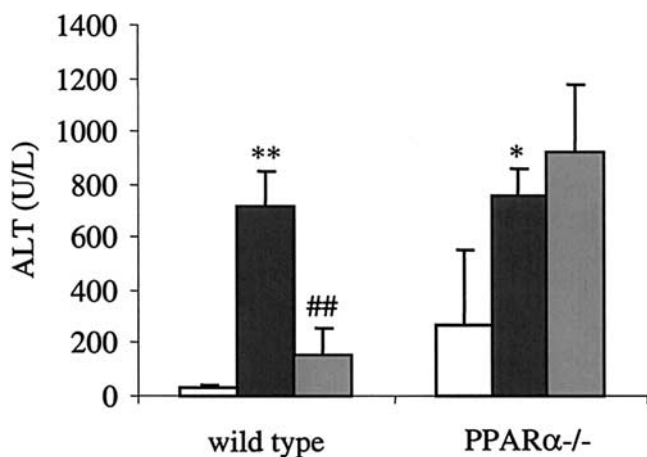


Fig. 1. Effect of MCD diet with or without Wy-14,643 on serum ALT levels in wt and PPAR $\alpha^{-/-}$ mice. Male wt or PPAR $\alpha^{-/-}$ mice were fed control diet (□), MCD diet (■), or MCD diet with Wy-14,643 (0.1% wt/wt) (▨) for 5 weeks. The graph represents mean $\pm$ SD for serum ALT, $n = 3-6$ /group (see Table 2). * $P < .01$, ** $P < .001$ compared with same genotype mice fed the control diet. ## $P < .001$ compared with same genotype mice fed the MCD diet.

mice, dietary feeding caused a marked elevation of serum ALT levels (Fig. 1), whereas serum triglyceride and cholesterol concentrations were reduced (Table 2). Liver histology showed moderate steatohepatitis. In addition to macrovesicular steatosis, neutrophil polymorphs and mononuclear cells were scattered diffusely throughout the hepatic parenchyma and also arranged in inflammatory foci (Fig. 2B).

Coadministration of the PPAR α agonist, Wy-14,643, with the MCD diet did not modify loss of weight compared with wt mice (Table 2). As expected from the known effects of PPAR α activation on hepatocyte and

peroxisome proliferation,²¹ both absolute and relative liver weights were increased in this group (Table 2).

Wy-14,643 prevented rather than increased liver injury induced by the MCD diet. Thus, in mice fed Wy-14,643 with the MCD diet, liver histology showed neither steatosis nor inflammation (Fig. 2C) and was considered histologically normal (Table 3). Peroxisome proliferation was evident as eosinophilic staining of hepatocyte cytoplasm, also seen in control diet-fed mice treated with Wy-14,643 (not shown). Consistent with these histologic findings, serum ALT levels were significantly lower in Wy-14,643-treated mice fed the MCD diet than in mice fed the MCD diet alone (Fig. 1).

Effects of Wy-14,643 on Serum and Hepatic Lipid Content, and Expression of Genes Involved With Fatty Acid Turnover. Feeding of the MCD diet lowered serum triglyceride levels in wt mice, and values were further reduced by coadministration of Wy-14,643. Conversely, Wy-14,643 treatment of MCD diet-fed mice restored serum cholesterol levels to values observed in mice fed the control diet (Table 2). Having noted these changes, we examined whether the histologic absence of steatosis in mice fed Wy-14,643 with the MCD diet could be attributed to altered hepatic triglyceride handling. Total hepatic triglyceride levels increased by a factor of 2 in MCD diet-fed mice. Wy-14,643 not only prevented triglyceride accumulation, but lowered hepatic triglyceride content to below levels observed in mice fed the control diet (Fig. 3).

We sought an explanation for the serum and hepatic triglyceride-lowering effects of Wy-14,643 by assessing the hepatic expression of genes involved in fatty acid turn-

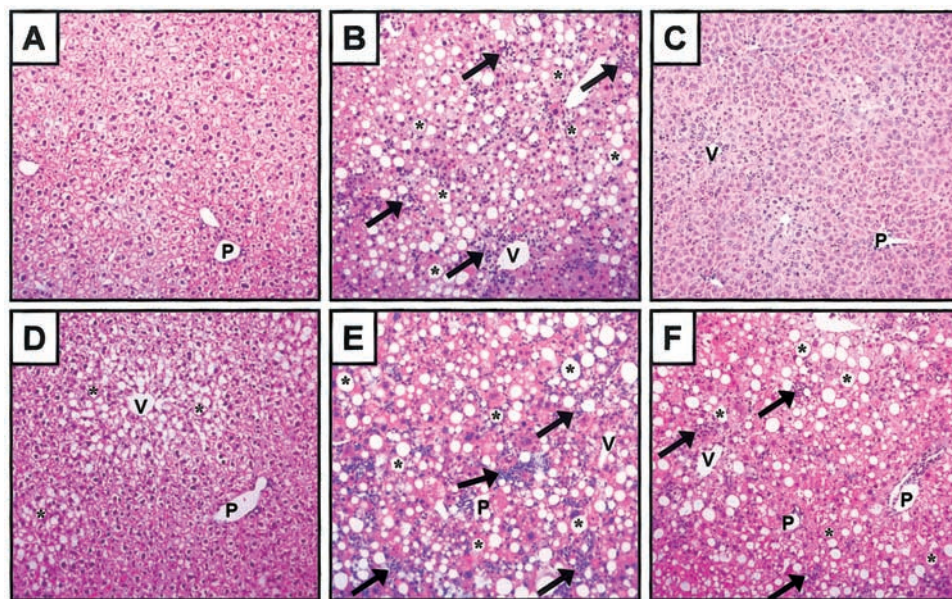


Fig. 2. Effect of the control diet, MCD diet, and MCD diet with Wy-14,643 on liver histology in wt and PPAR $\alpha^{-/-}$ mice. Hematoxylin and eosin-stained liver sections (original magnification $\times 100$) from wt (A, B, and C) and PPAR $\alpha^{-/-}$ (D, E, and F) mice fed the control (A and D), MCD (B and E), or MCD diet with 0.1% (wt/wt) Wy-14,643 (C and F) for 5 weeks. Representative sections show presence of macrovesicular fat droplets (*) and necroinflammatory foci (arrows) that together are characteristic of steatohepatitis. Macrovesicular steatosis in PPAR $\alpha^{-/-}$ mice is more severe around the central vein (V) than portal tracts (P).

Table 3. Effect of MCD Diet With or Without Wy-14,643 on Scores of Severity of Hepatic Steatosis and Necroinflammatory Lesions in wt and PPAR α ^{-/-} Mice

	wt			PPAR α ^{-/-}		
	Control	MCD		Control	MCD	
	-	-	Wy-14,643	-	-	Wy-14,643
Steatosis	0.5 $\pm$ 1.0	2.3 $\pm$ 0.3*	0.0 $\pm$ 0.0‡	2.1 $\pm$ 1.5§	3.0 $\pm$ 0.0§	3.0 $\pm$ 0.0
Necroinflammation	0.0 $\pm$ 0.0	2.3 $\pm$ 0.3†	0.0 $\pm$ 0.0‡	0.7 $\pm$ 0.6	3.0 $\pm$ 0.0‡§	3.0 $\pm$ 0.0

NOTE. The severity of hepatic steatosis and necroinflammation were each scored on a scale of 0-3 by 2 independent pathologists (P.H. and R.K.) as described in Materials and Methods. The data are mean $\pm$ SD, n = 3-6/group.

*P < .05, †P < .001 compared with same genotype mice fed control diet.

‡P < .001 compared with same genotype mice fed MCD diet, §P < .05 compared with wt mice fed the same diet.

over. Administration of the MCD diet reduced mRNA levels for LFABP and ACO (5- and 2.5-fold, respectively; Table 4). LFABP regulates uptake of fatty acids,²² whereas ACO is the rate-limiting enzyme in the peroxisomal β -oxidation pathway. Thus, at least two central regulators of hepatic fatty acid turnover are down-regulated after administration of the high-fat MCD diet. By contrast, Cyp4a14 mRNA was increased in mice fed the MCD diet compared with controls, showing that the regulatory mechanisms may differ between individual PPAR α target genes. Administration of Wy-14,643 together with the MCD diet induced LFABP mRNA 5-fold and mRNA for ACO, BIEN, and KT by 7-, 36-, and 10-fold, respectively, compared with mice fed the MCD diet alone (Table 4).

Induction of Cyp4a10 and Cyp4a14 by Wy-14,643 Is Not Associated With Increased Lipid Peroxidation In Vivo. Previous experiments indicated that an increase in hepatic expression of Cyp4a family members is a potential source of oxidative stress in the liver.^{10,11} In the present study, hepatic mRNA levels of Cyp4a14 were induced 2.7-fold in mice fed the MCD diet as compared with controls, whereas Cyp4a10 was unchanged. Administration of Wy-14,643 further induced hepatic Cyp4a14 expression and also increased Cyp4a10, both to 18-fold higher levels compared with MCD diet-fed mice (Table 4).

To determine whether increased levels of Cyp4a mRNA in mice fed the MCD diet were associated with increased formation of toxic lipoperoxides, total hepatic TBARS were measured. TBARS were increased 50-fold in mice fed the MCD diet compared with those fed the control diet; coadministration of Wy-14,643 with the MCD diet almost completely prevented the accumulation of lipid peroxides (Fig. 4), despite substantially up-regulating Cyp4a10 and Cyp4a14.

Do Microsomal Cyp4a Enzymes Catalyze Lipid Peroxidation in MCD Diet-Fed and Wy-14,643-Treated Mice? One reason why high levels of Cyp4a mRNA in Wy-14,643-treated mice might not lead to

increased levels of hepatic lipoperoxide is that induced Cyp4a proteins may not be catalytically active. We therefore assessed NADPH-dependent lipid peroxidation in hepatic microsomal fractions. Administration of the MCD diet with or without Wy-14,643 increased microsomal lipid peroxidation compared with mice fed the control diet (Table 5). In mice treated with Wy-14,643, immunoinhibition of CYP4A enzymes by anti-rat CYP4A1 antibody largely reduced the formation of lipoperoxides. A lower inhibition was obtained with CYP2E1 antibody (Table 5). Cyp4a enzymes are therefore potentially responsible for increasing *in vitro* microsomal lipid peroxidation in wt mice fed Wy-14,643 with the MCD diet.

Cyp4a enzymes also predominated over Cyp2e1 as a microsomal cause of lipid peroxidation in mice fed only the MCD diet (Table 5). Although a role for Cyp4a14 is

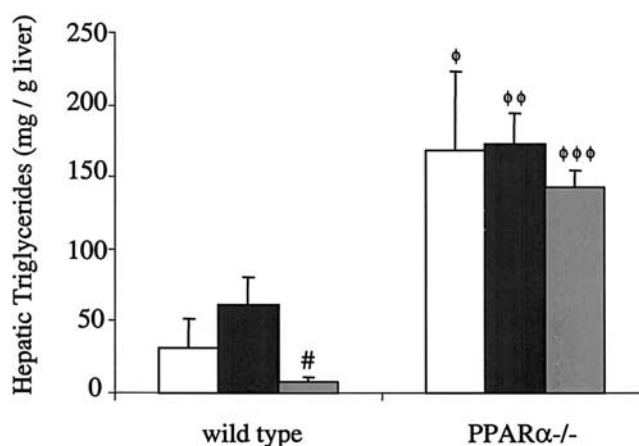


Fig. 3. Effect of Wy-14,643 and the MCD diet on liver triglyceride content in wt and PPAR α ^{-/-} mice. Hepatic triglyceride content was measured in wt and PPAR α ^{-/-} mice fed control diet (□), MCD diet (■), or MCD diet with Wy-14,643 (0.1% wt/wt) (▒) for 5 weeks. Data are mean $\pm$ SD with 3 animals in each group, each assayed in triplicate. #P < .01 compared with same genotype mice fed the MCD diet. φP < .05, φφP < .01, φφφP < .001 compared with wt mice fed the same diet.

Table 4. Effects of MCD Diet and Wy-14,643 on Hepatic mRNA Levels of PPAR α Target Genes in wt and PPAR α ^{-/-} Mice

	wt			PPAR α ^{-/-}		
	Control	MCD		Control	MCD	
	-	-	Wy-14,643	-	-	Wy-14,643
LFABP	1.0 $\pm$ 0.06	0.23 $\pm$ 0.04†	0.88 $\pm$ 0.09¶	0.48 $\pm$ 0.05	0.03 $\pm$ 0.01‡	0.09 $\pm$ 0.02§
ACO	1.0 $\pm$ 0.48	0.39 $\pm$ 0.15*	7.0 $\pm$ 1.5¶	0.95 $\pm$ 0.40	0.35 $\pm$ 0.17†	0.48 $\pm$ 0.10
BIEN	1.0 $\pm$ 0.24	0.90 $\pm$ 0.39	36 $\pm$ 5.4¶	0.77 $\pm$ 0.18	0.47 $\pm$ 0.19	0.76 $\pm$ 0.19
KT	1.0 $\pm$ 0.37	0.59 $\pm$ 0.48	9.7 $\pm$ 1.8¶	1.4 $\pm$ 0.39	0.58 $\pm$ 0.21†	0.59 $\pm$ 0.06
Cyp4a10	1.0 $\pm$ 0.09	0.97 $\pm$ 0.19	18 $\pm$ 7.4¶	0.68 $\pm$ 0.34	0.31 $\pm$ 0.18	0.17 $\pm$ 0.06
Cyp4a14	1.0 $\pm$ 0.08	2.7 $\pm$ 0.77†	48 $\pm$ 17¶	0.63 $\pm$ 0.20	0.27 $\pm$ 0.07	0.26 $\pm$ 0.09

NOTE. LFABP, BIEN, Cyp4a10, and Cyp4a14 mRNA levels were measured by RNase protection assay. ACO and KT mRNA levels were determined by Northern blotting. Specific mRNA values are normalized to the expression of internal control as detailed in the Materials and Methods section. Data are mean $\pm$ SD (n = 3-6/group) expressed relative to the values obtained in mice fed the control diet, which were arbitrarily assigned a value of 1.0.

* P < .05, † P < .01, ‡ P < .001 compared with same genotype mice fed control diet.

§ P < .05, || P < .01, ¶ P < .001 compared with same genotype mice fed the MCD diet.

expected given that it is induced 2.7-fold by the MCD diet (Table 4), the apparent dominant role for Cyp4a over Cyp2e1 differs from our previous observations in female mice.¹⁰ Cyp4a and Cyp2e1 are regulated in a sex-dependent fashion such that Cyp4a is more heavily induced by PPAR α agonists in male compared with female mice,²³⁻²⁵ whereas in some mouse strains, Cyp2e1 expression is higher in females compared with males.^{26,27} To test whether gender differences account for the diminished role of Cyp2e1 in male mice fed the MCD diet, female littermates were fed the MCD or control diet for 5 weeks and Cyp2e1 protein levels were assessed in hepatic microsomal fractions. Consistent with results obtained by Leclercq et al.,¹⁰ there was a 2.7-fold increase (P = .004) in Cyp2e1 protein in female mice fed the MCD diet compared with those fed the control diet, but there was no difference between Cyp2e1 protein levels in male

mice fed the control compared with the MCD diet (P = .9). Thus, while Cyp2e1 is the predominant microsomal source of oxidative stress in female mice fed the MCD diet, Cyp4a enzymes may be more pathogenically important in male mice fed the MCD diet.

PPAR α ^{-/-} Mice Fed the MCD Diet Develop Severe Steatohepatitis That Is Not Alleviated by Wy-14,643.

Given that PPAR α activation by Wy-14,643 prevented MCD diet-induced steatohepatitis, we reasoned that lack of the PPAR α signaling apparatus should worsen steatohepatitis. We therefore conducted parallel experiments in PPAR α ^{-/-} mice of the same age, gender, and genetic background as wt mice. PPAR α ^{-/-} mice are larger than age-matched wt mice, but administration of the MCD diet caused weight loss that, relative to body size, was of a

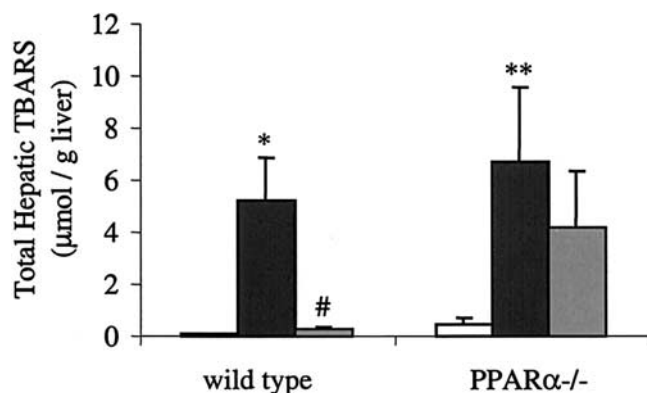


Fig. 4. Effect of Wy-14,643 and MCD diet on total liver lipoperoxide levels in wt and PPAR α ^{-/-} mice. Mice were fed the control diet (□), MCD diet (■), or MCD diet with Wy-14,643 (0.1% wt/wt) (▒) for 5 weeks, and total lipoperoxide levels were assessed in liver homogenate using the TBARS assay. Data are mean $\pm$ SD for each group (n = 3-6/group) obtained in duplicate. * P < .01, ** P < .001 compared with same genotype mice fed the control diet, # P < .01 compared with same genotype mice fed the MCD diet.

Table 5. NADPH-Dependent Lipid Peroxidation in Hepatic Microsomal Fractions From Male wt Mice Fed the Control or MCD Diet With or Without Wy-14,643 and Effects of CYP2E1 and CYP4A1 Antisera

	Microsomal NADPH-Dependent Lipid Peroxidation (nmol/mg protein/min)	% Inhibition of Lipid Peroxidation*	
		Anti-Rat CYP2E1†	Anti-Rat CYP4A1‡
Control diet	0.94 $\pm$ 0.69	45 $\pm$ 12§	78 $\pm$ 7§
MCD diet	2.76 $\pm$ 0.49¶	11 $\pm$ 9	44 $\pm$ 21
MCD + Wy-14,643	2.24 $\pm$ 0.26¶	17 $\pm$ 8	57 $\pm$ 20

NOTE. Values are mean $\pm$ SD from 3 animals per group performed in duplicate. Note that differences between this and a previous study (in female mice) are attributable to gender differences of Cyp2e1 and Cyp4a expression (see text).

*Results are expressed as percentage inhibition of lipid peroxidation obtained when sample incubated with specific antiserum compared with nonspecific antibody (see methods).

†Ratio of anti-rat CYP2E1 IgG to microsomal protein is 3:1.

‡Ratio of anti-rat CYP4A1 IgG to microsomal protein is 2:1.

§Note that, due to nonspecific effects of protein binding, inhibition by anti-CYP2E1 and anti-CYP4A1 antisera does not necessarily add up to $\leq$ 100%.

|| P < .05 compared with mice fed the control diet.

similar proportion to that observed in wt mice (Table 2). Serum triglyceride and cholesterol levels were comparable between PPAR $\alpha^{-/-}$ and wt mice fed the control diet, and were reduced following intake of the MCD diet (Table 2).

ALT levels in PPAR $\alpha^{-/-}$ mice fed the control diet were moderately elevated (Fig. 1), indicative of mild liver injury. This was confirmed histologically (Fig. 2D). Hepatic steatosis and mild parenchymal inflammation might be the consequence of the high fat content of this diet²⁸ as age-matched PPAR $\alpha^{-/-}$ mice fed standard mouse chow had normal liver histology and ALT levels (data not shown). Administration of the MCD diet to PPAR $\alpha^{-/-}$ mice for 5 weeks increased serum ALT levels (Fig. 1) to values observed in MCD diet-fed wt mice. Marked steatohepatitis (Fig. 2E) was histologically graded as significantly more severe than that observed in wt mice (Table 3).

Consistent with the severe steatosis in PPAR $\alpha^{-/-}$ mice fed MCD diet, intrahepatic triglyceride levels were 3-fold higher than those of MCD diet-fed wt mice (Fig. 3), while expression of fatty acid metabolizing enzymes was greatly reduced (Table 4). MCD diet-induced steatohepatitis in PPAR $\alpha^{-/-}$ mice was accompanied by elevation of lipoperoxide levels to values similar to those observed in wt mice fed the MCD diet (Fig. 4). Cyp2e1 protein levels in the control diet-fed PPAR $\alpha^{-/-}$ mice were similar to those obtained in wt mice (0.72 ± 0.47 compared with control diet-fed wt mice, $P = .6$) and were not increased by MCD dietary feeding (0.89 ± 0.19 , $P = .07$). Consistent with this finding and reduced levels of Cyp4a10 and Cyp4a14 mRNA (Table 4) in PPAR $\alpha^{-/-}$ mice, NADPH-dependent lipid peroxidation in microsomal fractions from PPAR $\alpha^{-/-}$ mice fed the control or MCD diet were low (0.29 ± 0.22 and 0.28 ± 0.09 nmol TBARS/mg protein/min, respectively). Microsomal Cyp proteins are therefore unlikely to be an important source of oxidative stress pertinent to the pathogenesis of steatohepatitis in PPAR $\alpha^{-/-}$ mice.

As expected, Wy-14,643 did not affect liver histology in PPAR $\alpha^{-/-}$ mice. Liver histology (Fig. 2F), serum ALT (Fig. 1), serum lipid levels (Table 2), and hepatic expression of PPAR α -regulated genes, excluding LFABP (Table 4) were unchanged compared with PPAR $\alpha^{-/-}$ mice fed the MCD diet alone, confirming the dominant requirement for PPAR α in mediating these effects.

Discussion

In this study, we tested the hypothesis that increasing Cyp4a lipid oxidases would enhance the development of steatohepatitis. However, we instead showed that pharmacologic activation of PPAR α -dependent pathways involved in hepatic triglyceride turnover actually prevents

steatohepatitis. This occurred despite marked induction of the two major Cyp4a proteins in murine liver, Cyp4a10 and Cyp4a14. Conversely, lack of PPAR α rendered the liver more susceptible to MCD diet-induced steatohepatitis. Severe steatosis with minor lobular inflammation developed in PPAR $\alpha^{-/-}$ mice fed the control diet while superimposing methionine and choline deficiency led to very severe steatohepatitis. Thus, the most important finding of this experimental study is the powerful negative correlation between extent of PPAR α activity, accumulation of intrahepatic lipids, and steatohepatitis severity. While induction of microsomal oxidases that can catalyze lipid peroxidation *in vitro* may play a role in the pathogenesis of steatohepatitis, our data strongly suggest that this role is subservient to prior accumulation of supranormal levels of hepatic fatty acids.

Other studies have shown in rats fed a high-fat,²⁹ high-fructose,³⁰ or choline-deficient diet,³¹ and in the A-ZIP/F-1 mouse,³² that administration of a PPAR α agonist reduces or prevents the development of hepatic steatosis. Although rats fed a choline-deficient diet may develop a mild form of steatohepatitis,^{31,33} the present study shows that a PPAR α agonist can prevent steatohepatitis in a model characterized by moderate inflammatory changes, hepatocellular degeneration, and ultimately progressive pericellular fibrosis.³⁴

Some of the end products of lipid peroxidation have proinflammatory properties: 4-hydroxynonenal promotes prostaglandin production via induction of cyclooxygenase-2 expression³⁵ and increases neutrophil chemotaxis,³⁶ whereas malondialdehyde activates hepatic stellate cells.³⁷ The microsomal CYPs 2E1 and 4A are increased in the livers of humans and rodents with steatohepatitis.^{8,9} Both enzymes are potential sources of ROS and have been proposed as initiating factors for steatohepatitis induced by the MCD diet in wt mice¹⁰ or associated with ACO deficiency.^{11,15} However, neither of these microsomal enzymes were induced in MCD diet-fed PPAR $\alpha^{-/-}$ mice despite accumulation of hepatic lipoperoxides and severe steatohepatitis. This infers that extra-microsomal sources of ROS, such as mitochondria and inflammatory cells, are likely to account for increased oxidative stress in these mice. In MCD diet-fed wt mice treated with Wy-14,643, Cyp4a was massively up-regulated, yet hepatic lipoperoxide was low and steatohepatitis was absent. Interpretation of the role of Cyp4a in these animals is complicated by its coregulation with other PPAR α -regulated genes. Thus, activation of PPAR α dramatically reduces hepatic triglyceride content by stimulating hepatic fatty acid disposal, and we propose that this depletes the liver of substrate for lipid peroxidation. As a result, an increase in ROS of any origin, in the absence of

elevated intrahepatic triglyceride levels, is insufficient to cause accumulation of lipid peroxides.

Further evidence to support a critical role for hepatic triglycerides in creating an environment conducive to inflammatory processes was provided by the present studies in PPAR $\alpha^{-/-}$ mice. In addition to LFABP and the enzymatic pathways involved in fatty acid turnover studied here (peroxisomal ACO, BIEN, KT, and Cyp4a enzymes), PPAR α also regulates the expression of mitochondrial β -oxidation enzymes and proteins involved in fatty acid trafficking including fatty acid transport protein and apolipoproteins A-I and A-II.^{14,38,39} The main physiologic function of the PPAR α system in rodents, appears to be coordinating the switch to utilize fatty acids as the main hepatic fuel source. As the corollary to this function, PPAR $\alpha^{-/-}$ mice develop hepatic steatosis and hypertriglyceridemia and are unable to increase ketogenesis in response to circumstances that require fatty acids as the major fuel source, such as fasting or diabetes.^{14,40} In this study, PPAR $\alpha^{-/-}$ mice fed a high-fat but otherwise nutritionally adequate diet developed severe hepatic steatosis and in some cases mild hepatic necroinflammatory changes. It seems likely that lipid overload occurs to a threshold for progression to steatohepatitis in the absence of other aggravating factors so that PPAR α -dependent intrahepatic fatty acid combustion reduces the susceptibility to steatohepatitis as well as steatosis. Consistent with this proposal, we showed that expression of the PPAR α -regulated genes LFABP and ACO were reduced following administration of the MCD diet to wt mice. Wan et al. have reported similar observations in ethanol-fed mice.⁴¹ Conversely, activation of PPAR α by Wy-14,643 in wt mice increased fatty acid uptake into the liver and stimulated their hepatic β -oxidation. This most likely accounts for prevention of hepatic steatosis and reduced serum triglyceride levels in wt mice fed the MCD diet and in other models of hepatic steatosis and increased resistance to development of MCD diet-induced steatohepatitis.

An alternative explanation for the prevention of dietary steatohepatitis by Wy-14,643 is that activation of PPAR α has other, direct effects on inflammation. PPAR α mediates repression of nuclear factor κ B (NF- κ B) through formation of inhibitory complexes with NF- κ B proteins⁴² and by increased expression of the NF- κ B inhibitory protein, I κ B α .⁴³ In addition, CYP4A is involved in the degradation fatty acid derivatives, which may act as inflammatory mediators. It has been implicated in the inactivation of 4-hydroxynonenal⁴⁴ and leukotriene B₄,⁴⁵ an eicosanoid that increases vascular permeability.

Determining whether the preventative effects of PPAR α activation on experimental steatohepatitis occur

through modulation of fatty acid turnover prior to, or in conjunction with, its effects on inflammatory processes has potentially important clinical implications. Expression of PPAR α in human liver is much less than in mice, and treatment of hypertriglyceridemia with fibrates, which are PPAR α ligands, does not cause peroxisome proliferation or up-regulate ACO.^{46,47} Functionality of the human PPAR α gene and protein has been shown *in vitro*⁴⁸ and *in vivo*.⁴⁹ Furthermore, the L162V polymorphism in the human PPAR α gene that increases PPAR α activity has been associated with increased sensitivity of type II diabetic patients to the lipid-lowering effects of fibrates.⁵⁰ Until the role of PPAR α in humans and mode of fibrate action are discerned, it can be assumed that the effect of PPAR α activation in humans is less than in rodents, and that the dramatic effect of potent PPAR α activation in preventing steatohepatitis in this rodent model is unlikely to be replicated in humans with risk factors for NASH. However, a better understanding of whether the prevention of steatohepatitis by PPAR α agonists in this and other models is the direct consequence of depleted intrahepatic lipid, or results from enhanced degradation of lipid inflammatory mediators will be valuable in the design of effective therapeutic strategies for clinically relevant forms of steatohepatitis.

In conclusion, the development of steatohepatitis in mice fed a high-fat, methionine and choline deficient diet is dependent on the activation of pathways for hepatic lipid turnover, which are largely governed in rodents by PPAR α . Thus, PPAR $\alpha^{-/-}$ mice develop mild steatohepatitis during intake of a high-fat but otherwise nutritionally adequate diet, whereas steatohepatitis provoked by methionine and choline deficiency is more severe. Pharmacologic induction of hepatic pathways for fatty acid catabolism with the potent PPAR α agonist, Wy-14,643, prevented the development of steatohepatitis in wt mice fed the high-fat MCD diet despite induction of Cyp4a-dependent lipid oxidases. It is therefore clear that, in the absence of hepatic triglyceride accumulation, these enzymes are not associated with retention of lipid peroxides in the liver and recruitment of the inflammatory response. Taken together, these findings bolster the concept that hepatic steatosis is seminal and essential for steatohepatitis to occur. Although hepatic lipid turnover in the human liver is likely to be controlled extensively by non-PPAR α mechanisms, the finding that downstream pathways of cellular injury and inflammatory recruitment in steatohepatitis are critically dependent on lipid accumulation in the liver has conceptual and practical implications for the prevention and treatment of humans with steatohepatitis.

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